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Full Length Research Paper

Sinomenine enhanced aclarubicin-induced apoptosis by blocking NF-κB pathway in HL- 60 cells

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Sinomenine (SN) a component from Sinomenium acutum, widely used as an anti-inflammatory agent in Chinese traditional medicine, was reported to have anti-tumor activities. This study aims to examine the anti-leukemic activity of SN with or without aclarubicin (Acla) on leukemia cells and its molecular mechanism. HL-60 cells were treated with SN (5 to 20 ng/ml) with or without Acla (0.05 to 0.25 µg/ml). To study apoptosis, the cells were stained with Annexin V - propidium iodide (PI) and assessed by flow cytometry. To investigate the molecular mechanism, Caspase 3, Caspase 9 and cyclooxygenase-2 (Cox-2) were assessed by Western blot, NF-kB activity and prostaglandin E2 (PGE2) were tested by ELISA. Exposure to Acla (even at 0.25 µg/ml) alone or SN alone (20 ng/ml) for 20 h did not induce significant apoptosis. However, SN (5 to 20 ng/ml) promoted apoptosis induced by 0.1 µg/ml Acla in a dose-dependent manner. Increased Caspase 3 and 9 protein expression correlated positively with SN concentration when combined with 0.1 µg/ml Acla. In addition, SN significantly inhibited Acla-induced NF-kB activation, Cox-2 gene expression and PGE2 production. In summary, SN most likely enhances apoptosis by suppressing NF-kB activation, and Cox-2 and PGE2 expression. In the future, this natural agent may provide a new avenue for anti-leukemia treatment.

Key words: Sinomenine, Aclarubicin, apoptosis, nuclear factor-kappa B, prostaglandin E2 (PGE2), cyclo-oxygenase (Cox)-2.

INTRODUCTION

Inflammatory pathways, characterized by over-expression of nuclear factor κB (NF-kB) and cyclooxygenase-2 (Cox-2), and prostaglandin E2 (PGE2) production, are implicated in the development of several types human cancer, such as colon, breast, prostate and leukemia (Fernandez et al., 2007; Choi et al., 2008). NF-kB signaling is an important regulatory pathway for cell proliferation and apoptosis. Cox-2, which promotes cell proliferation and may contribute to carcinogenesis, catalyzes PGE2 production. PGE2 production has been linked to proliferation and metastasis of tumor cells (Adhami et al., 2007). Because of their roles in carcinogenesis, apoptosis, and angiogenesis, these are excellent targets for treating human cancers, including leukemia (Park et al., 2007).

Sinomenine (7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinane-6-one, SN), extracted from the Chinese medical plant Sinomenium acutum, has been used successfully for centuries to treat rheumatoid arthritis patients in China (Wang et al., 2005; Li et al., 2006; Shu et al., 2007; Park et al., 2007) with minimal side effects (Nishida and Satoh, 2006). SN has immunomodulatory properties in a cardiac allograft transplantation model (Tang et al., 2006), protects mice from endotoxin-induced fulminate hepatitis, and exerts synergistic effects with cyclosporin A to inhibit immune responses and prolong cardiac allograft survival in rats (Zeng et al., 2007; Feng et al., 2006; Zhao et al., 2007). Previous studies show that SN inhibits lymphocyte

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Table 1. Groups of HL-60 cells treated differently in this study.

Group	A	B	C	D	E	F	G	H
Acla (µg/ml)	0	0.05	0.1	0.25	0	0.1	0.1	0.1
SN (ng/ml)	0	0	0	0	20	5	10	20

SN: sinomenine; Acla: aclarubicin

proliferation, macrophage production of pro-inflammatory factors, and NF-kB activity (Wang et al., 2005; Li et al., 2006; Shu et al., 2007).

Anti-inflammatory drugs induce apoptosis in human leukemia cells *in vitro*; inhibiting NF-kB activation alone can be sufficient to induce cell death. In addition, NF-kB may be involved in chemoresistance due to its constitutive activation, such as is found in human acute myelogenous leukemic cells (Subhashini et al., 2005). However, little is known about the ability of SN to induce tumor cell apoptosis. Previous studies have demonstrated that Caspase 3 is critical in tumor cell apoptosis induced by chemotherapeutic agents (He et al., 2005; Ma et al., 2007). In addition, studies *in vitro* have identified Caspase 9 and Cytochrome c as participants in a complex important for Caspase 3 activation (Ougolkov et al., 2007), and reduced apoptosis in Caspase 9-deficient mice (Mathieu et al. 2006).

Leukemia patients usually are treated with aclarubicin (Acla), However, the therapeutic dose has many side effects. Reducing the Acla dose by including an adjuvant therapy may avoid some of these serious complications. In this study, we demonstrate that SN combined with low Acla concentration effectively induces apoptosis in the human leukemia cell line HL-60. We also explored the molecular mechanisms by examining Cox-2 expression and PGE2 generation, as well as activation of Caspases 3 and 9, as well as the transcription factor NF-kB. The effector caspase, Caspase 3, and the initiator caspase, Caspase 9, currently are considered the main executors of apoptosis. Caspase 9 functions in the mitochondrial pathway, which results in activation of Caspase 3. The transcription factor NF-kB, and p65, one of its subunits, are key molecules controlling the apoptotic process, acting mainly as an apoptosis inhibitor.

MATERIALS AND METHODS

Materials

Cell culture medium components were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise noted. The human myeloid leukemia cell line HL-60 was obtained from ATCC (Manassas, VA, USA). Acla (Sigma, St Louis, MO, USA) was 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). SN (7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinane-6-one, C19H23NO4) was purchased from Sigma (St. Louis, MO, USA) with a purity ≥ 98.5% and was dissolved in dimethylsulfoxide (DMSO, Invitrogen, Carlsbad, CA, USA) at 100 µg/ml and stored at -30˚C. Immediately, it was diluted to 5, 10, or 20 ng/ml in complete DMEM (DMEM with FBS).

Cell culture and treatments

HL-60 cells were cultured in complete DMEM at 37°C in a humidified atmosphere containing 5% CO_2. The cells were treated for 20 h with Acla (0.05, 0.1, or 0.25 µg/ml) or SN (5, 10, or 20 ng/ml) with 0.1 µg/ml Acla (Table 1). Complete DMEM alone was added to the control group. The culture supernatants were collected and kept at -20°C.

Flow cytometry analysis

The cells were harvested and apoptosis assessed using reagents from BD Pharmingen (San Diego, CA, USA). Briefly, the cells were washed with ice-cold PBS, and stained with FITC-coupled Annexin V and propidium iodide (PI) for 20 min. Flow cytometry was performed with a 488 nm laser coupled to a FacsCalibur cell sorter (BD Biosciences, San Jose, CA, USA). The cells stained with Annexin V positive and PI negative were considered apoptotic.

Cell viability assay (MTS)

Cell viability was quantified on the basis of metabolic activity with the MTS assay (Promega, Madison, Wisconsin), according to manufacturer's protocol. HL-60 cells were seeded at a density of 5 $\times 10^4$/well in a 96-well plate in DMEM with 5% fetal bovine serum. The cultures were incubated in serum-free medium containing 20 µl/well of the MTS tetrazolium compound for 3 h at 37°C. The absorbance of formazan products was photometrically measured at 490 nm with a microplate reader. The cell viability was expressed as absorbance (OD).

Assay for PGE2 production

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

Activity assay for NFκB p65

NF-κB p65 transcription factor assay kits (Active Motif, California, USA), which combine all the benefits of fast, sensitive and specific assays were used to monitor NF-κB activation. 5 µg of cell extracts/well were added to a 96-well plate coated with an oligonucleotide probe containing the site for NF-κB binding. Each well was then incubated with primary antibody specific for the active form of bound transcription factor. Wells were then incubated with

Table 2. HL-60 cells treated with sinomenine combined with Acla for 20 h. The effect of inducing-apoptosis induction and PGE2 production were correlated positively with sinomenine concentration.

Treatment group (μg/ml)	Apoptotic cells (%)	PGE2 (μg/L)
(A) SN 0 ng/ml + Acla 0	1.53 ± 0.22	16.72 ± 2.53
(B) SN 0 ng/ml + Acla 0.05	2.43 ± 0.41	15.07 ± 3.04
(C) SN 0 ng/ml + Acla 0.1	3.35 ± 0.22	13.90 ± 2.25
(D) SN 0 ng/ml + Acla 0.25	8.12 ± 0.52#	10.17 ± 1.91#
(E) SN 20 ng/ml + Acla 0	8.75 ± 0.39#	9.75 ± 1.14#
(F) SN 5 ng/ml + Acla 0.1	10.11 ± 1.44#	8.67 ± 1.75#
(G) SN 10 ng/ml + Acla 0.1	20.41 ± 3.21#▲	5.63 ± 0.93#▲
(H) SN 20 ng/ml + Acla 0.1	33.05 ± 5.02#▲	2.43 ± 0.77#▲

SN: sinomenine; Acla: aclarubicin; # $P < 0.05$ compared to A group; ▲ $P < 0.05$ compared to D group.

an HRP-conjugated secondary antibody. The plate was washed three times at each step, and 100 μl of standard developing solution (TMB) was added to each well. The absorbance results were read using a spectrophotometer (Biotek-Elx800) at 450 nm with a reference wavelength of 650 nm. Both positive control wells and blank wells were set for this assay. Specificity of binding was determined by prior addition of a 20-fold excess of unlabeled competitor consensus oligonucleotide.

SDS PAGE and Western blot analysis

Cell lysates were prepared for Western blot analysis of Cox-2, p-NF-kB, p65 (phospho-p65, Ser529), cleaved-caspase 3, and cleaved-Caspase 9 using protein and whole cellular protein extraction kits repectively (Active Motif Inc. Lake Placid, New York, USA). The protein concentration in each cell lysate was determined by BCA-protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA; Sigma) as the standard. An equal amount of protein (40 μg) from each sample was loaded on a 10% SDS polyacrylamide gel for electrophoresis prior to transfer to a 0.45 μm nitrocellulose membrane (Millipore, Billerica, MA, USA). Nonspecific binding was blocked with 5% nonfat dry milk in TBS (25 mM Tris-HCl, 150 mM sodium chloride, pH 7.2) for 1 h at room temperature. The membranes were incubated with mouse-anti-Cox-2, rabbit-anti-p-NF-κB p65, mouse-anti-cleaved-Caspase 3, cleaved-Caspase 9, or rabbit-anti-β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1,000 dilution in TBST-5% milk at 4°C overnight. The membranes were washed three times with TBST and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz) at 1:2000 in TBST-5% milk) for 2 h at room temperature. Bound antibodies were detected using enhanced chemiluminescence (ECL) reagents (Super Signal Dura kit, Pierce, IL, USA) according to the manufacturer's instructions. Blots were scanned and signal intensities were calculated relative to β-actin.

Statistical analysis

All data were derived from at least three independent experiments. Statistical analyses were performed with Sigma Plot software; values are presented as the mean ± the standard deviation (SD). Significant differences between the groups were determined using the unpaired Student's t-test. A p value of less than 0.05 was considered statistically significant.

RESULTS

To quantify precisely Acla-induced apoptosis in HL-60 cells, apoptotic cells were detected by Annexin V-FITC. Table 2 and Figure 1 show a slight increase in the percentage of apoptotic HL-60 cells following treatment with Acla from 0.05 to 0.1 μg/ml (3.09 ± 0.29%) compared with blank control group(1.88 ± 0.20%, $P > 0.05$). To determine the effect of SN on Acla-induced apoptosis, cells were treated with 0.1 μg/ml, the dose that did not induce significant apoptosis. While treatment with 20 ng/ml SN alone induce slight apoptosis (8.77 ± 0.36%), co-treatment with SN at 5, 10, or 20 ng/ml significantly increased Acla-induced apoptosis.

To evaluate the effect of Acla with or without SN on the viability of HL-60 cells. The cytotoxic effects were evaluated 20 h by the MTS assay after the treatment of cells to determine the responsiveness of SN combined with Acla, 0.1μg/ml Acla was chosen for its little effect on viability as shown previously. Figure 2 shows coaddition of SN (5 to 20 ng/ml) and Acla (0.1 μg/ml) result in significant reduction of viability whereas. Acla alone induced only a slight decrease even at 0.25 μg/ml.

Next, we examined the effect of SN on Acla-induced PGE2 production. A low Acla dose was chosen for its minimal effect on PGE2 production as previously shown. Acla (0.1 μg/ml) alone slightly decreased PGE2 production, even at 0.25 μg/ml, but the difference was not significant. Table 2 shows that cotreatment with SN (5, 10, or 20 ng/ml) and Acla (0.1 μg/ml) significantly reduces PGE2 generation, while either Acla (0.25 μg/ml) or SN (20 ng/mll) alone induced only a slight decrease (Table 2).

Once we confirmed that SN sensitizes leukemia cells to Acla-induced apoptosis, we investigated the potential mechanism. First, we examined Caspases 3 and 9 protein expression. Figure 3 shows that treatment with Acla alone (0 to 0.1 μg/ml), slightly increased Caspases 3 and 9 expression in HL-60 cells. With either 0.25 μg/ml Acla or 20 ng/mll SN alone, Caspases 3 and 9 expression increased significantly. Cotreatment with SN (5, 10,

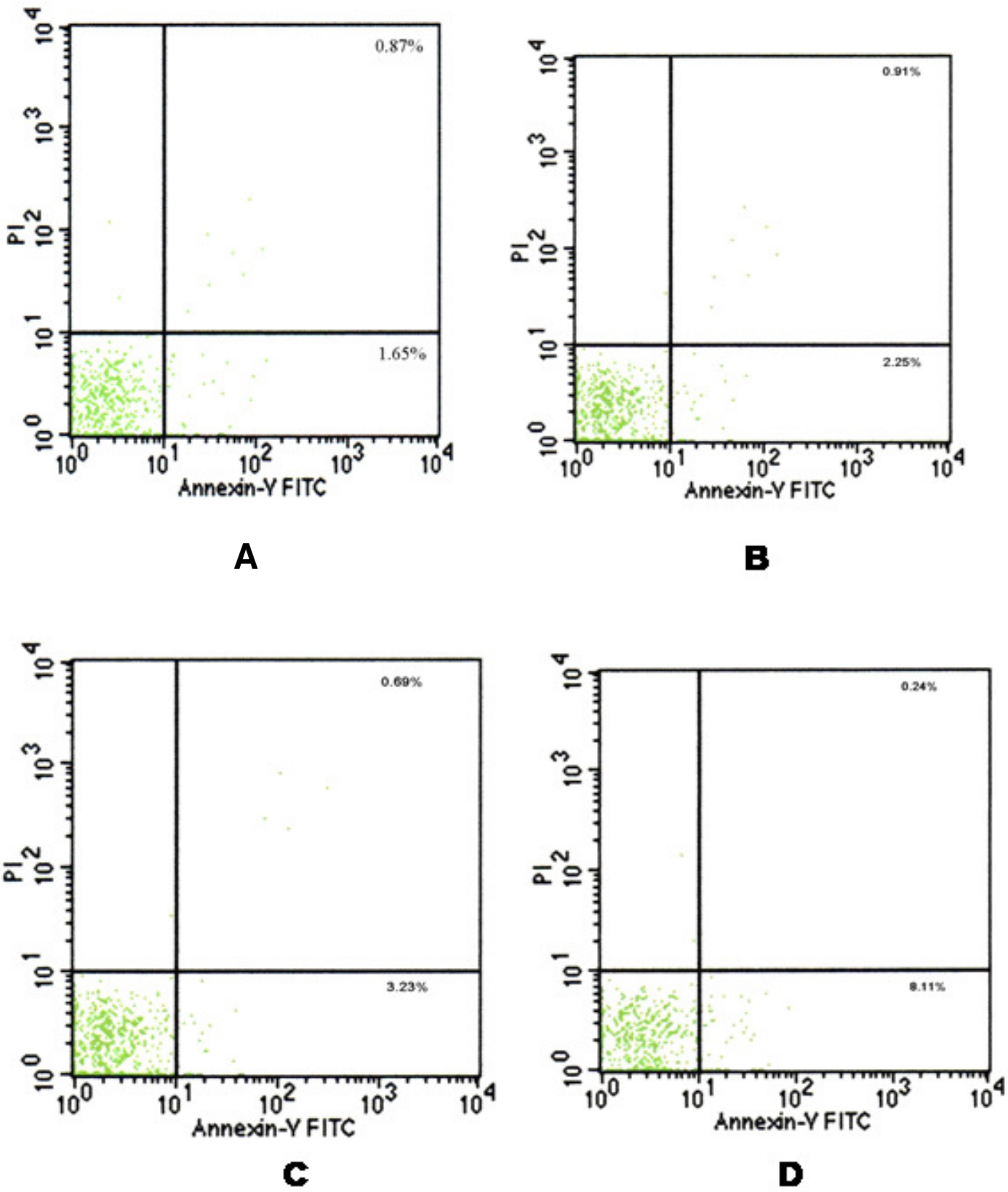


Figure 1. Sinomenine combined with Acla increased apoptotic HL-60 cells detected by flow cytometry. Apoptotic cells were detected by Annexin V and PI double staining. In the present of sinomenine and Acla, apoptosis induction increased significantly in a dose-dependent manner. A, control; B, 0.05 μg/ml 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.

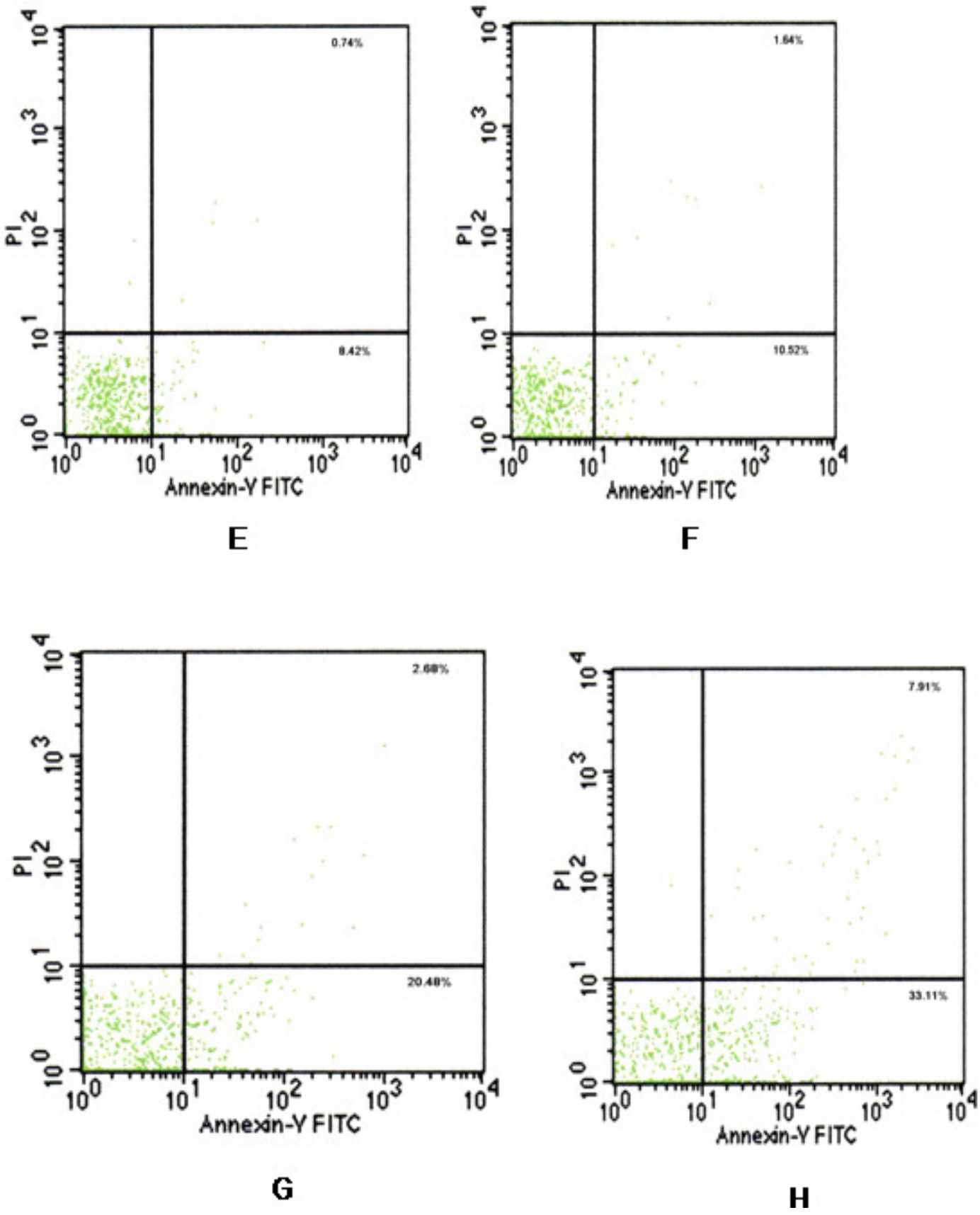
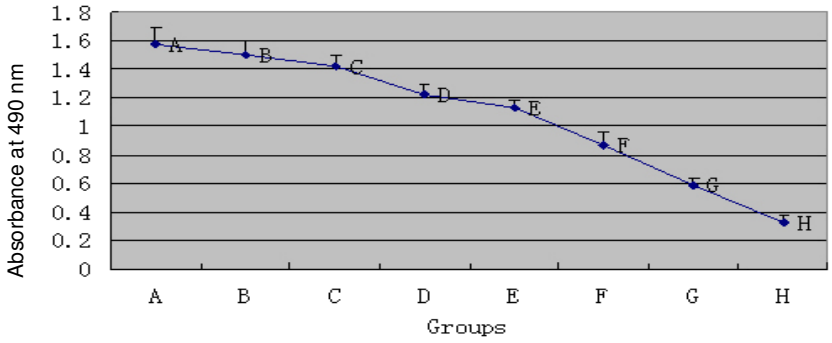
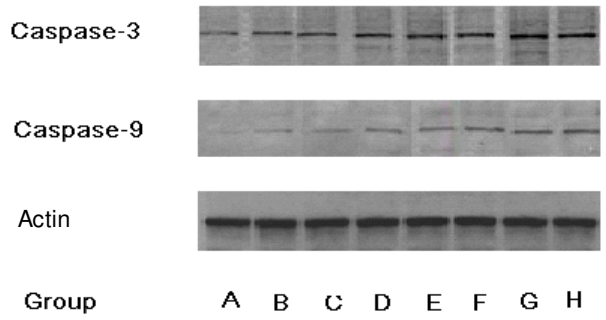


Figure 1. Continued.

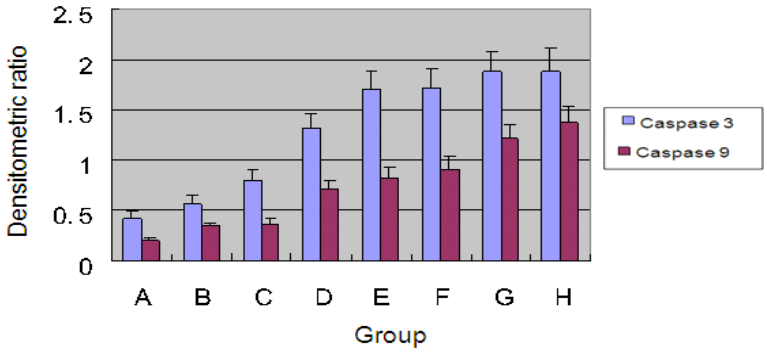


Acla(μg/ml)	0	0.05	0.1	0.25	0	0.1	0.1	0.1
SN(ng/ml)	0	0	0	0	20	5	10	20

Figure 2. The effect of Acla combined with SN on the viability of HL-60 cells.Treatment of cells with concentrations of Acla ranging from 0 μg/ml to 0.25μg/ml. No cytotoxicity was evident at concentrations below 0.1μg/ml, However, there was a dose-dependent significantly increase with SN from 5 ng/ml to 20 ng/ml combined with Acla at 0.1 μg/ml.



3A



Acla (μg/ml)	0	0.05	0.1	0.25	0	0.1	0.1	0.1
SN (ng/ml)	0	0	0	0	20	5	10	20

3B

Figure 3. Sinomenine combined with Acla increases cleaved Caspase-3 and cleaved Caspase-9 protein expression in HL-60 cells were detected by Western-blot. (A) Sinomenine combined with Acla significantly increased Caspase-3 and Caspase-9 protein expression in HL-60 cells, in a dose-dependent manner. Densitometric analysis (B) was performed as described in SDS PAGE and Western blot analysis.

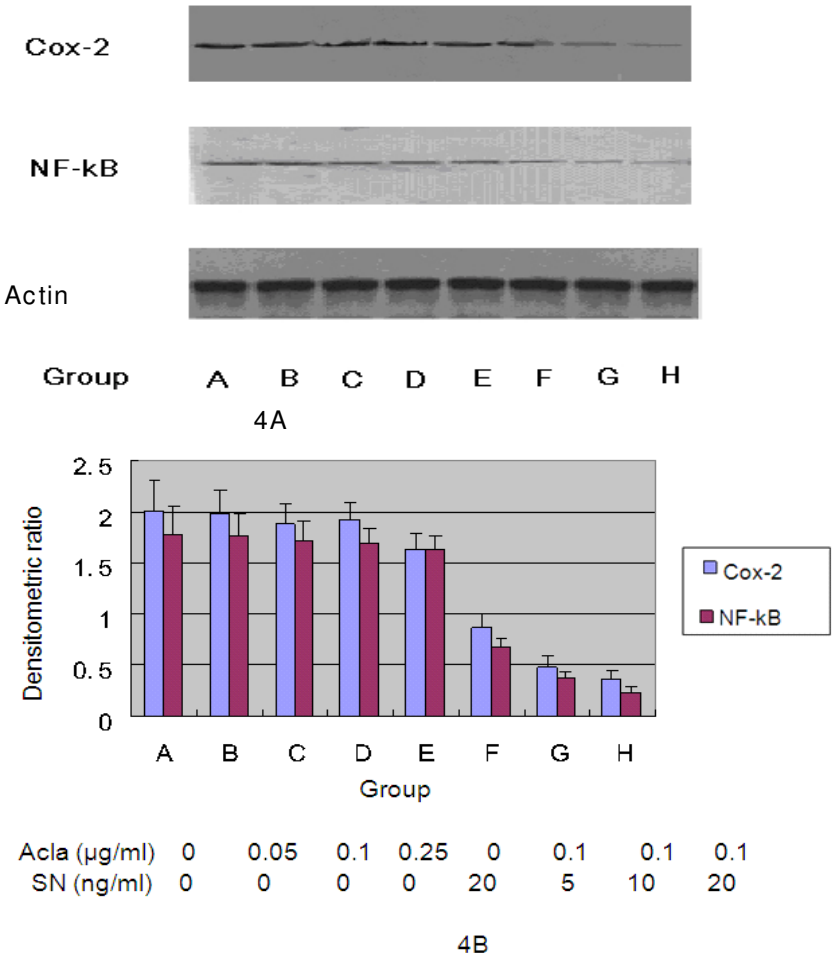


Figure 4. Sinomenine combined with Acla inhibits Cox-2 and NF-κB protein expression in HL-60 cells. The expression of Cox-2 and NF-κB (p65) in HL-60 cells was detected by Western-blot (A). Sinomenine combined with Acla significantly decreased Cox-2 and NF-κB protein expression in HL-60 cells, an effect which correlated positively with SN concentration. Densitometric analysis (B) was performed as described in SDS PAGE and Western blot analysis.

or 20 ng/ml) and Acla (0.1 μg/ml) significantly enhanced Caspases3 and 9 protein expression.

As expected, changes in Cox-2 and NF-kB protein expression in HL-60 cells paralleled the changes in PGE2 production. Treatment with different concentration of SN in the presence of 0.1 μg/ml, Acla abrogated Cox-2 and NF-kB in HL-60 cells; the inhibition positively correlated with the dose of SN (Figure 4).

The promoter region of COX-2 contains an NF-kB element that is known to be an induction target of COX-2. To examine the molecular mechanism of SN and/or Acla on COX-2 expression, NF-kB p65/ p50 transcription factor assay kits which based on ELISA (non-radioactive) detecting oligonucleotide probe were used to measure NF-kB activation. As shown in Figure 5, NF-kB activation was consistence with its corresponding COX-2 expression. NF-kB activities were significantly reduced by combined SN with Acla, which disappeared when high dose of SN in combination with Acla was compared with SN or Acla treatment alone.

DISCUSSION

Sinomenine extracted from the Chinese medical plant, has been used successfully to treat tumor combned with chemotherapy (Lu et al., 2010). The present study demonstrated that SN induced HL-60 cell apoptosis in a dose-dependent manner *in vitro*. Acla, a common treatment for leukemia patients, has unfortunate side effects at the therapeutic dose, such as cardiac and hematopoietic system toxicity. Therefore, supplementing Acla (used at a lower dose) could avoid serious compli-cations (Dartsch et al., 2002). To test this hypothesis, low

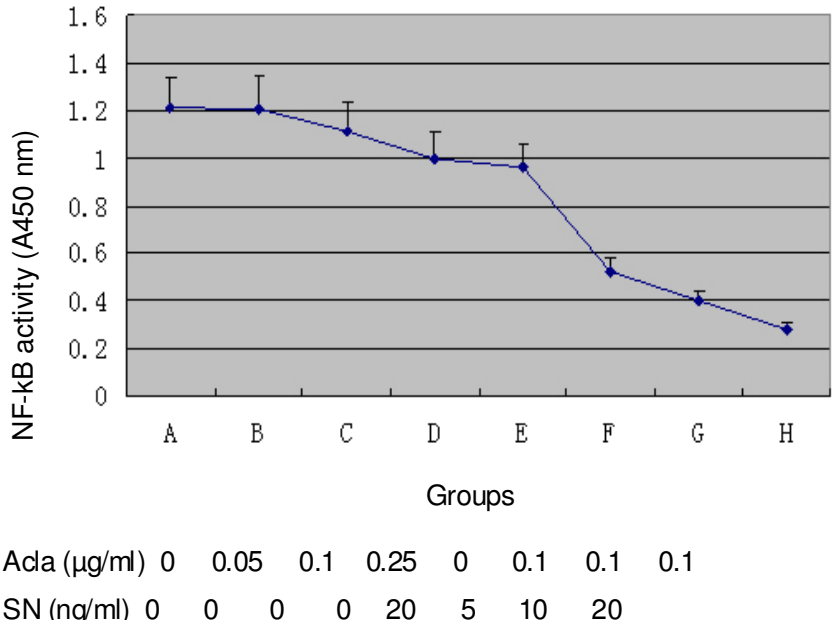


Figure 5. Sinomenine combined with Acla inhibits the NF-κB activation. NF-κB p65 transcription factor assay kits which based on ELISA (non-radioactive) detecting oligonucleotide probe were used to measure NF-κB activation. NF-κB activities were significantly reduced by combined SN with Acla, which disappeared when high dose of SN combining with Acla compared with SN or Acla treating alone.

concentrations of Acla were combined with SN to treat leukemia cells. Interestingly, low SN concentrations combined with Acla induced more apoptosis than either Acla or SN alone. Treatment of leukemia cells with Acla (0 - 0.1 μg/ml) alone increased Caspases 3 and 9 protein expression slightly. Treatment with SN (5, 10, or 20 ng/ml) and 0.1 μg/ml Acla, significantly increased Caspases 3 and 9 expression in a dose-dependent manner. While 0.1 μg/ml Acla or 20 ng/ml SN alone did not increased apoptosis, it was not correlated with apoptosis. This was a similar results reported by Mathieu et al. (2006). Thus, our findings demonstrate that both Caspases 9 and 3 are involved in apoptosis induced by SN combined with Acla in HL-60 cells.

Recently, it was reported that NF-kB, which up-regulates the transcription of proteins that promote cell survival, stimulate growth, and reduce susceptibility to apoptosis, is constitutively activated in the majority of primary leukemia specimens (Ougolkov et al., 2007; Mathieu et al., 2006). NF-kB can promote cell survival; reducing its activity leads to apoptosis (Lee et al., 2010). NF-kB activation also induces drug resistance in leukemia cells (Qin et al., 2007). Thus, strategies to inhibit NF-kB, may represent a useful approach to more tolerable leukemia therapy (Yuan, 2010). Several reports have shown that NF-kB inhibitors act as potent enhancers of chemotherapy-induced apoptosis. SN inhibits NF-kB activity (He et al., 2005; Ma et al., 2007). In this study, SN induced apoptosis in a dose-dependent manner when combined with low concentration of Acla.

Furthermore, a number of previous experiment support that PGE2 can stimulate tumor growth and metastasis by promoting migratory, invasive, and angiogenic abilities of tumor cells, which may also be triggered by activation of Cox-2 (Johnson et al., 2004; Lala and Chakraborty, 2001; Brandao et al., 2001). Up-regulation of PGE2 has been reported in a variety of different malignancies, including colorectal cancer and leukemia (Banerjee et al., 2002; Ouyang et al., 2007). As we know that the expression of Cox-2 and its production depend on NF-kB activation (Liu et al., 2009).

The lower Acla concentration did not affect NF-kB activity, Cox-2expression, or PGE2 production. In addition, SN alone, even at the highest dose tested, had no significant effect on NF-kB activation, Cox-2 expression, and PGE2 production in leukemia cells. However, when combined with Acla, SN suppressed NF-kB activity in a dose-dependent manner. These results suggest that SN enhances the sensitivity of leukemia cells to apoptosis combined with a low dose of Acla, thereby facilitating the anti-leukemia effect of Acla.

Conclusion

Cotreatment of HL-60 cells with SN and Acla results in significant apoptosis, with caspase 3 and 9 expression correlated with Cox-2 expression, PGE2 generation and

NF-kB activation. These results suggest that SN is a potential agent for anti-leukemia therapy when combined with chemotherapy.

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