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Full Length Research Paper

Parthenolide sensitizes HL-60 cells to aclarubicin-induced apoptosis by inhibiting NF-kappa band COX-2 activation

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Inducing leukemia cell apoptosis is a major therapeutic strategy. Herein we investigate the enhancing effect of the herbal constituent parthenolide on aclarubicin-induced apoptosis of human HL-60 leukemia cells. HL-60 cells were incubated with aclarubicin in the absence or presence of different doses of parthenolide. Apoptosis was assessed by flow cytometry using annexin V-propidium iodide double staining. To investigate the molecular mechanism by which parthenolide enhances aclarubicin-induced HL-60 apoptosis, caspase 3 and caspase 9 expression, as well as Cox-2 and NF-κB activity, were assessed by western blot. Following exposure to aclarubicin for 20 h, the percentage of cells undergoing apoptosis was highly correlated with dose. However, there was no significant apoptosis at a low aclarubicin concentration (0.1 µg/ml). Combined treatment at low aclarubicin and parthenolide concentration had a dose-dependent effect on apoptosis. In addition, combined parthenolide and aclarubicin treatment had a significant synergistic inhibitory effect on caspase 3, caspase 9 and Cox-2, NF-κB activity. In conclusion, parthenolide sensitizes leukemia cells to aclarubicin-induced apoptosis through increasing expression of cleaved caspase 3 and caspase 9, maybe by suppress Cox-2 and NF-κB activation, suggesting a new avenue of treatment of leukemia.

Key words: Parthenolide, leukemia cells, apoptosis, NF-κB, Cox-2.

INTRODUCTION

Aclarubicin is a common treatment for leukemia patients. However, its clinical use is restricted due to side effects (Dartsch et al., 2002). Recently, increasing attention has been paid to primitive medicinal plants to find substances with potentially useful biological activities. Parthenolide, the major bioactive molecule of the feverfew (Tanacetum parthenium), has a complicated role in regulating the life and death of cells (Li-Weber et al., 2005). Many studies have proposed that parthenolide has proapoptotic activity, either by preventing Cox-2 pathway by suppress NF-κB activation or through a NF-κB-independent pathway. Thus parthenolide is regarded as an anti-inflammatory and anti-tumoral agent (Beranek, 2003; Li-Weber et al., 2002; Nakshatri et al., 2004; Pozarowski et al., 2003; Wen et al., 2002). However, other studies argued that parthenolide might prevent apoptosis by suppressing apoptotic receptor expression. In addition, parthenolide has also been reported to affect the intracellular redox state by regulating the GSH activity (Herrera et al., 2005).

In this study, we demonstrated, for the first time, that parthenolide combined with aclarubicin could effectively inhibit leukemia cells growth by inducing apoptosis. We further explored the molecular mechanisms, such as caspase 3, 9 and Cox-2, NF-κB activity, suggesting a potential anti-leukemia effect.

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MATERIALS AND METHODS

Materials

Cell culture medium components were purchased from Invitrogen Life Technologies (San Diego, California) unless otherwise noted. The human leukemia cell line HL-60 was obtained from ATCC (Manassas, VA). Aclarubicin and parthenolide (with a purity ≥ 98.5%) were purchased from Sigma (St Louis, MO). Aclarubicin prepared immediately before use in phosphate buffered saline (PBS; Hyclone, Utah, USA) prior to dilution to 0.05, 0.1, or 0.25 µg/ml in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum (FBS; Hyclone). Parthenolide was dissolved in dimethylsulfoxide (DMSO) supplemented with 10% fetal bovine serum (FBS; Hyclone) at 50.0 mM. After storage at -30°C, it was diluted to a final concentration of 10, 100, or 500 µM. Antibodies used in western blot analysis included monoclonal antibodies recognizing caspase 3 and NF-κB(p-p65, phosphorylation sites is Ser276) (Santa Cruz Biotechnology, Santa Cruz, CA) and caspase 9 (Oncogene, Boston, MA) and a rabbit polyclonal antibody against GAPDH (Santa Cruz Biotechnology, Santa Cruz CA).

Cell culture and treatment

The HL-60 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were treated with aclarubicin (0.05, 0.1, or 0.25 µg/ml) for 20 h. For parthenolide, cells were treated with 10, 100, or 500 µM for 2 h, after which 0.1 µg/ml aclarubicin was added, and then the cells were incubated for a further 18 h. The culture supernatants were collected and kept at -20°C.

Flow cytometry analysis

Annexin V binding and propidium iodide (PI) staining were determined by flow cytometry, using reagents from BD Pharmingen (USA San Diego County). The cells were washed with ice-cold PBS, and double stained with FITC-coupled annexin V and PI for 20 min. Flow cytometry was performed with a 488 nm laser coupled to a cell sorter (FacsCalibur; BD Biosciences, San Jose, CA). Cells stained with both PI and annexin V were considered necrotic, and the cells stained only with annexin V were considered apoptotic.

Assay for PGE2 production

The amount of PGE2 in culture supernatants was determined using a competitive ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The lower detection limit of the assay was 36.2 pg/ml.

Western blot analysis

Cytoplasmic extracts were prepared in lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.9), 0.5% Triton X-100, 0.6% NP-40, and protease inhibitors, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 2 µg/ml aprotinin)). The protein contents were determined using the DC protein assay kit (Bio-Rad, Hercules, CA). Protein samples (40 µg) were mixed with 2×SDS sample buffer and separated on a 10% polyacrylamide gel, and blotted on a nitrocellulose membrane (Bio-Rad). The blots were blocked for 2 h in blocking buffer (PBS with 7.5% non-fat dry milk, 2% bovine serum albumin, 0.1% Tween), Blots were incubated with anti-Cox-2 and anti-NFκB phospho-p65, anti-caspase-3, caspase-9, and anti-β-actin specific rabbit polyclonal IgG primary antibody (Santa Cruz) at 1:500 dilution at 37°C for 2 h. Subsequently, the membranes were washed in washing buffer (PBS with 0.1% Tween-20), incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Pierce; 1:10000 in blocking buffer) for 1 h at room temperature, washed in PBS, and developed using the ECL chemiluminescence detection system (Super signal Dura kit, Pierce, Rockford, IL). In all experiments, Ponceau staining was carried out to detect equal loading.

Statistical analysis

All experiments were performed at least three times. The results are expressed as mean ± the standard deviation of the mean (SD). Statistical significance was analyzed by one-way analysis of variance (ANOVA). A P value less than 0.05 was considered statistically significant.

RESULTS

Flow cytometry analysis of HL-60 cells apoptosis

To quantify the induction of apoptosis by aclarubicin at different concentrations, the percentage of apoptotic cells was detected by annexin V-FITC and PI double staining (Figure 1). A slight increase of the percentage of apoptotic HL-60 cells treated with 0.25 µg/ml aclarubicin was observed compared with 0.05 µg/ml (5.09 ± 0.67% vs. 2.58 ± 0.42, $P > 0.05$) (Table 1). To determine the effect of parthenolide combined with aclarubicin, 0.1 ug/ml aclarubicin was chosen for its minimal effect. The results show that aclarubicin (0.05, 0.1, or 0.25 µg/ml) alone did not increase the rate of apoptosis when compared to the aclarubicin (0.1 µg/ml) combination with different dose of pathenolide (PN) (10, 100, or 500 µM).

PGE2 production assayed by ELISA

For PGE2 production, a slight decrease treated with 0.25 µg/ml aclarubicin was observed compared with 0.05 µg/ml. To determine the effect of parthenolide combined with aclarubicin, 0.1 ug/ml aclarubicin was chosen for its minimal effect. Figure 2 shows that parthenolide (10, 100, or 500 µM) with aclarubicin (0.1 µg/ml) significantly decreased PGE2 production, while either Acla (0.1 µg/ml) or PN (500 µM) alone induced only a slight decrease.

Western blot detection of caspase 3 and 9

Once we confirmed that parthenolide sensitizes leukemia cells to aclarubicin-induced apoptosis, we examined the expression of the effector caspase 3 and the initiator caspase 9. Figure 3 shows that aclarubicin increased caspase 3 and caspase 9 protein immunocontent slightly in HL-60 cells. Parthenolide (10, 100, or 500 µM) with aclarubicin (0.1 µg/ml) significantly enhanced caspase

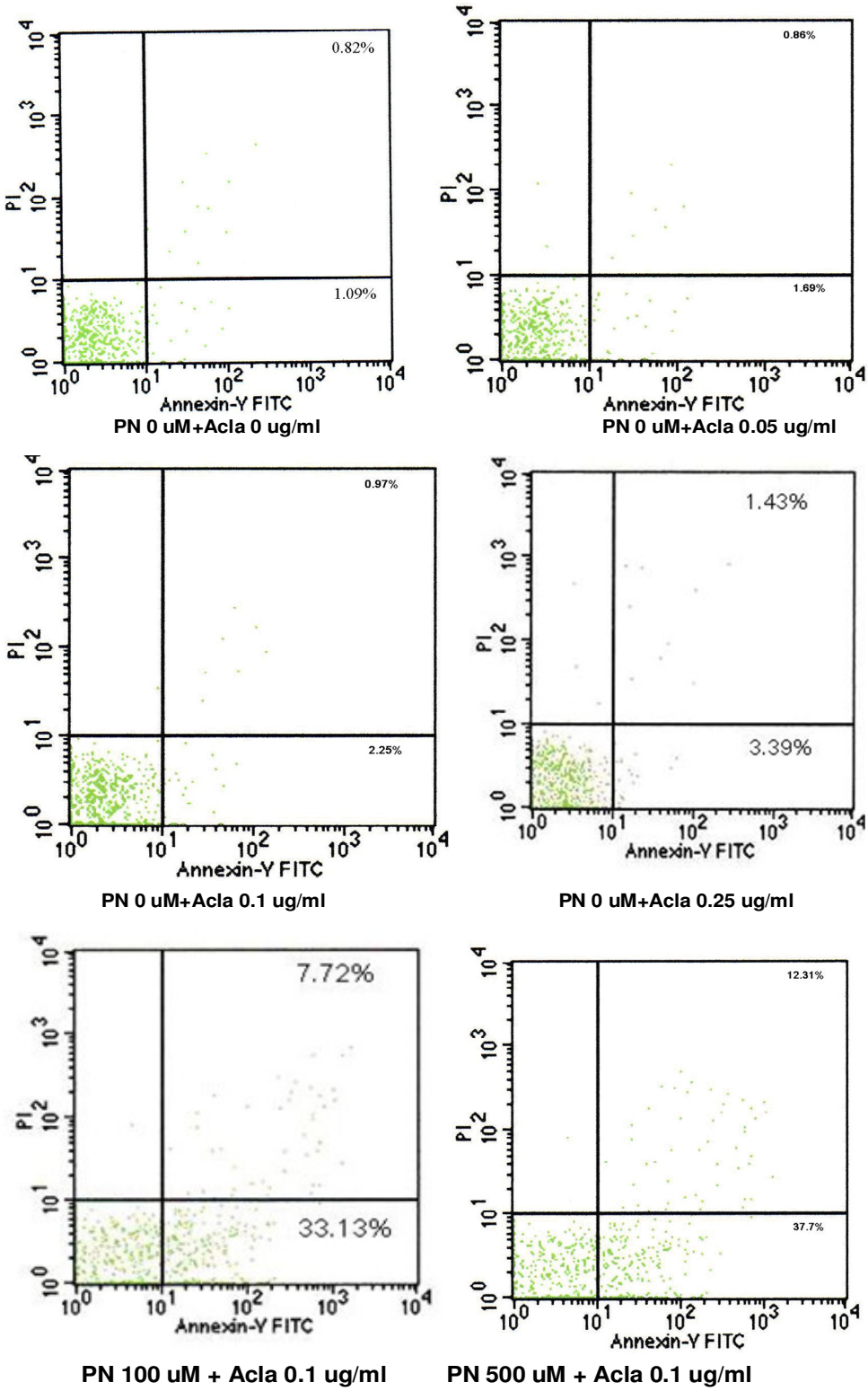

Figure 1. Flow cytometry shows increased apoptotic HL-60 cells after treatment with parthenolide combined with aclarubicin. Apoptotic cells were detected by Annexin V and PI double staining as previously described in the materials and methods. Apoptotic cells were detected by Annexin V and PI double staining. In the present of sinomenine and Acla, apoptosis induction increased significantly in a dose-dependent manner. A, control; B, 0.05 µg/ml Acla; C, 0.1 µg/ml Acla; D, 0.25 µg/ml Acla; E, 20 ng/ml sinomenine; F, 5 ng/ml sinomenine + 0.1 µg/ml Acla; G, 10 ng/ml sinomenine + 0.1 µg/ml Acla; H, 20 ng/ml sinomenine + 0.1 µg/ml Acla.

Table 1. HL-60 cells treated with parthenolide combined with aclarubicin for 20 h.

Group	Apoptotic rate (%)
PN 0 uM + Acla 0 ug/ml	1.90±0.06
PN 0 uM + Acla 0.05 ug/ml	2.58±0.42
PN 0 uM + Acla 0.1 ug/ml	3.73±0.49
PN 0 uM + Acla 0.25 ug/ml	5.09±0.67
PN 500 uM + Acla 0 ug/ml	16.37±0.80#
PN 10 uM + Acla 0.1 ug/ml	18.43±0.79#
PN 100 uM + Acla 0.1 ug/ml	39.83±1.18#
PN 500 uM + Acla 0.1 ug/ml	49.16±1.46#

#:*P* < 0.01 compared to the untreated control. PN, Pathenolide; Acla, aclarubicin. Apoptosis induction correlated positively with parthenolide concentration (X±S).

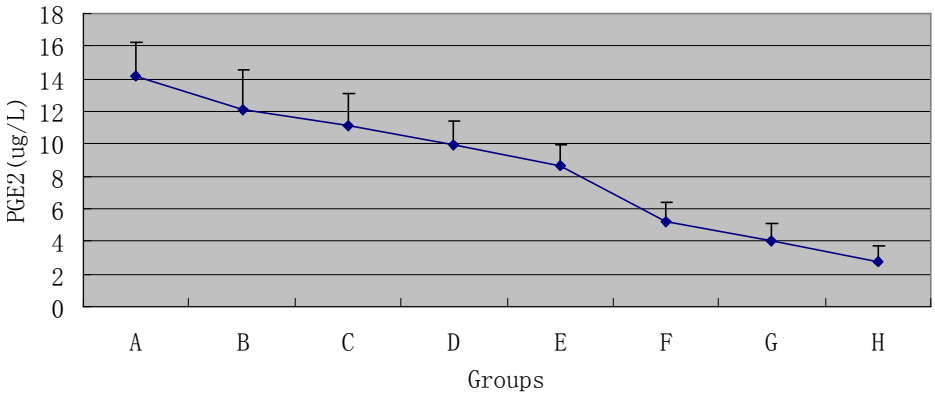

Figure 2. PGE2 production tested by ELISA. PGE2 slightly decreased treated by PN (10, 100, or 500 µM) and Acla (0.1µg/ml) alone, even at 500 µM, cotreatment with Acla (0.1 µg/ml) significantly reduces PGE2 generation. PGE2 production tested by ELISA. (A) PN 0 uM + Acla 0 ug/ml (B) PN 0 uM + Acla 0.05 ug/ml; (C) PN 0 uM + Acla 0.1 ug/ml; (D) PN 0 uM + Acla 0.25 ug/ml; (E) PN 500 uM + Acla 0 ug/ml; (F) PN 10 uM + Acla 0.1 ug/ml; (G) PN 100 uM + Acla 0.1 ug/ml; (H) PN 500 uM + Acla 0.1 ug/ml.

expression, whereas aclarubicin alone induced only a slight increase in caspase expression, even at 0.25 µg/ml.

Inhibition of p-p65 NF-κB and COX-2 at protein level in HL-60 treated

NF-kB is an ideal target for anti-cancer drug development as it has been shown to block apoptosis and promote proliferation. Caspases, a family of cysteine proteases, play a critical role in the execution of apoptosis (Liu et al., 2010) which are modulated by several upstream genes, especially cytochrome-c; NF-□B-mediated Bcl-2 down-regulation, release of mitochondrial cytochrome-c to the cytoplasm and activation of caspase-9 and caspase-3. To examine the molecular mechanism of aclarubicin and/or parthenolide, phophorylated NF-κB was measured by detecting p-p65. As shown in Figure 4, phophorylated NF-κB was significantly reduced by aclarubicin with parthenolide; coaddition of PN (10, 100, or 500 µM) and Acla (0.1 µg/ml) result in significant reduction of viability whereas PN alone induced only a slight decrease even at 500µM. Phophorylated NF-κB was consistent with the corresponding caspase 3 and caspase 9 protein content. Cox-2 expression was consistent with the phophorylated NF-κB. Cox-2 reduced significantly when treated with aclarubicin (0.1µg/ml) combined with parthenolide (10, 100, or 500 µM). The decrease had dose-dependent property of parthenolide.

DISCUSSION

Previous study demonstrated that parthenolide suppresses H_2O_2-induced HLE cell death in a dose-dependent manner *in vitro* (Yao et al., 2007). The present study showed that parthenolide can sensitize tumor cells

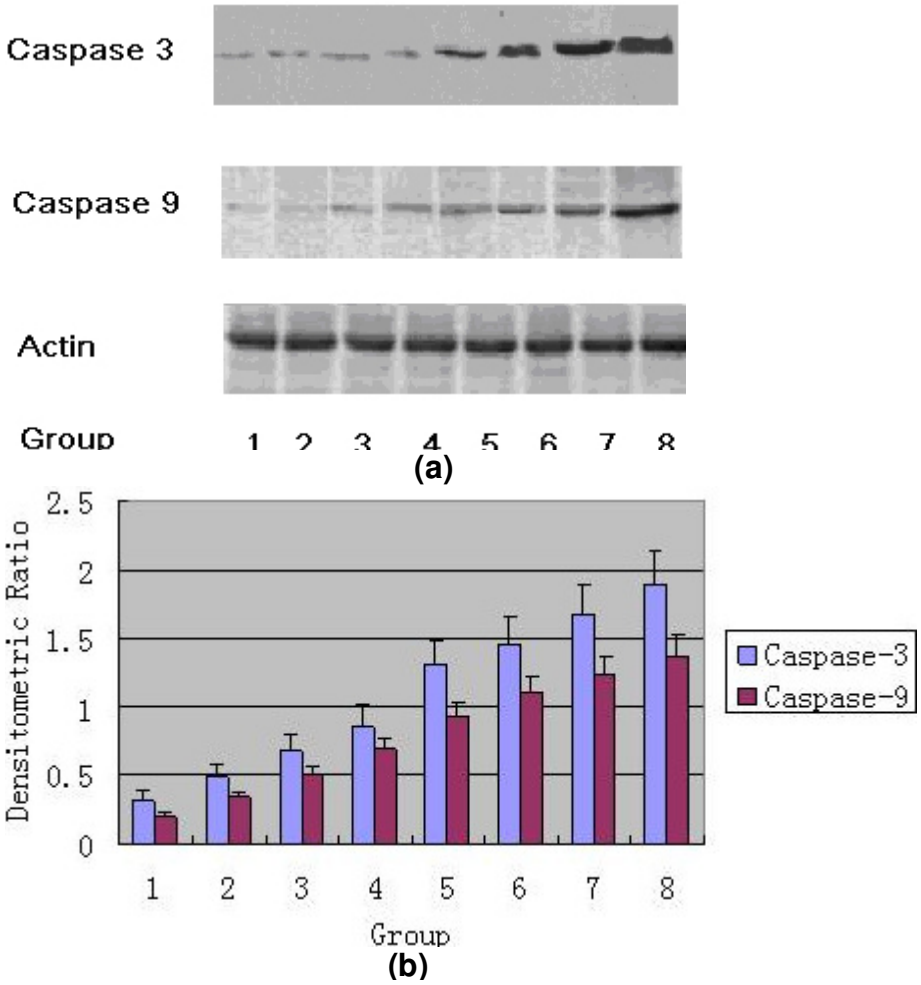

Figure 3. Parthenolide combined with aclarubicin inhibits the protein content of caspase 3 and caspase 9 at the protein level in HL-60 cells. The expression of caspase 3 and caspase 9 in HL-60 cells was detected by western blot (A) as described in the Materials and methods section. (b) Densitometric analysis was performed using a software program from Kodak Digital Science. The net intensity of each band was compared to that of the housekeeping gene β-actin and the ratio is shown. Bars show the SD. The protein content of caspase 3 and caspase 9 detected by western blot (1) PN 0 uM + Acla 0 ug/ml; (2) PN 0 uM + Acla 0.05 ug/ml; (3) PN 0 uM + Acla 0.1 ug/ml; (4) PN 0 uM + Acla 0.25 ug/ml; (5) PN 500 uM + Acla 0 ug/ml; (6) PN 10 uM + Acla 0.1 ug/ml; (7) PN 100 uM + Acla 0.1 ug/ml; and (8) PN 500 uM + Acla 0.1 ug/ml.

to apoptosis. In recent years, published reports have shown that parthenolide can induce leukemia cell apoptosis (Zunino et al., 2007; Guzman et al., 2005). Until now, little is known about the anti-leukemia mechanism. We demonstrated that aclarubicin induced apoptosis in a dose-dependent manner *in vitro*. Aclarubicin is commonly used for patients with leukemia. However, due to the side effects at the therapeutic dose, substituting or supplementing aclarubicin with safer drugs may avoid serious complications (Zunino et al., 2007). To test this hypothesis, low concentrations of aclarubicin were combined with parthenolide to treat HL-60 leukemia cells. Interestingly, low aclarubicin concentrations, combined with parthenolide, induce more apoptosis, as measured by flow cytometry.

Apoptotic cell death can be induced through either the death receptor or the mitochondria-mediated signaling pathways (Yang et al., 2002). Caspase-3 is an effector protease which can be activated by mitochondrial or receptor-mediated cell death; caspase 9 plays a key role in tumor cell apoptosis induced by chemotherapeutic agents (Saito et al., 2004; Koceva-Chyła et al., 2005). In addition, *in vitro* studies have identified caspase 9, Apaf1 and cytochrome c as participants in a complex important for caspase 3 activation (Yao et al., 2003). Caspase 9 deficient mice have reduced levels of apoptosis

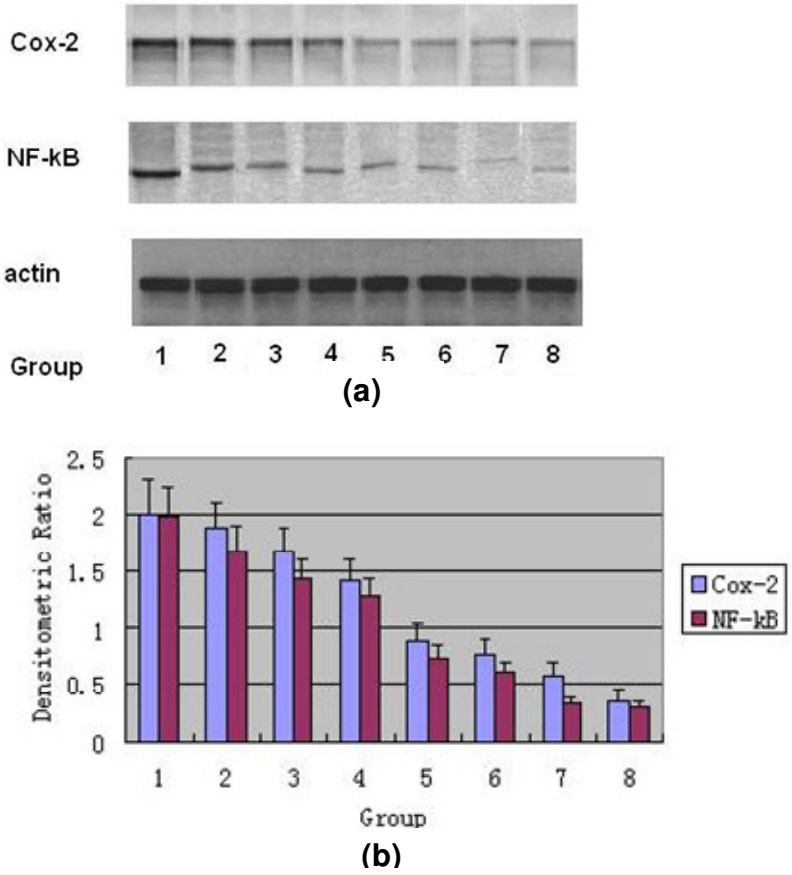

Figure 4. Parthenolide combined with aclarubicin inhibits the expression of Cox-2 and NF-κB at the protein level in HL-60 cells. (a) The expression of Cox-2 and NF-κB in HL-60 cells was detected by western blot as pr5eviously described in the 'materials and methods' (b) Densitometric analysis was performed using a software program from Kodak Digital Science. The net intensity of each band was compared to that of the housekeeping gene β-actin and the ratio is shown reported. Bars show the mean ± SD. The Cox-2 and phophorylated NF-κB assayed by western blot (1) PN 0 uM + Acla 0 ug/ml; (2) PN 0 uM + Acla 0.05 ug/ml; (3) PN 0 uM + Acla 0.1 ug/Ml; (4) PN 0 uM + Acla 0.25 ug/ml; (5) PN 500 uM + Acla 0 ug/mL; (6) PN 10 uM + Acla 0.1 ug/ml; (7) PN 100 uM + Acla 0.1 ug/ml; and (8) PN 500 uM + Acla 0.1 ug/ml.

(Li-Weber et al., 2005). Treatment of leukemia cells with aclarubicin resulted in slight increase of caspase 3 protein levels. In the presence parthenolide (10, 100, or 500 µM), caspase 3 levels were significantly increased in a dose-dependent manner. Similar results were observed for caspase 9. Thus, our findings point to a dependence on both caspase 9 and caspase 3 during apoptosis of leukemia cells treated with aclarubicin and parthenolide.

Nuclear factor-κB (NF-κB) is a transcription factor that has a critical role in the inappropriate survival of various types of malignant cells; it is closed correlated with caspase 9 and 3 activation (Pickering et al., 2007; Takigawa et al., 2006). In the present study, we demonstrated that parthenolide suppressed NF-κB activity in a dose-dependent manner. The lower concentration of aclarubicin (0.1 µg/ml) did not affect NF-κB activity. Interestingly, low concentrations of aclarubicin combined with parthenolide significantly inhibited NF-κB activity in a dose-dependent manner, and the inhibition correlated with the increased caspase 3 and caspase 9 expressions. These results suggest that parthenolide enhances the anti-leukemia effect of aclarubicin.

Cox-2 is a pro-inflammatory mediator that can suppress apoptosis, while inhibition of both Cox-2 and NF-κB has shown great potential as an anti-inflammation treatment for cancer therapy (Ghosh et al., 2007), while over expression of PGE2 has been been reported in a variety of malignancies, including colorectal cancer and lung cancer (Fernández-Martínez et al., 2007). PGE2 has been shown to stimulate tumor growth and metastasis by promoting the migration, invasion, and angiogenesis of tumor cells, which may also be triggered by activation of

Cox-2 (Ouyang et al., 2007). Furthermore, previous studies have demonstrated that expression of Cox-2 may be regulated by NF-κB (Ohshima et al., 2005), which can also up-regulate the transcription of proteins that promote cell survival, stimulate cell growth, and reduce susceptibility to apoptosis in prostate cancer and leukemia (Liu et al., 2009)

In conclusion, combined treatment with parthenolide and aclarubicin significantly increases apoptosis, compared to aclarubicin or parthenolide alone. This effect involves both NF-κB activation and increased caspase 3 and caspase 9 expressions. The effect of sensitive leukemia cells are sensitized to apoptosis most likely through suppressing NF-κB activity. These results suggest that pathenolide may be an effective adjuvant therapy to aclarubicin treatment of leukemia.

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