

Sanguinarine inhibits growth of human cervical cancer cells through the induction of apoptosis

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Received May 29, 2012; Accepted July 25, 2012

DOI: 10.3892/or.2012.2024

Abstract. Sanguinarine, a natural benzophenanthridine alkaloid, has been shown to possess anticancer activity *in vitro* and *in vivo*. In the present study, we demonstrated that sanguinarine caused a dose-dependent inhibition of growth in HeLa and SiHa human cervical cancer cells, i.e., 2.43 $\mu\text{mol/l}$ (IC_{50}) in HeLa cells and 3.07 $\mu\text{mol/l}$ in SiHa cells. Cell cycle analysis revealed that sanguinarine significantly increased the sub-G1 population, from 1.7 to 59.7% in HeLa cells and from 1.7 to 41.7% in SiHa cells. Sanguinarine caused a dose-dependent decrease in Bcl-2 and NF- κB protein expression and a significant increase in Bax protein expression. Our findings indicate that sanguinarine as an effective anticancer drug candidate inhibits the growth of cervical cancer cells through the induction of apoptosis.

Introduction

Despite the advances in combinatorial chemistry, 60% of the agents used for cancer treatment are substances of natural origin or derivatives (1). Sanguinarine [13-methyl(1,3) benzodioxolo(5,6-c)-1,3-dioxolo(4,5) phenanthridinium] (Fig. 1), derived from the root of *sanguinaria canadensis* and other poppy-fumaria species, is the most widely-used benzophenanthridine alkaloid. It has been used in many over-the-counter products such as toothpaste, mouthwash, cough and cold remedies due to its antimicrobial, antioxidant and anti-inflammatory properties (2,3). The cytotoxic and cytostatic effects of sanguinarine on a variety of human cancer cells, including human epidermoid carcinoma, erythroleukemia, prostate cancer, pancreatic carcinoma, colon cancer, breast cancer, lung cancer, promyelocytic leukemia and bone

cancer (4-13), have been reported. Sanguinarine exhibits the highest cytotoxicity among benzophenanthridine alkaloids (14). Markedly, sanguinarine results in antiproliferative effects on human epidermoid carcinoma cells (A431), but not in the normal human epidermal keratinocytes (4). This different antiproliferative response is also observed in a study where mouse lymphocytic leukemic cells were significantly more sensitive to sanguinarine compared with normal spleen cells (15). Thus, sanguinarine has gained increasing attention as a potential agent in the treatment of cancer.

Cervical cancer is the second most common cancer in women worldwide, and its incidence is disproportionately high (>80%) in the developing countries (16). The major cause of mortality from this disease is metastatic cancer cells that fail to respond to chemoradiation therapy (17). Since sanguinarine possesses the potential ability for cancer treatment, the availability of sanguinarine in advanced, recurrent, metastatic cervical cancer merits further research. To the best of our knowledge, the effect of sanguinarine on cervical carcinoma has yet to be studied extensively, despite discovering that sanguinarine caused apoptosis in HeLa cells (18,19) when compared with other benzophenanthridine alkaloids. Thus, a systemic study is required to elucidate the mechanism by which sanguinarine inhibits cell growth in some types of cervical cancer cells.

Sanguinarine-induced apoptosis, which is the process of programmed cell death, is the key issue in cancer cells mentioned above. The Bax and Bcl-2 proteins belong to the Bcl-2 family, which is the best-characterized group of apoptosis-regulating factors. The Bcl-2 family can be divided into two main groups according to their functional properties: the anti-apoptotic proteins such as Bcl-2 and Bcl-XL, and the pro-apoptotic proteins, such as Bax, Bak and Bad. Sanguinarine decreased anti-apoptotic Bcl-2 and increased the pro-apoptotic Bax protein in a dose-dependent pattern in K562 erythroleukemia cells (5,6), human pancreatic carcinoma AsPC-1 and BxPC-3 cells (8), and immortalized human HaCaT keratinocytes (20).

The transcription factor NF- κB is retained normally in an inactive form in the cytoplasm through interaction with I κB (inhibitor κB) family proteins and can be activated by various inflammatory and stress stimuli (21). NF- κB activation requires nuclear translocation. Active nuclear form of the NF- κB transcription factor complex is composed of two DNA

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Key words: sanguinarine, cervical cancer, apoptosis, Bcl-2, Bax, NF- κB

binding subunits, NF- κ B p65 and p50. Studies have indicated that NF- κ B promotes cell survival by inhibiting apoptosis and NF- κ B has been recognized as an anti-apoptotic regulator. The decreased expression of NF- κ B by sanguinarine has been observed in K562 erythroleukemia cells (5) and human myeloid ML-1a cells (21).

During the last decade, the incidence of cervical adenocarcinoma has increased, particularly in young women (22). In the present study, the antiproliferative and apoptosis-inducing effects of sanguinarine were investigated in cervical adenocarcinoma HeLa and cervical squamous cell carcinoma SiHa cells.

Materials and methods

Reagents and antibodies. Sanguinarine (>99.0% pure), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), protein lysis buffer, RNase A and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sanguinarine was dissolved to a stock solution of 20 mmol/l in DMSO and directly diluted in medium to appropriate concentrations before the experiments. Primary antibodies against Bcl-2, Bax and NF- κ B and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and treatment. HeLa and SiHa cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (D-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 4 mmol/l L-glutamine, and nonessential amino acids at 37°C in a 5% CO₂ humid environment. Unless otherwise indicated, cells were plated in 100-mm Petri dishes at a density of 2x10⁶ cells/dish.

MTT assay. Cell viability was determined by MTT assay. Cells were plated at 1x10⁵ cells/well in 200 μ l D-MEM containing sanguinarine in a 96-well microtiter plate. Each concentration was repeated in 6 wells. After 12, 24, 36, 48, 60 h of incubation at 37°C in a humidified chamber, 20 μ l MTT (5 mg/ml in PBS) was added to each well and further incubated for 4 h. Then, the microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 min at 4°C. MTT solution was removed from the wells by aspiration and formazan crystals were dissolved in 150 μ l DMSO. Absorbance was measured in an ELISA reader at 570 nm, with the absorbance at 630 nm to correct for background. The effect of sanguinarine on growth inhibition was assessed as percentage inhibition in cell growth where untreated cells were taken as 100%.

Clonogenic assay. Cell survival was assessed using the colony formation assay. Briefly, cells were treated with 0, 0.5, 1, 2 and 3 μ mol/l sanguinarine for 12 h and collected immediately by trypsinization. The cells were counted and re-plated in 60-mm tissue culture dishes in 2 sets of triplicates for each concentration with 500, 1000, 2000, 5000 cells/well. Sufficient numbers were seeded to ensure that 50~100 macroscopic colonies would appear in untreated cells at the end of 14 days. Media were replaced every 3 days. At the end, cells were stained with 0.5% crystal violet in 1:1 methanol-water. Fifty cells were used

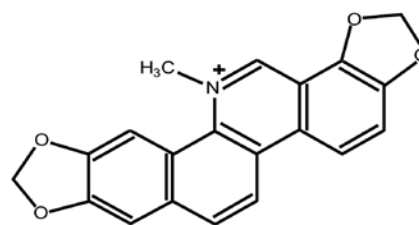


Figure 1. Chemical structure of sanguinarine.

as minimum number to define a colony and colonies were counted under a light microscope.

Cell morphology. Alteration of cell morphology was observed by an inverted microscope (Olympus Corporation, Tokyo, Japan). The key morphological criterion for the detection of apoptosis was the formation of apoptotic bodies according to other reports (4,5,18).

TUNEL assay. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was carried out using the In Situ Cell Death Detection kit according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA). Briefly, cells were fixed in 4% paraformaldehyde and permeabilized in ice-cold 0.1% triton X-100, 0.1% sodium citrate. TUNEL reaction mix was added at 1:5 dilution of TUNEL reaction mix and incubated for 1 h at 37°C in the dark under humidified conditions. TUNEL-Peroxidase (POD) converter was then added and incubated for an additional hour. Fluorescein-based TSA fluorescence system (PerkinElmer Life Sciences, Waltham, MA, USA) was added for 20 min before visualizing with a Nikon microscope using a 20x0.50 objective and photographed with a camera. TUNEL-positive cells were counted using particle analysis and cell counter plug-ins with Image software.

DNA fragmentation gel electrophoresis assay. Briefly, cells were exposed to sanguinarine for 24 h and then lysed with 200 μ l lysis buffer [10 mM Tris-HCl (pH 7.6), 100 mmol/l EDTA, and 20 mmol/l NaCl] for 30 min. Following centrifugation, the supernatant was transferred into new tubes and incubated by adding 20 μ l SDS and 200 μ l RNase A (10 mg/ml) for 2 h at 56°C, 30 μ l proteinase K (50 mg/ml) was added to each tube. The cell lysates were incubated for another 2 h at 37°C. The DNA was finally precipitated with the addition of 10 μ l of 10 M potassium acetate and 1 ml 100% ethanol at -80°C for 30 min. The extracted DNA samples were centrifuged and washed with 70% ethanol. Pure DNA was finally loaded and run on a 1% agarose gel at 80 V in running buffer (89 mmol/l Tris-acetate, 2 mmol/l Na₂EDTA, and 89 mmol/l boric acid). The gels were stained with 0.1 μ g/ml ethidium bromide and photographed. A DNA molecular weight ladder (Invitrogen) was included as a molecular size marker. Gel images were evaluated for typical ladder patterns of low molecular weight DNA fragments in multiples of 180-200 base pairs, a hallmark of apoptosis. The bands were visualized under a UV transilluminator (Model TM-36; UVP Inc., San Gabriel, CA, USA) followed by Polaroid photography in MP-4 Photographic System (Fotodyne Inc., Hartland, WI, USA).

Cell cycle analysis. Cells were harvested, centrifuged, incubated with 5 μ l RNase (20 μ g/ml final concentration) for 30 min and propidium iodide (50 μ g/ml final concentration) for 1 h. Cell cycle analysis was carried out by FACScan benchtop cytometry (BD Biosciences, San Jose, CA, USA), Cell Quest software (BD Biosciences) and ModFit LT software (Verity Software House, Topsham, ME).

Western blot assay. Western blot assay was performed as previously described (23). Briefly, cells were harvested and lysed with a lysis buffer containing [50 mmol/l HEPES (pH 7.5), 150 mmol/l NaCl, 1.5 mmol/l MgCl, 1 mmol/l EDTA, 10% glycerol and 1% Triton X-100] containing protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and trypsin inhibitor). Protein concentration was determined by Bio-Rad protein assay (Invitrogen). Total lysates (50 μ g) were separated on SDS-PAGE and electroblotted onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Following overnight incubation with the appropriate primary antibodies, blots were treated with horseradish peroxidase conjugated secondary antibodies. Chemiluminescence detection was finally performed with a chemiluminescence (ECL) system (Amersham Biosciences, Amersham, UK).

Statistical analysis. Assays were performed in triplicate for each sample. Average value was determined and expressed as the mean \pm SD. Statistical analysis was performed by means of multifactorial ANOVA and SPSS software for Windows (11.0, Chicago, IL, USA). $P < 0.05$ was considered to indicate statistically significant differences.

Results

Sanguinarine inhibits viability of HeLa and SiHa cells. We first assessed the effect of sanguinarine on the proliferation of two human cervical cancer cell lines, HeLa and SiHa, by MTT assays. As shown in Fig. 2, a significant dose-dependent inhibition of cell proliferation was observed in HeLa and SiHa cells exposed to sanguinarine (24 h), >90% cells lost their viability following 24 exposure to 5 μ mol/l sanguinarine in both cell lines. An IC_{50} value was 2.62 ± 0.21 μ mol/l in HeLa cells and 3.07 ± 0.23 μ mol/l in SiHa cells ($P < 0.05$), respectively, indicating that HeLa cells were slightly more sensitive than SiHa cells. Similar results were obtained by trypan blue dye exclusion assay, which was used to enumerate the proportion of live and dead cells in a population (data not shown).

Sanguinarine inhibits cell colony formation. To assess the reproductive potential and long-term cell survival, the effect of sanguinarine on cell ability to form colonies was detected. As shown in Fig. 3, sanguinarine resulted in a dose-dependent inhibition of colony formation in HeLa and SiHa cells. For example, 1.0 μ mol/l sanguinarine clearly decreased the colony formation to 53.5% in HeLa cells and to 61.0% in SiHa cells. Similarly, sanguinarine was more effective in HeLa cells than in SiHa cells ($P < 0.05$).

Sanguinarine induces apoptosis in HeLa and SiHa cells. We determined whether the decrease of cell viability and colony formation by sanguinarine was via the occurrence of apoptosis.

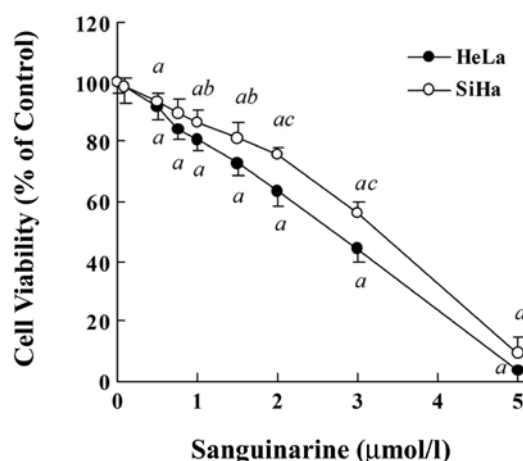


Figure 2. Sanguinarine decreases cell viability. Cells were exposed to different doses of sanguinarine for 24 h and then subjected to MTT assay as described in Materials and methods. a, $P < 0.01$ compared with corresponding untreated control in each cell line. b, $P < 0.05$, and c, $P < 0.01$, compared with HeLa cells.

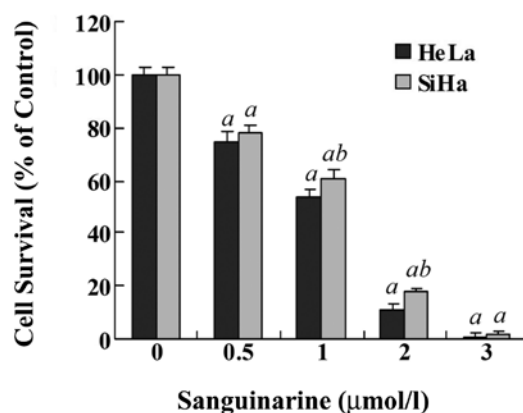


Figure 3. Sanguinarine inhibits colony formation. Cells were exposed to different doses of sanguinarine for 12 h and subjected to colony formation assay. a, $P < 0.01$ compared with corresponding untreated control in each cell line. b, $P < 0.05$, compared with HeLa cells. Data are expressed as the mean \pm SE of three experiments.

For this purpose, apoptotic cell death was examined by cell morphology, DNA fragmentation and flow cytometry assays. Using light microscopy, apoptotic bodies were observed in HeLa and SiHa cells treated with sanguinarine at various doses. The cytosolic condensation and nuclear condensation, typical of apoptotic cell death, were observed in the cells treated with sanguinarine at all doses tested (data not shown). Moreover, sanguinarine induced a significant increase in the number of TUNEL-labeled apoptotic cells in a dose-dependent manner, and the change of TUNEL-positive cell numbers in HeLa cells was evident compared with SiHa cells following sanguinarine treatment at the same dose (Fig. 4).

Apoptosis was also assessed by a classical DNA fragmentation assay. As shown in Fig. 5, fragmented DNAs were observed in HeLa cells treated with 2.0 μ mol/l sanguinarine and became more obvious in cells treated with 3.0 μ mol/l sanguinarine. DNA fragmentation was also observed in SiHa cells treated with sanguinarine at 3.0 μ mol/l, but not at 2.0 μ mol/l.

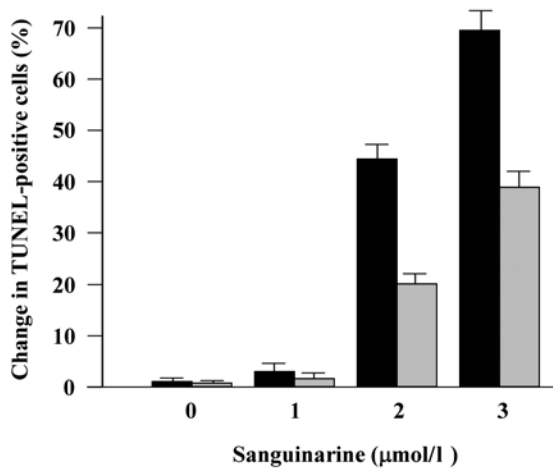


Figure 4. Sanguinarine induces apoptosis in a dose-dependent manner. HeLa and SiHa cells were plated at 200,000 cells/well in an 8-well chamber slide, treated for 24 h with sanguinarine at indicated doses and harvested for TUNEL assay. Data are expressed as the percent of TUNEL-positive cells normalized to the untreated control cells from three independent experiments, with at least four fields counted in each sample. * $P<0.05$ denotes statistically significant differences compared with the respective controls.

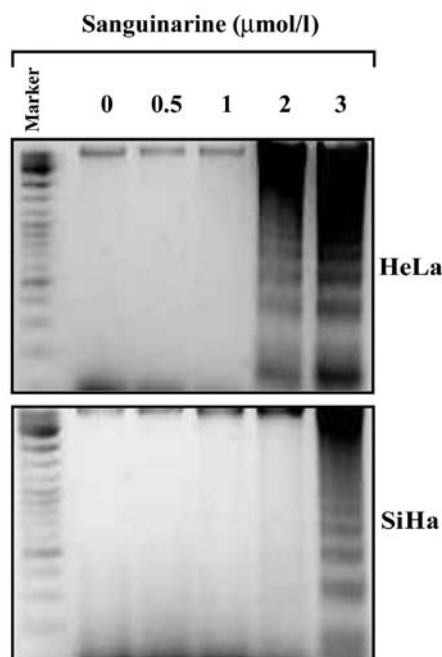


Figure 5. Sanguinarine causes DNA fragmentation. Cells were treated with sanguinarine at indicated doses for 24 h and then subjected to agarose gel electrophoresis followed by visualization of bands as described in Materials and methods. Data shown are from a representative experiment repeated three times with similar results.

Additionally, as evidenced by flow cytometry, a significant increase of sub-G1 fraction, a characteristic of apoptotic cells, on a DNA histogram was also observed in both HeLa and SiHa cell lines exposed to sanguinarine (Fig. 6). A larger Sub-G1 population was seen in HeLa than in SiHa cells at the same concentration of sanguinarine ($P<0.01$); for example, in HeLa cells treated with 2.0 and 3.0 $\mu\text{mol/l}$ sanguinarine, the sub-G1 fraction increased from 1.7% (untreated control) to 42.5 and 59.7% ($P<0.01$), respectively.

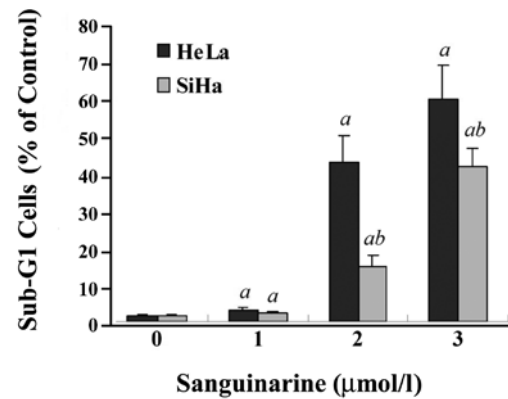


Figure 6. Sanguinarine increases sub-G1 fraction. Cells were exposed to different doses of sanguinarine for 24 h and subjected to flow cytometry. a, $P<0.01$ compared with corresponding untreated control; b, $P<0.01$, compared with HeLa cells.

Effects of sanguinarine on apoptosis-related proteins. To investigate the potential mechanism(s) involved in sanguinarine-induced apoptosis, we determined the impact of sanguinarine on Bcl-2 and Bax, two key apoptosis regulator proteins. As shown in Fig. 7, 24 h of exposure to sanguinarine resulted in a dose-dependent increase of pro-apoptotic protein Bax expression and a concomitant decrease of anti-apoptotic protein Bcl-2 levels. After treatment with 2.0 and 3.0 $\mu\text{mol/l}$ sanguinarine, the Bax/Bcl-2 ratio, which favors apoptosis, significantly increased in HeLa cells ($P<0.01$) and in SiHa cells ($P<0.05$ for 2.0 $\mu\text{mol/l}$ sanguinarine; $P<0.01$ for 3.0 $\mu\text{mol/l}$ sanguinarine).

To investigate the alteration of NF- κ B in sanguinarine-induced apoptosis, the p65 subunit of NF- κ B was determined by western blot assay. As shown in Fig. 8, a dose-dependent decrease of NF- κ B protein expression was detectable in HeLa and SiHa cells. Moreover, such reduction of NF- κ B was more evident in HeLa cells compared to SiHa cells.

Discussion

In the present study, we demonstrated that sanguinarine significantly inhibited cell viability and colony formation in HeLa and SiHa human cervical cancer cells in a dose-dependent manner. The IC_{50} of sanguinarine was 2.62 ± 0.21 $\mu\text{mol/l}$ in HeLa cells and 3.07 ± 0.23 $\mu\text{mol/l}$ in SiHa cells, respectively, which is consistent with the results of previous studies (14). Other studies reported that IC_{50} of sanguinarine is 0.72 ± 0.33 $\mu\text{mol/l}$ in HL-60 human leukemia cells (12) and 5.2 $\mu\text{mol/l}$ in L1210, a mouse lymphocytic leukemic cell line (15). Thus, the anticancer ability of sanguinarine may be cell-type dependent. The clonal adaptation and growth of cancer cells are crucial for tumor growth (23), hence, suppression of cell colony formation in the present study may suggest potential anticancer use of sanguinarine at different stages of carcinogenesis.

It has been reported that cervical adenocarcinoma is chemo- and radio-resistant and patients with adenocarcinoma of uterine cervix exhibit significantly poorer recurrence-free survival than those with squamous cell carcinoma (24,25). The inhibitory effect of sanguinarine on chemo-resistant cells

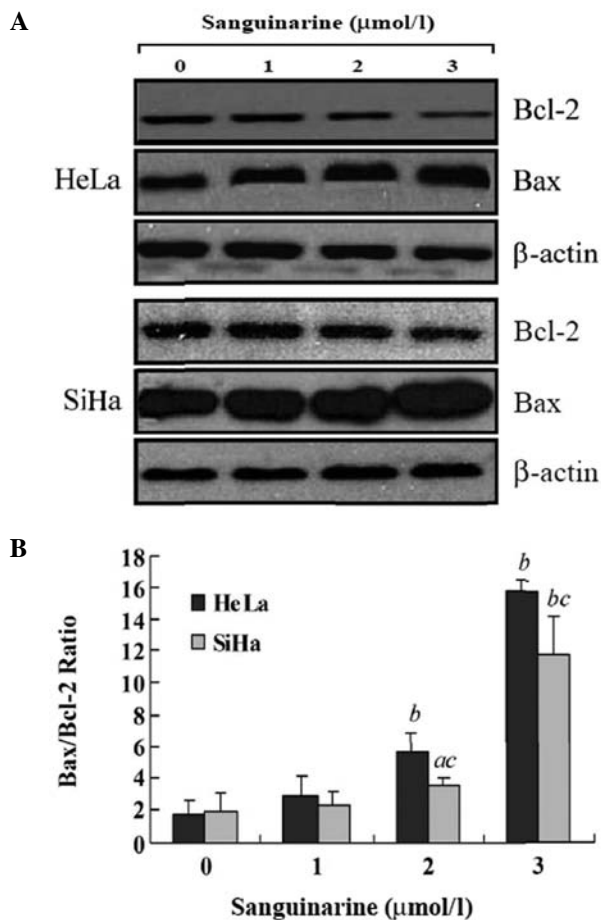


Figure 7. Effect of sanguinarine on Bcl-2 and Bax expression. (A) Cells were treated with the indicated doses of sanguinarine for 24 h and subjected to western blot assay. Equal loading was confirmed by stripping the blot and reprobing for β -actin. (B) A Bax/Bcl-2 ratio is shown. a, $P < 0.05$, b, $P < 0.01$ compared with corresponding untreated control; c, $P < 0.01$, compared with HeLa cells.

was also observed using multidrug resistance (MDR) cells to human papillomavirus type 16-immortalized endocervical cells (18). Similar sanguinarine sensitivity of HeLa (cervical adenocarcinoma) cells to SiHa (squamous cell carcinoma) cells may suggest that sanguinarine is a promising agent for possible development as an anticancer drug for cervical cancer, particularly for radio-chemo-resistant cervical adenocarcinoma.

Both normal and cancer cells within a living system are affected by the rate of apoptosis, which is regarded as an ideal way to eliminate damaged cells. Agents that induce apoptosis may be used for the management and therapy of cancer by modulating the steady-state cell population. To determine whether the decrease of viability and colony formation in cervical cells exposed to sanguinarine was due to apoptosis, we performed morphological observation by light microscopy and found a significant change of apoptotic morphology following treatment with sanguinarine. The induction of apoptosis by sanguinarine was also evident from the assay of DNA fragmentation using classical DNA ladder assay since fragmentation of DNA by endonucleases is considered a hallmark of apoptotic cell death (26). Sanguinarine provoked the DNA fragmentation in cervical cancer cells, which is in agreement with findings of previous studies in human epidermoid carcinoma

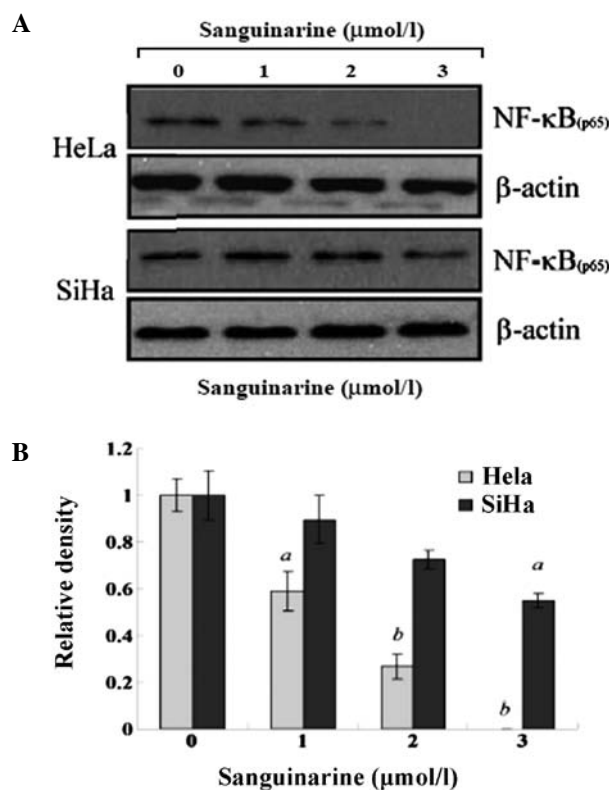


Figure 8. Effect of sanguinarine on NF- κ B (p65) expression. (A) Cells were treated with the indicated doses of sanguinarine for 24 h and subjected to western blot assay. (B) Protein bands were quantitated by densitometry, and the data are expressed as the mean \pm SE from three experiments. a, $P < 0.05$; b, $P < 0.01$.

(A431), human breast cancer cells (MDA-231), KB cancer cells and primary effusion lymphoma cells (4,10,27,28). However, Slunská *et al* found that compared with other benzophenanthridine alkaloids, sanguinarine did not induce significant DNA ladder formation in HL-60 cells (19). Weerasinghe *et al* also found that human erythroleukemia cells treated with a low level (4.1 μ mol/l) of sanguinarine showed the morphology of apoptosis in ~96% of cells, and sanguinarine at a high dose of 34.4 μ mol/l resulted in necrotic cell death morphology in over 90% of cells (5,6).

The appearance of sub-G1 peak on a DNA histogram, as determined by flow cytometry, represents apoptotic cells with fractional DNA content which underwent activation-induced apoptosis (29). Our current results provide additional evidence that sanguinarine results in dose-dependent apoptosis induction in cervical cancer cells as the percentage of sub-G1 gradually increased after exposure to sanguinarine, which is consistent with other studies (4,12,19,27,30,31).

Members of the Bcl-2 protein family regulate and execute many cell intrinsic apoptotic pathways; for example, Bcl-2 has been shown to function as an inhibitor of apoptosis. The increased expression of Bcl-2 confers drug resistance, which is a major obstacle to successful chemotherapy (32). By contrast, Bax, another member of the Bcl-2 family, is found to be involved in the apoptotic cell death process (33). Bax has been shown to significantly increase the sensitivity to chemotherapeutic drugs such as gemcitabine and 5-Fu in pancreatic cancer AsPC-1 cells (34). Our results also show an

upregulation of the Bax protein and a downregulation of the Bcl-2 protein in response to sanguinarine, further suggesting a role of apoptosis induction in controlling growth of cervical cancer cells by sanguinarine. On the other hand, the imbalance between pro-apoptotic and anti-apoptotic proteins is involved in the distinctive biological features of cancer cells. An increase of Bax/Bcl2 ratio favors apoptosis induction and enhances chemotherapeutic effects, which has been found in human pancreatic carcinoma cells, K562 erythroleukemia cells, immortalized human keratinocytes (HaCaT) (5,7,20) and human cervical cancer cells used in our study following treatment with sanguinarine. Bax expression increases sensitivity of cancer cells to chemotherapeutic drugs (34) and Bcl-2 increase is related to drug resistance (32).

NF- κ B is a nuclear transcription factor that regulates expression of a large number of genes involved in the regulation of apoptosis, tumorigenesis, inflammation, atherosclerosis, viral replication and several autoimmune diseases (35). NF- κ B is commonly upregulated in cancer cells and several attempts are currently underway to block its activation in order to render cells more susceptible to anticancer drugs (35,36). Chaturvedi *et al* first reported that the tumor necrosis factor (TNF)-induced appearance of p65 was blocked by sanguinarine and the inhibition of NF- κ B activation by sanguinarine is not cell-type specific (21). In our present study, sanguinarine reduced the expression of the NF- κ B protein. This inhibitory effect may be specific to sanguinarine and may not be observed in other structural analogues of sanguinarine (21,37). Our results are also supported by other studies where the decrease of NF- κ B expression in K562 erythroleukemia cells was observed following treatment with 4.5 μ mol/l of sanguinarine (5).

In conclusion, sanguinarine in the range of observed concentrations, significantly inhibits proliferation and induces apoptosis in both HeLa and SiHa human cervical cancer cell lines. The increase of apoptotic bodies, DNA fragmentation and sub-G1 phase by sanguinarine was more obvious in HeLa cells than in SiHa cells. Furthermore, sanguinarine decreased the expression of anti-apoptotic proteins Bcl-2 and transcription factor NF- κ B and increased the pro-apoptotic protein Bax in both cell lines, particularly in HeLa cells. Therefore, the higher sensitivity to sanguinarine in HeLa cells compared to SiHa cells is contributed to the higher susceptibility of HeLa cells to sanguinarine-induced apoptosis. These observations further support the possible use of sanguinarine in the treatment of cervical cancer, although further studies are required to explore the exact mechanism(s) of the responses evoked by sanguinarine as well as to verify their effectiveness in an animal model system.

Acknowledgements

This study was supported by grants from the NNSFC (81071906 and 81172127), the Program for Scientific Innovation Research of College Graduate in Jiangsu Province (CX10B-0452); the Outstanding Doctoral Thesis Project of Soochow University (233209749); the Medical Scientific Research Project of Soochow University (HZ200905) and the Program for Preliminary Scientific Research of Soochow University (SDY2011A45).

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