

*Full Length Research Paper*

# Sinomenine induces apoptosis of prostate cancer cells by blocking activation of NF-kappa B

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**Sinomenine (SN) is an alkaloid that has previously been shown to have a role in such varied processes as inflammation, angiogenesis, arthritis and immunosuppression. Therefore, in this study, possible anti-tumor effects of SN on the human prostate cancer cell lines, PC-3 and DU-145, was investigated. After treatment with varying doses of SN, apoptosis was measured by flow cytometry. Prostaglandin E2 production was detected by ELISA, and expression levels of cyclooxygenase-2 and nuclear factor-kappa B were evaluated by western blot. In both PC-3 and DU-145 cells, SN treatment induced apoptosis, inhibited *in vitro* production of prostaglandin E2, and decreased activation of cyclooxygenase-2 and nuclear factor-kappa B. Growth inhibition assays were also performed to determine whether cell viability was responsible for the decrease in the activation observed. In combination, these results suggest that SN can induce an anti-tumor response by suppressing the activation of cyclooxygenase-2 and nuclear factor-kappa B signaling pathways.**

**Key words:** Sinomenine, apoptosis, cyclooxygenase 2, nuclear factor-kappa B, prostaglandin E2, prostate cancer.

## INTRODUCTION

Signaling involved in the process of inflammation includes over expression of cyclooxygenase-2 (Cox-2), nuclear factor-kappa B (NF-κB) and prostaglandin E2 (PGE2). Over expression of these molecules has also been associated with several types of human cancers such as colon, breast cancer, prostate cancer and leukemia, and appears to control many cellular processes (Fernández-Martínez et al., 2007; Liu et al., 2002). Based on their roles in carcinogenesis, apoptosis and angiogenesis, Cox-2, NF-κB and PGE2 have the potential to be valuable targets in the development of new drugs to selectively inhibit human cancers (Adhami et al., 2007; Park et al., 2007).

Sinomenine (SN) is an immunosuppressive compound derived from the Chinese medicinal plant, *Sinomenium acutum*, and has been successfully applied to the treatment of various autoimmune diseases (Park et al., 2007). Previous studies have also indicated that SN can inhibit lymphocyte proliferation, production of pro-inflammatory factors by macrophage, and NF-κB activity (Wang et al., 2005; Li et al., 2006; Shu et al., 2007). Although, *in vitro* models of leukemia treated with anti-inflammatory drugs have been shown to induce apoptosis (Subhashini et al., 2005) little is known about the mechanism by which SN may induce the apoptosis of tumor cells. In this study, two types of human prostate cells, PC-3 and DU-145, were treated with SN and the effects on Cox-2 and NF-κB were evaluated. The results suggest that this Chinese medicinal plant represents a potential therapeutic agent for prostate cancer.

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## MATERIALS AND METHODS

### Sample

Human prostate cancer cell lines, PC-3 and DU-145, were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. SN [7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methyl-morphinan-6-one, (C<sub>19</sub>H<sub>23</sub>NO<sub>4</sub>)] was purchased from Sigma (St. Louis, MO, USA) with a purity of  $\geq 98.5\%$  and was dissolved in dimethylsulfoxide (DMSO, Invitrogen, Carlsbad, CA, USA) at 100  $\mu\text{g}/\text{mL}$  and stored at  $-30^\circ\text{C}$ . For the experiments, stock solutions of SN were diluted in Dulbecco's modified essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) to achieve final concentrations of 2, 5, 10, 20, 50, and 100  $\text{ng}/\text{mL}$ .

### Cell culture and treatment

PC-3 and DU-145 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Utah, USA) at  $37^\circ\text{C}$  in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were treated with various concentrations of SN for 20 h before supernatants were collected and stored at  $-20^\circ\text{C}$ .

### Flow cytometry analysis

Cells were harvested using trypsin/collagenase I, washed with ice-cold PBS, then double-stained with FITC-annexin V and propidium iodide (PI) (FacsCalibur; BD Biosciences, San Jose, CA, USA) for 20 min in the dark at RT. Flow cytometry was performed using a FacsCalibur (BD Biosciences, CA, USA).

### Assay for PGE2 production

The levels of PGE2 in the culture supernatants were detected 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  $\text{pg}/\text{mL}$ .

### Western blot analysis

Cell lysates were collected using whole cellular protein extraction kits (Active Motif, CA, USA), and the concentration of each cell lysate was determined using a BCA-protein assay kit (Pierce, IL, USA). Bovine serum albumin (BSA; Sigma, MO, USA) was used as the standard. The samples of total protein (40  $\mu\text{g}$ ) were separated by 10% SDS-PAGE, was transferred to nitrocellulose membranes (0.45  $\mu\text{m}$ , Millipore, MA, USA), and blocked in 5% BSA in TBS (25 mM Tris-HCl, 150 mM sodium chloride, pH 7.2) for 1 h at RT. Blots were then incubated with rabbit polyclonal IgG primary antibodies (1: 500) specific for Cox-2, phospho-NF- $\kappa\text{B}$  p65 (pp65), or  $\beta$ -actin (Santa Cruz, CA, USA) and incubated at  $37^\circ\text{C}$  for 2 h. Blots were washed three times with PBS and then was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (diluted 1:2000) for 2 h at RT. 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  $\beta$ -actin.

### [<sup>3</sup>H]-Thymidine incorporation assays

PC-3 and DU-145 cells ( $1 \times 10^4/\text{well}$ ) in RPMI-1640 supplemented with 10% FBS were plated in 96-well plates. After 54 h, [<sup>3</sup>H]-

Thymidine (1  $\mu\text{Ci}/\text{well}$ ) (Easton Turnpike Fairfield, USA) was added to each well and cells were incubated for an additional 18 h. Plates were then washed three times with PBS and cells were fixed in 5% trichloroacetic acid at  $4^\circ\text{C}$  for 20 min. After three washes with trichloroacetic acid, radioactivity was measured using liquid scintillation counting.

### Statistical analysis

Experiments were performed in triplicate and the results were expressed as the means  $\pm$  SD. Statistical analyses were performed using the ANOVA analysis of variance test for continuous variables (SPSS 13.0 software), and differences were considered statistically significant when P values were  $\leq 0.05$ .

## RESULTS

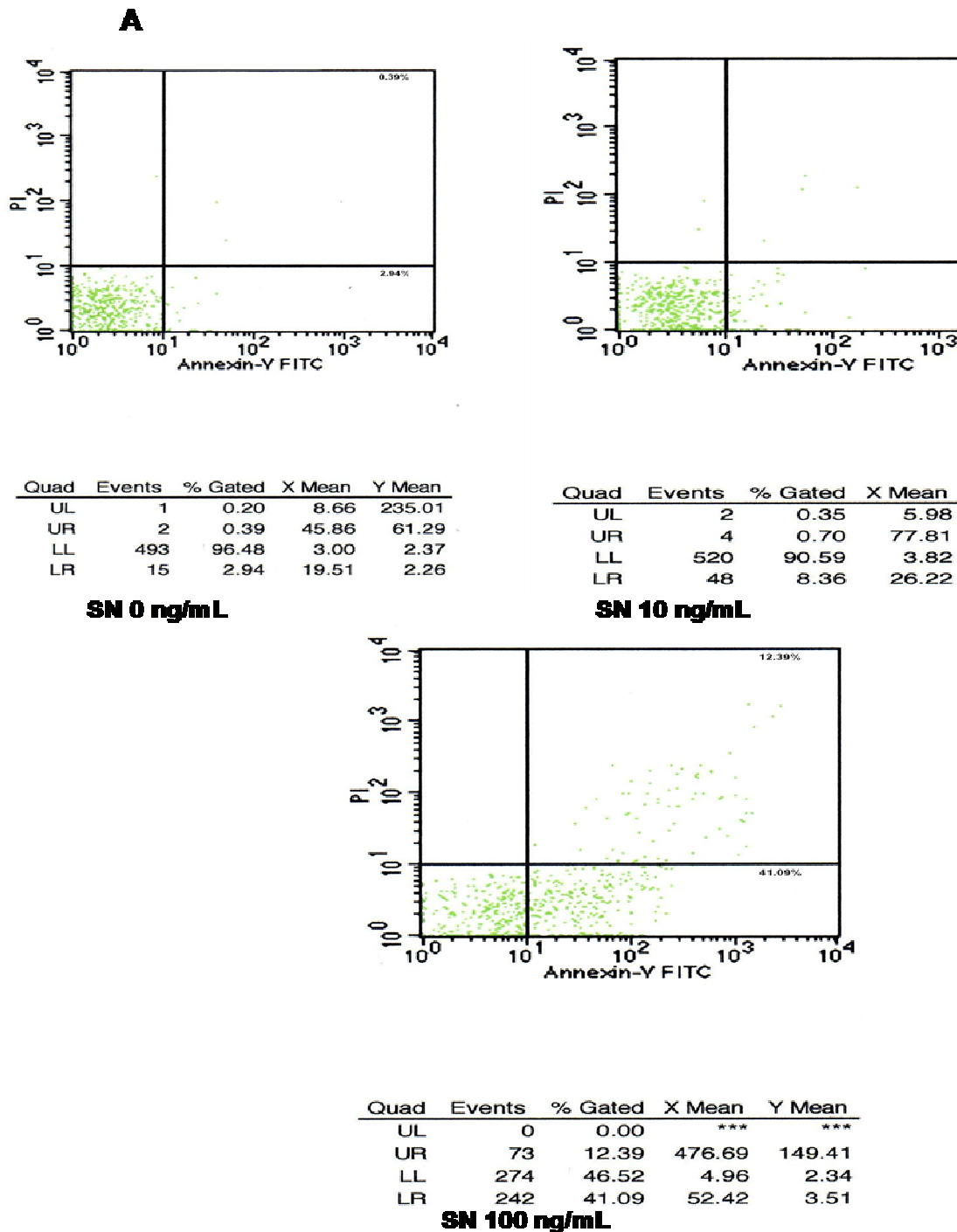
To determine whether SN induced apoptosis in PC-3 and DU-145 cells, cells were treated with varying concentrations of SN (2, 5, 10, 20, 50, and 100  $\text{ng}/\text{mL}$ ), and the percentage of apoptotic cells were detected. The toxicity of IC<sub>50</sub> at about 40  $\text{ng}/\text{mL}$  was tested. Cells that were only stained with annexin V, and cells that were stained with both PI and annexin V, were considered apoptotic. In contrast, cells that were only stained with PI were considered necrotic. As shown in Figures 1 and 2, a significant increase in the percentage of apoptotic PC-3 cells and DU-145 cells treated with 100  $\text{ng}/\text{mL}$  SN was observed ( $53.14 \pm 2.89\%$  and  $4.88 \pm 0.14\%$ , respectively;  $P < 0.01$ ), and were compared with cells treated with 5  $\text{ng}/\text{mL}$  SN ( $50.38 \pm 4.28\%$  and  $5.48 \pm 0.61\%$ , respectively;  $P < 0.05$ ).

For PGE2 production, a dose-dependent increase was detected in both PC-3 and DU-145 cells treated with varying concentrations of SN (2-100  $\text{ng}/\text{mL}$ ) ( $r^2 = 0.712$ ,  $P < 0.01$ ,  $r^2 = 0.784$ ,  $P < 0.01$ , respectively), with a greater response detected at concentrations  $> 10 \text{ ng}/\text{mL}$ .

Cox-2 expression in the PC-3 and DU-145 cell lines following different treatments were also found parallel to the trends in PGE2 levels detected in cell supernatants (Figures 3 and 4), and the inhibition effects positively correlated with the concentrations of SN administered. Significant inhibition of NF- $\kappa\text{B}$  was detected when the concentration of SN administered was  $> 10 \text{ ng}/\text{mL}$ .

The promoter region of Cox-2 contains an NF- $\kappa\text{B}$  element that is known to be an activator of Cox-2 transcription. Therefore, to determine whether SN effects Cox-2 expression, NF $\kappa\text{B}$  pp65 was measured. As shown in Figures 3 and 4, NF- $\kappa\text{B}$  pp65 activation correlated with Cox-2 expression and activation of NF- $\kappa\text{B}$  pp65 was observed to significantly decrease following treatment with SN. The down-regulation observed also positively correlated with the concentration of SN administered.

To investigate whether the decrease in expression of Cox-2, NF- $\kappa\text{B}$  pp65 and PGE2 was based on cell viability, [<sup>3</sup>H] Thymidine incorporation assays were performed. IC<sub>50</sub> was detected in PC-3 cells and DU-145 cells treated with SN at about 40  $\text{ng}/\text{mL}$ .



**Figure 1.** Flow cytometry showing the increased apoptotic PC-3 and DU-145 cells after treatment with sinomenine. A, PC-3 cells; 1B, DU-145 cells. The apoptotic cells were detected by Annexin V and Propidium iodide double staining methods. In the present of different doses of sinomenine, the induction of apoptosis was significantly increased in a dose-dependant manner.

**DISCUSSION**

Sinomenine (SN) is a compound extracted from the Chinese medical plant, *Sinomenium acutum*, which has been used for centuries in China to successfully treat

rheumatoid arthritis with minimal side effects (Wang et al., 2005; Li et al., 2006; Shu et al., 2007; Nishida and Satoh, 2006). SN has also been shown to have immunomodulatory properties in a cardiac allograft transplantation model (Tang et al., 2006), to protect mice from

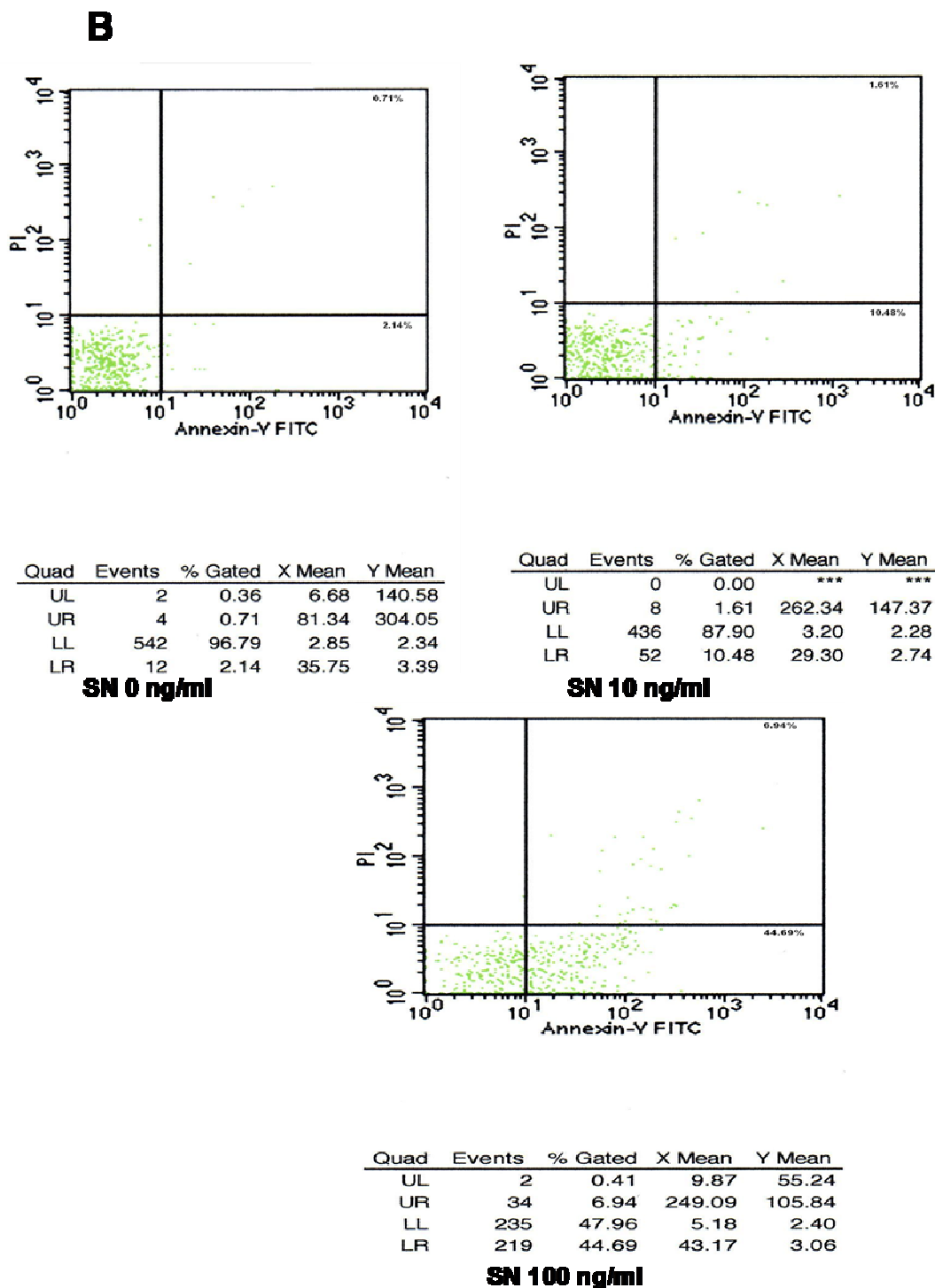
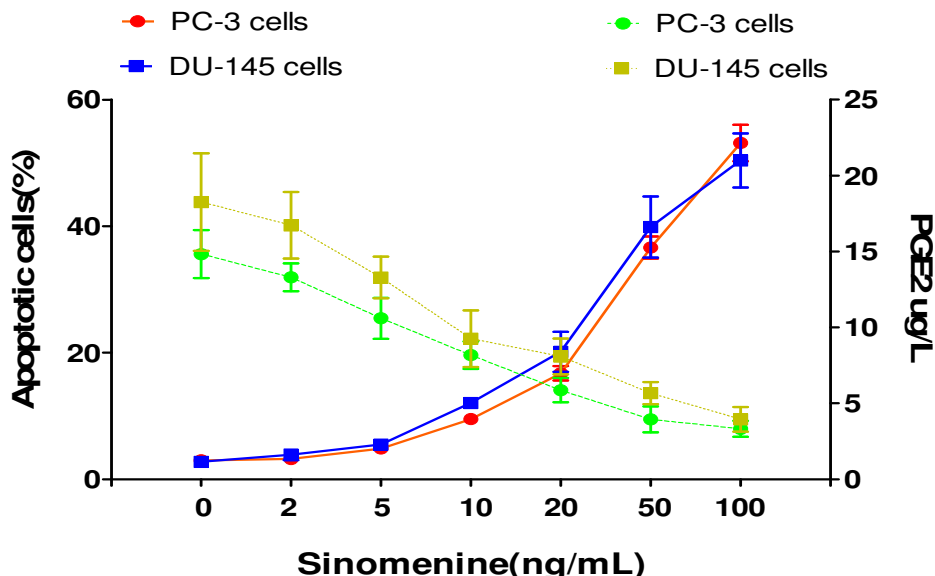


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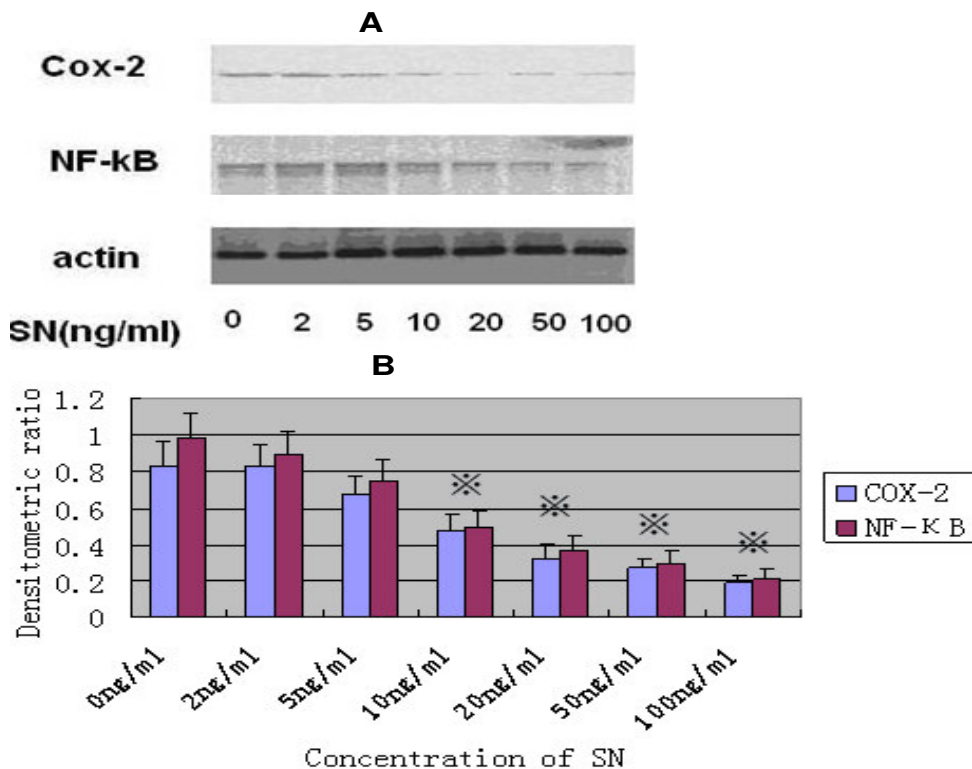
endotoxin-induced fulminate hepatitis, and exert synergistic effects when combined with sub-optimal doses of Cyclosporine A (CsA) to inhibit immune responses and prolong cardiac allograft survival in rats (Zeng et al., 2007; Feng et al., 2006; Zhao et al., 2007). Therefore, SN appears to be a promising immune-

suppressive drug, and its mechanism of action have been hypothesized to include regulation of the inflammatory pathway.

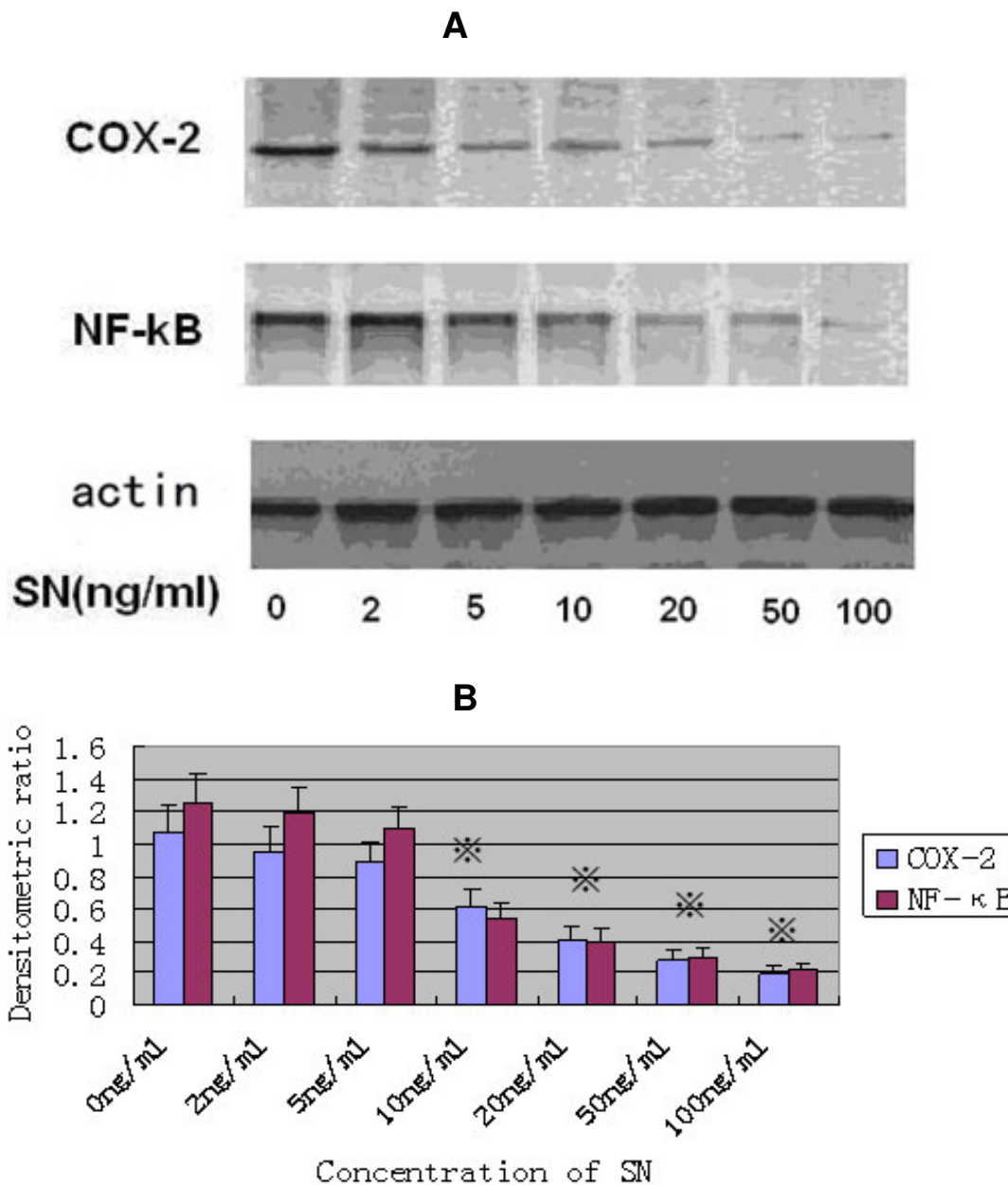
Previously, inflammation has been shown to affect the angiogenesis and invasion of certain types of tumors (Peters et al., 2009; Mantovani et al., 2010). Cox-2 is a



**Figure 2.** Dose-dependent SN induced apoptosis and PGE2 reduction in PC-3 and DU-145 cells. The red and blue line indicate the SN induced apoptotic cells (%) plotted on the left y-axis in PC-3 and DU-145 cells, respectively. The green and yellow broken line indicate the SN induced PGE2 reduction plotted on right y-axis in PC-3 and DU-145 cells, respectively.



**Figure 3.** Sinomenine inhibition of the expression of Cox-2 and NF- $\kappa$ B at the protein level in PC-3 cells. A, the expression of Cox-2 and NF- $\kappa$ B in PC-3 cells was detected by Western-blot. 40  $\mu$ g of protein was loaded per lane. Sinomenine decreased significantly the protein expression of Cox-2 and NF-kappa B in PC-3 cells, and the inhibition effect was positively correlated with the concentrations of sinomenine; B, densitometric analysis was performed by software program Kodak digital science, the net intensity of each band was compared to that one of the housekeeping gene  $\beta$ -actin and their ratio are reported. Bars show the mean  $\pm$  SD. Data represent mean  $\pm$  SEM (n=3); p < 0.05 vs 0 ng/ml group.



**Figure 4.** Sinomenine inhibition of the expression of Cox-2 and NF- $\kappa$ B at the protein level in DU-145 cells. A, Effect of SN on the production of the Cox-2 and NF- $\kappa$ B in DU-145 cells. The harvest cells were subjected to western blot analysis for Cox-2 and NF- $\kappa$ B. 40  $\mu$ g of protein was loaded per lane; B, densitometric analysis was performed by software program Kodak digital science. The net intensity of each band was compared to that one of the housekeeping gene  $\beta$ -actin and their ratios are reported. Bars show the mean  $\pm$  SD. Data represent mean  $\pm$  SEM (n = 3); p < 0.05 vs 0 ng/ml group.

pro-inflammatory mediator that can suppress apoptosis, while inhibition of both Cox-2 and NF- $\kappa$ B has shown great potential as an anti-inflammation treatment for cancer therapy (Ghosh et al., 2007; Han et al., 2005). Over-expression of Cox-2 and NF- $\kappa$ B has been associated with several types of human cancers such as colon, breast cancer, prostate cancer and leukemia (Fernández-Martínez et al., 2007; Liu et al., 2002), while over

expression of PGE<sub>2</sub> has been reported in a variety of malignancies, including colorectal cancer and lung cancer (Banerjee et al., 2002; Ouyang et al., 2007). PGE<sub>2</sub> 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 (Ohshima et al., 2005; Johnson et al., 2004; Lala and Chakraborty, 2001). Furthermore,

previous studies have demonstrated that expression of Cox-2 may be regulated by NF- $\kappa$ B (Liu et al., 2009; Lee et al., 2010), 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 (Min et al., 2006; Vykhoanets et al., 2008; Christensen et al., 2007).

SN has previously been shown to inhibit NF- $\kappa$ B activity and reverse multidrug resistance (MDR) (Ma et al., 2007; He et al., 2005). Similarly, in a recent study of fermented ginseng, another compound of Chinese medicine associated with anti-inflammatory effects, inhibition of NF- $\kappa$ B activity was detected (Yuan and Chung, 2010). Anti-tumor agents have also been shown to induce apoptosis by modulating NF- $\kappa$ B expression and activity (Pandey et al., 2007). Thus, strategies to inhibit NF- $\kappa$ B may provide a cancer therapy applicable to many types of carcinomas (Zhang et al., 2007; Zheng et al., 2007; Paule et al., 2007; Nonn et al., 2007). In this study, SN suppressed NF- $\kappa$ B activity and PGE2 production in a dose-dependent manner. While concentrations of SN  $\leq$  5 ng/mL did not affect NF- $\kappa$ B activation or the expression of Cox-2 and PGE2, significant inhibition of NF- $\kappa$ B was detected when the concentration of SN administered was  $>$  10 ng/mL. Furthermore, treatment with SN resulted in a decrease in expression of Cox-2, which also correlated with the extent of apoptosis detected. Based on these results, it can be hypothesized that SN can enhance the sensitivity of prostate cancers to anti-cancer drugs.

## Conclusion

In conclusion, SN had a significant apoptotic effect on PC-3 and DU-145 human prostate cancer cell lines, which was dose-dependent. It can be hypothesized that this inhibitory effect was mediated by the suppression of different inflammatory factors including NF- $\kappa$ B, Cox-2 and PGE2. While further study is needed, these results provide valuable insight into the mechanisms and potential targets for the treatment of patients with prostate cancer.

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