Molecular Cancer Therapeutics

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Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery

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Abstract

Prostate cancer is the second leading cause of cancer-related deaths in males in the United States. This warrants the development of novel mechanism-based strategies for the prevention and/or treatment of prostate cancer. Several studies have shown that plant-derived alkaloids possess remarkable anticancer effects. Sanguinarine, an alkaloid derived from the bloodroot plant *Sanguinaria canadensis*, has been shown to possess antimicrobial, anti-inflammatory, and antioxidant properties. Previously, we have shown that sanguinarine possesses strong antiproliferative and proapoptotic properties against human epidermoid carcinoma A431 cells and immortalized human HaCaT keratinocytes. Here, employing androgen-responsive human prostate carcinoma LNCaP cells and androgen-unresponsive human prostate carcinoma DU145 cells, we studied the antiproliferative properties of sanguinarine against prostate cancer. Sanguinarine (0.1–2 μmol/L) treatment of LNCaP and DU145 cells for 24 hours resulted in dose-dependent (1) inhibition of cell growth [as evaluated by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay], (2) arrest of cells in G_0-G_1 phase of the cell cycle (as assessed by DNA cell cycle analysis), and (3) induction of apoptosis (as evaluated by DNA ladder formation and flow cytometry). To define the mechanism of antiproliferative effects of sanguinarine against prostate cancer, we studied the effect of sanguinarine on critical molecular events known to regulate the cell cycle and the apoptotic machinery. Immuno-blot analysis showed that sanguinarine treatment of both LNCaP and DU145 cells resulted in significant (1) induction of cyclin kinase inhibitors p21/WAF1 and p27/KIP1; (2) down-regulation of cyclin E, D1, and D2; and (3) down-regulation of cyclin-dependent kinase 2, 4, and 6. A highlight of this study was the fact that sanguinarine induced growth inhibitory and antiproliferative effects in human prostate carcinoma cells irrespective of their androgen status. To our knowledge, this is the first study showing the involvement of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery during cell cycle arrest and apoptosis of prostate cancer cells by sanguinarine. These results suggest that sanguinarine may be developed as an agent for the management of prostate cancer. [Mol Cancer Ther 2004;3(8):933–40]

Introduction

Prostate cancer is a common malignancy and, next only to lung cancer, is the second leading cause of cancer-related deaths of males in the United States (1). According to an estimate of the American Cancer Society, a total of 230,110 men will be diagnosed with prostate cancer in the United States in the year 2004 and 29,900 prostate cancer-related deaths are predicted for 2004 (1). The major cause of mortality from this disease is metastasis of hormone-refractory cancer cells that fail to respond to hormone ablation therapy (2, 3). Because surgery and current treatment options have proven to be inadequate in curing or controlling prostate cancer, the search for novel agents for the management of this disease has become a priority. In the recent past, agents obtained from herbs and plants have gained considerable attention for the prevention and/or treatment of certain cancer types including prostate cancer (4).

Naturally occurring plant-based agents often provide opportunities for the management of cancer and other diseases (ref. 5 and references therein). 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 a benzophenanthridine alkaloid and a structural homologue of chelerythrine. It has been shown to possess antimicrobial, antioxidant, anti-inflammatory, and antitumor properties (6). It is widely used in toothpaste and mouthwash for the prevention/treatment of gingivitis and other inflammatory conditions (7–9). There is a suggestion for the antitumor properties of this alkaloid (6, 10–16). In a recent study, we have shown that sanguinarine, at micromolar concentrations, imparts cell growth inhibitory responses in human squamous carcinoma (A431) cells via an induction of apoptosis (10). The important observation of this
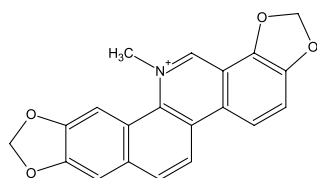
Figure 1. Chemical structure of sanguinarine (13-methyl[1,3]benzo-dioxolo[5,6-*c*]-1,3-dioxolo[4,5]phenanthridinium).

study was that sanguinarine treatment did not result in apoptosis of the normal human epidermal keratinocytes at similar dose (10). In another recent study, we showed that sanguinarine causes an apoptotic death of immortalized human keratinocytes (HaCaT) via modulations in the mitochondrial pathway and the Bcl-2 family of proteins (11). The present work is our mechanism-based effort to assess if sanguinarine could be developed as an agent for the management of prostate cancer. We assessed the antiproliferative effects of sanguinarine on growth/proliferation of human prostate cancer cells and the involvement of cell cycle regulatory events as the mechanism of this response.

Uncontrolled cellular proliferation is a hallmark of all cancers, and the blockade of the cell cycle is regarded as an effective strategy for eliminating cancer cells (17–23). In fact, at present, various cell cycle inhibitors are being evaluated as therapeutic tools for the management of cancer in preclinical and clinical studies. The cell cycle in eukaryotes is controlled (at least in part) by a family of protein kinase complexes wherein each complex is composed of a catalytic subunit, the cyclin-dependent kinase (cdk), and its essential regulatory subunit, the cyclin (24–27). These complexes are activated at specific intervals during the cell cycle and can also be regulated by exogenous factors (26). The cyclin-cdk complexes are subject to inhibition via binding to a class of proteins known as the cyclin kinase inhibitors (cki). Anticancer agents may alter one or more regulatory events in the cell cycle resulting in blockade of cell cycle progression, thereby reducing the growth and proliferation of the cancer cells. Cell cycle blockade may ultimately lead to a programmed death (i.e., apoptosis of cancer cells). The ability of tumor cells to evade apoptosis plays a significant role in their resistance to conventional therapeutic regimens (28). Therefore, search for novel agents designed to impart cell cycle arrest and induction of apoptosis in cancer cells is being earnestly pursued.

In the present study, we show that sanguinarine imparts antiproliferative effects against androgen-responsive (LNCaP) and androgen-unresponsive (DU145) human prostate cancer cells and that this effect is mediated through dysregulation of cell cycle and induction of apoptosis. To our knowledge, this is the first study showing the modulation of cell cycle regulatory events by sanguinarine.

Materials and Methods

Reagents

Sanguinarine (>98% pure) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). The antibodies (p21; p27; cyclin E, D1, and D2; and cdk 2, 4, and 6) used in this study were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Apo-direct apoptosis kit was obtained from Phoenix Flow Systems (San Diego, CA). The DC protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Novex precast Tris-glycine gels were obtained from Invitrogen (Carlsbad, CA).

Cell Culture

The androgen-responsive human prostate carcinoma cells LNCaP and androgen-unresponsive human prostate carcinoma cells DU145 were obtained from American Type Culture Collection (Rockville, MD). DU145 cells were cultured in MEM with 2 mmol/L L-glutamine and Eagle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, and 1% penicillin-streptomycin. LNCaP cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 2 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, and 1% penicillin-streptomycin. The cells were maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment.

Treatment of Cells

Sanguinarine (dissolved in ethanol) was employed for the treatment of cells. The cells (70% to 80% confluent) were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours in complete cell culture medium. Cells that were used as controls were incubated with the maximum used amount of ethanol only.

Cell Growth/Cell Viability

The effect of sanguinarine on the viability of cells was determined by MTT assay. The cells were plated at 2×10^5 cells per well in 200 μL DMEM containing 0.1, 0.2, 0.5, 1, and 2 μmol/L sanguinarine in a 96-well microtiter plate. Each concentration of sanguinarine was repeated in 10 wells. The cells were incubated for 24 hours at 37°C in a humidified chamber. Following 24 hours of incubation, MTT reagent (4 μL, 5 mg/mL in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at 4°C. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150 μL). Absorbance was recorded on a microplate reader at 540 nm wavelength. The effect of sanguinarine on growth inhibition was assessed as percentage inhibition in cell growth wherein vehicle-treated cells were taken as 100%.

Detection of Apoptosis by DNA Ladder Assay

The LNCaP and DU145 cells were grown to ∼70% confluency and treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours. Following this treatment, the

cells were washed twice with PBS [10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 5 mmol/L MgC1$_2$, 0.5% Triton X-100], left on ice for 15 minutes, and pelleted by centrifugation (14,000 × g) at 4°C. The pellet was incubated with nuclear lysis buffer [10 mmol/L Tris (pH 7.5), 400 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100] for 30 minutes on ice and centrifuged at 14,000 × g at 4°C. The supernatant obtained was incubated overnight with RNase (0.2 mg/mL) at room temperature and with proteinase K (0.1 mg/mL) for 2 hours at 37°C. DNA was extracted using phenol/chloroform (1:1) and precipitated with 95% ethanol for 2 hours at −80°C. The DNA precipitate was centrifuged at 14,000 × g at 4°C for 15 minutes, and the pellet was air dried and dissolved in Tris-EDTA buffer [20 μL, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA]. Total amount of DNA was resolved over 1.5% agarose gel containing 0.3 μg/mL ethidium bromide in 1× Tris-borate EDTA buffer (pH 8.3; 89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L EDTA; BioWhittaker, Inc., Walkersville, MD). The bands were visualized under UV transilluminator (Model TM-36, UVP Inc., San Gabriel, CA) followed by Polaroid photography (MP-4 Photographic System, Fotodyne Inc., Hartland, WI).

Quantitiation of Apoptosis by Flow Cytometry

The cells were grown at a density of 1 × 10^6 cells in 100 mm culture dishes and treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged dUTP nucleotide and propidium iodide using Apo-direct apoptosis kit (Phoenix Flow Systems) as per the manufacturer's protocol. Labeled cells were analyzed by flow cytometry.

DNA Cell Cycle Analysis

The cells (70% confluent) were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) in complete medium for 24 hours. The cells were trypsinized thereafter, washed twice with cold PBS, and centrifuged. The cell pellet was resuspended in 50 μL cold PBS to which cold methanol (450 μL) was added and the cells were incubated for 1 hour at 4°C. The cells were centrifuged at 1,100 rpm for 5 minutes, pellet washed twice with cold PBS, suspended in 500 μL PBS, and incubated with 5 μL RNase (20 μg/mL final concentration) for 30 minutes. The cells were chilled over ice for 10 minutes and incubated with propidium iodide (50 μg/mL final concentration) for 1 hour and analyzed by flow cytometry.

Preparation of Cell Lysates and Western Blot Analysis

The cells were harvested at 24 hours following sanguinarine treatment as described above and washed with cold PBS (10 mmol/L, pH 7.4). The cells were incubated in ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na$_3$VO$_4$, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) over ice for 30 minutes. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21.5 G needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14,000 × g for 15 minutes at 4°C, and the supernatant (total cell lysate) was collected, aliquoted, and stored at −70°C. The protein content in the lysates was measured by DC protein assay (Bio-Rad Laboratories) as per the manufacturer's protocol.

For Western blot analysis, protein (20–50 μg) was resolved over 8% to 12% SDS-PAGE gels and transferred onto a nitrocellulose membrane. The nonspecific sites were blocked by incubating the blot with 5% nonfat dry milk in buffer (containing 10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween 20) for 1 hour at room temperature or overnight at 4°C. The blot was washed with wash buffer (10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween 20) for 2 × 10 minutes and incubated overnight with appropriate primary antibody specific for the protein to be assessed. The antibodies were used at dilutions specified by the manufacturer. The blot was washed for 2 × 10 minutes followed by an incubation with the corresponding secondary antibody horseradish peroxidase conjugate (Amersham Life Science, Inc., Arlington Heights, IL) at 1:2,000 dilution for 1 hour at room temperature. The blot was washed in wash buffer twice for 10 minutes each and four times for 5 minutes each. The protein was detected by chemiluminescence using enhanced chemiluminescence kit (Amersham Life Science) and autoradiography with XAR-5 film (Amersham Life Science). For every immunoblot, equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody.

Statistical Analysis

Results were analyzed using a two-tailed Student's t test to assess statistical significance. Values of $P < 0.05$ were considered statistically significant.

Results and Discussion

Prostate cancer in humans progresses from an androgen-responsive to an androgen-unresponsive state, and at the time of clinical diagnosis, most prostate cancers represent a mixture of androgen-responsive and androgen-unresponsive cells (29). Whereas androgen-responsive cells undergo rapid apoptosis on androgen ablation, androgen-unresponsive cells evade apoptosis during androgen withdrawal, although they retain the molecular machinery for apoptosis. Mortality from prostate cancer generally occurs from the proliferation and invasion of these androgen-unresponsive cells, which fail to undergo apoptosis culminating into hormone-refractory prostate cancer for which no cure but only palliative treatment is available (3). Therefore, there is an urgent need to intensify our efforts for a better understanding of this disease and for the development of novel mechanism-based approaches for its prevention and treatment (30).

Earlier studies in cell culture system from our laboratory showed that sanguinarine treatment resulted in an apoptotic death of A431 carcinoma cells (10). In fact, this report was the first systematic study showing the anticancer effect of sanguinarine. In the present study, we assessed the

anticancer effects of this plant-based alkaloid against prostate cancer. For this study, we employed two human prostate cancer cell lines DU145 and LNCaP. The choice of these two cells lines was based on the fact that LNCaP cells are androgen responsive and DU145 cells are androgen unresponsive and that, at the time of clinical diagnosis, most prostate cancers present as a mixture of androgen-responsive and androgen-unresponsive cells. Therefore, eliminating both cell types seems to be an effective approach for the management of prostate cancer. In the first set of experiments, we evaluated whether sanguinarine treatment imparts antiproliferative effects in human prostate cancer cells. Employing the MTT assay, we observed that sanguinarine (0.1–2 μmol/L) treatment of DU145 and LNCaP cells resulted in dose-dependent decrease in the growth of both cell types (Fig. 2). Interestingly, an IC_{50} of ~1 μmol/L was observed for both cell types. Sanguinarine has been shown to induce apoptosis in certain types of cancer and transformed cells (10–16). Studies have shown that, at low concentrations, sanguinarine treatment of cancer cells induced apoptosis distinguished by cell surface blebbing whereas, at higher concentrations, sanguinarine caused a second mode of cell death, oncosis, distinguished by cell surface blistering (13–16). In this study, we determined if the observed growth inhibition of LNCaP and DU145 cells by low concentrations of sanguinarine is mediated via apoptosis. As shown in Fig. 3, our data showed that sanguinarine
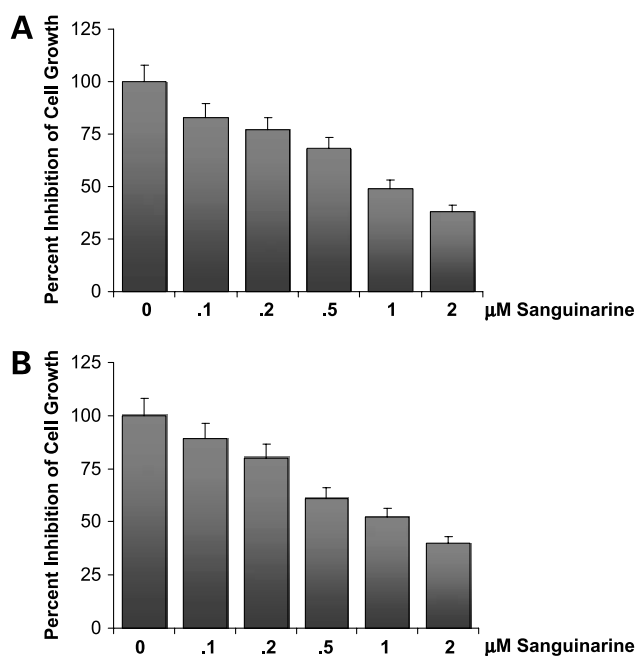

Figure 2. Effect of sanguinarine on the growth of prostate cancer cells LNCaP (**A**) and DU145 (**B**). Cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L), and the percentage inhibition of cell growth was determined by MTT assay in a 96-well ELISA plate as detailed in Materials and Methods. *Columns,* mean of three separate experiments wherein each treatment was repeated in 10 wells; *bars,* SE.
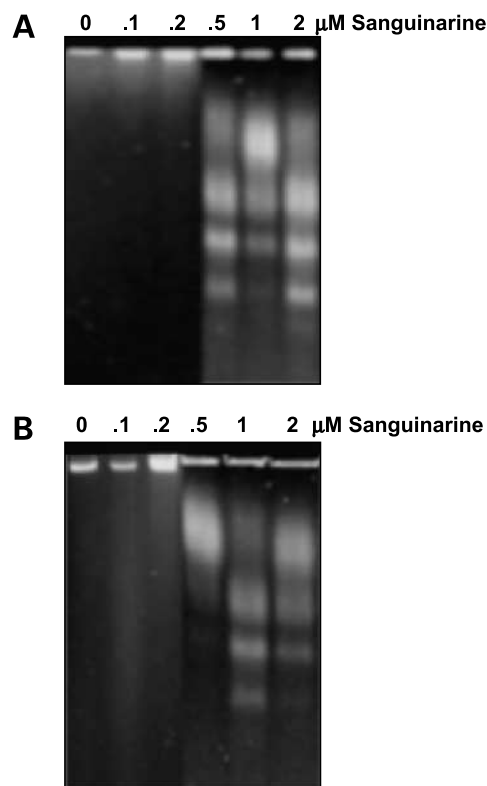

Figure 3. Effect of sanguinarine on DNA fragmentation in prostate cancer cells LNCaP (**A**) and DU145 (**B**) as analyzed by DNA ladder formation. Cells were grown to 70% confluency and treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours. The DNA was isolated and resolved over 1.5% agarose gel followed by visualization of bands as described in Materials and Methods. Data are representative of an experiment repeated three times with similar results.

Table 1. Effect of sanguinarine on apoptosis in prostate cancer cells LNCaP and DU145 as analyzed by flow cytometry

Treatment	% Apoptosis	
	LNCaP Cells	DU145 Cells
Control	2.7 ± 0.03	3.1 ± 0.02
Sanguinarine (μmol/L)		
0.1	4.4 ± 0.25	5.3 ± 0.36
0.2	6.1 ± 0.08	5.9 ± 0.08
0.5	12.2 ± 0.38*	13.2 ± 0.68*
1.0	19.0 ± 2.34[†]	18.6 ± 3.11[†]
2.0	41.1 ± 2.47[‡]	43.7 ± 4.13[‡]

Cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours and labeled with dUTP using terminal deoxynucleotidyl transferase and propidium iodide. Cells showing dUTP fluorescence above that of the control population are considered as apoptotic and their percentage population is shown. Data are means ± SE of three experiments done in triplicate.
*$P < 0.05$.
[†]$P < 0.01$.
[‡]$P < 0.001$.
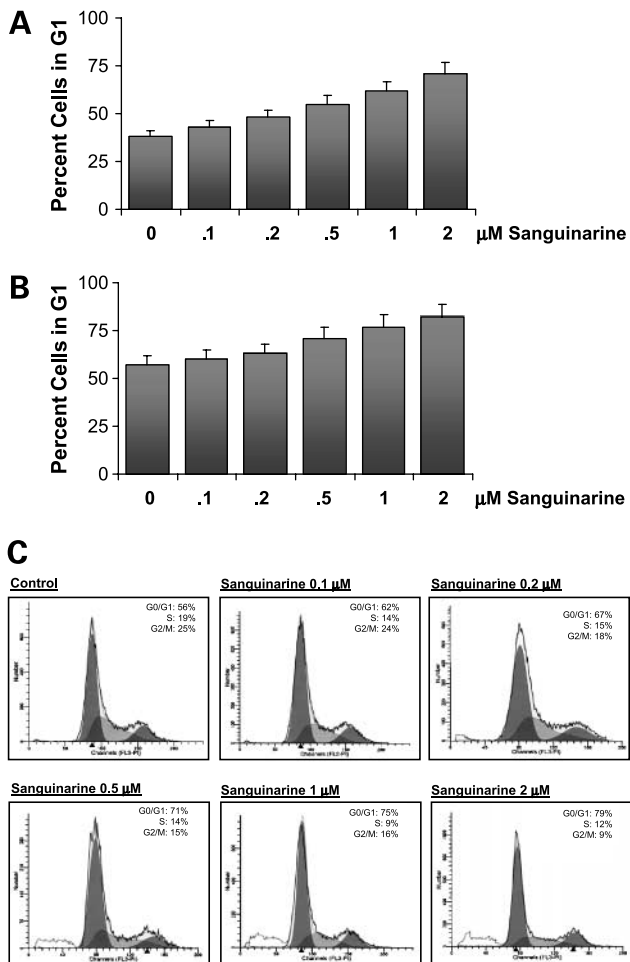
A

B

C

Figure 4. Effect of sanguinarine on cell cycle in prostate cancer cells. The growing cells (~60% confluent) were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours, and the DNA cell cycle analysis was done as described in Materials and Methods. *Columns,* mean of three separate experiments conducted in triplicate with LNCaP cells (**A**) and DU145 cells (**B**); *bars,* SE. **C,** data are representative of actual analysis of cell cycle distribution for DU145 cells done in triplicate with similar results.

treatment of both androgen-responsive LNCaP cells and androgen-unresponsive DU145 cells resulted in the formation of DNA ladder, a hallmark of apoptosis. These results were further verified by terminal deoxynucleotidyl transferase–mediated nick end labeling assay.

As shown by the data in Table 1, sanguinarine treatment to both cell lines resulted in a dose-dependent increase in terminal deoxynucleotidyl transferase–mediated nick end labeling positive (apoptotic) cells. Apoptotic cell death is the consequence of a series of precisely regulated events that are frequently altered in tumor cells. This provides an opportunity for selective clinical intervention to induce a programmed death of the cancer cells, ideally without affecting the normal cells (28). Apoptosis is a physiologic process that involves elimination of cells with DNA

damage (31) and represents a distinct form of cell death that differs from necrotic cell death (32). Hence, agents that can modulate apoptosis may be useful in the management and therapy of cancer (33, 34).

Several studies have shown that the induction of apoptosis may be cell cycle dependent (refs. 35–39 and references therein). Therefore, in our next series of experiments, we tested the hypothesis that sanguinarine-caused apoptosis of LNCaP and DU145 cells is mediated via cell cycle blockade. We therefore did DNA cell cycle analysis to assess the effect of sanguinarine treatment on the distribution of cells in the cell cycle. As shown in Fig. 4, compared with vehicle treatment, sanguinarine treatment was found to result in dose-dependent accumulation of DU145 cells in G_1 phase of the cell cycle. Similar results were observed when LNCaP cells were treated with increasing dose of sanguinarine (Fig. 4). This observation is important because the molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (40, 41). Therefore, in recent years, inhibition of the cell cycle has been appreciated as a target for the management of cancer (42, 43).

We next studied the involvement of cki-cyclin-cdk machinery in G_1-phase cell cycle arrest of human prostate cancer cells by sanguinarine. The journey of cells through the cell cycle in eukaryotes is coordinated by a family of
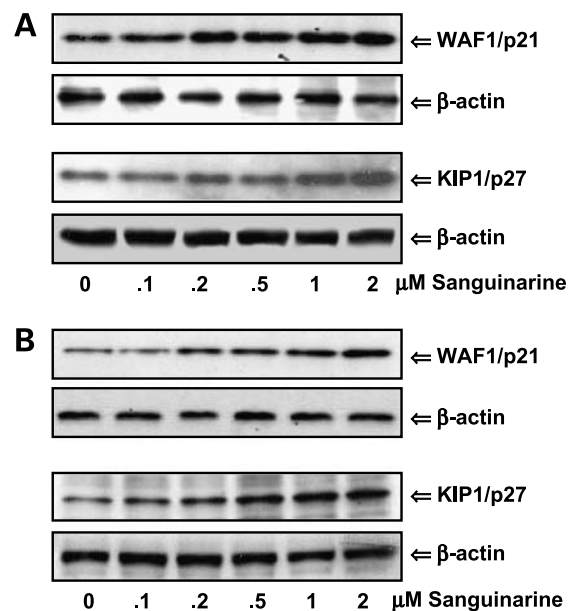

Figure 5. Effect of sanguinarine on the protein expression of p21/WAF1 and p27/KIP1 in prostate cancer cells LNCaP (**A**) and DU145 (**B**). The cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) and harvested at 24 hours following the treatment. Total cell lysates were prepared and protein (50 μg) was subjected to SDS-PAGE followed by Western blot analysis using specific antibodies and secondary horseradish peroxidase–conjugated antibodies. The protein was detected by chemiluminescence. Details are described in Materials and Methods. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Data are representative of a typical experiment repeated three times with similar results.
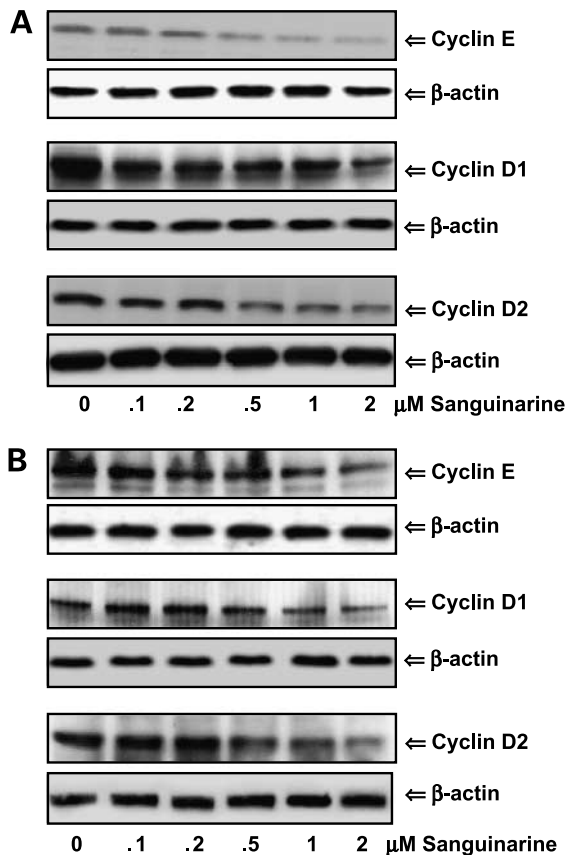
Figure 6. Effect of sanguinarine on the protein expression of cyclin E, D1, and D2 in prostate cancer cells LNCaP (**A**) and DU145 (**B**). The cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) and harvested at 24 hours following the treatment. Total cell lysates were prepared and protein (20 μg) was subjected to SDS-PAGE followed by Western blot analysis using specific primary antibodies and secondary horseradish peroxidase–conjugated antibodies. The proteins were detected by chemiluminescence. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Details are described in Materials and Methods. Data are representative of a typical experiment repeated three times with similar results.

protein kinase complexes. Each complex is composed minimally of cyclins (regulatory subunit) that bind to cdks (catalytic subunit) to form active cyclin-cdk complexes. These complexes are activated at various checkpoints after specific intervals during the cell cycle and can also be regulated by several exogenous factors (40). However, in transformed cells, cell cycle progression could be a mitogenic signal-dependent or mitogenic signal-independent process (44, 45). Cdk activity is additionally regulated by small proteins known as ckis. Ckis include the p21/WAF1 and p27/KIP1 family of proteins. Therefore, we studied the modulation in cell cycle regulatory events operative in the G_0-G_1 phase as a mechanism of sanguinarine-mediated cell cycle dysregulation and apoptosis in human prostate cancer cells. As shown by Western blot analysis (Fig. 5A), sanguinarine treatment (0.2–2.0 μmol/L for 24 hours) of LNCaP cells resulted in significant dose-

dependent up-regulation of the ckis p21/WAF1 and p27/KIP1. Interestingly, similar results were obtained with DU145 cells (Fig. 5B). Many studies have shown that these ckis regulate the progression of cells in the G_0-G_1 phase of the cell cycle, and an induction of these molecules causes a blockade of G_1-S transition, thereby resulting in a G_0-G_1 phase arrest (46). Further, studies have also shown that loss of functional cki in different human cancers and derived cell lines leads to uncontrolled cell proliferation due to an increase in the levels of cdk-cyclin complex (47). p21/WAF1/CIP is an important cki and is shown to be almost a universal inhibitor of cdks (48, 49). Many studies have shown that certain exogenous stimuli may result in a p53-dependent and p53-independent induction of p21/WAF1, which in turn may trigger a series of events, ultimately resulting in a cell cycle arrest and/or apoptosis
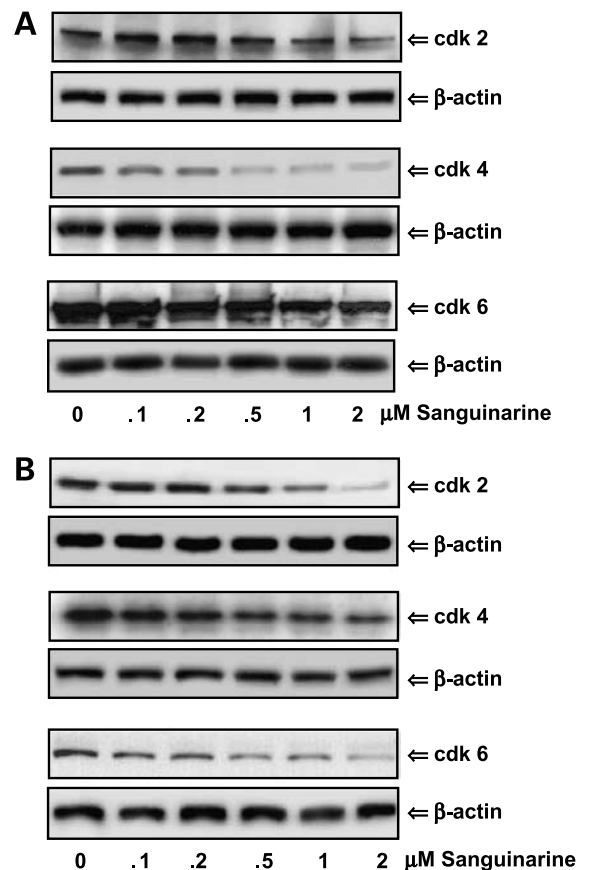

Figure 7. Effect of sanguinarine on the protein expression of cdk 2, 4, and 6 in prostate cancer cells LNCaP (**A**) and DU145 (**B**). The cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) and harvested at 24 hours following the treatment. Total cell lysates were prepared and protein (20 μg) was subjected to SDS-PAGE followed by Western blot analysis using appropriate primary antibodies and secondary horseradish peroxidase–conjugated antibodies. The proteins were detected by chemiluminescence. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Details are described in Materials and Methods. Data are representative of a typical experiment repeated twice with similar results.
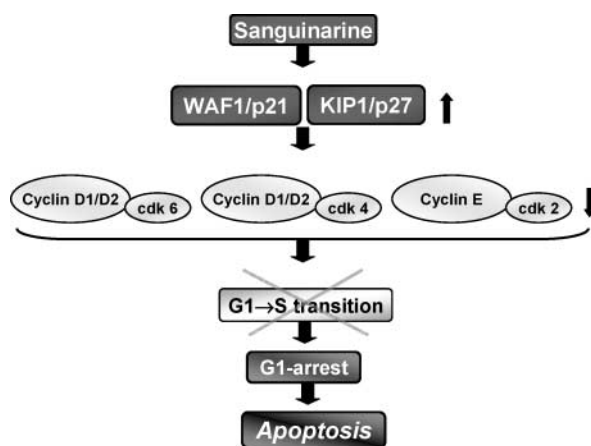
Figure 8. Proposed model for sanguinarine-mediated cell cycle arrest and apoptosis of cancer cells.

(46–49). Our data showing an induction of p21/WAF1 by sanguinarine seem to be p53 dependent in LNCaP cells (with wild-type p53) because sanguinarine treatment to these cells was found to result in a concentration-dependent increase in protein levels of p53 (data not shown). Further, the induction of p21/WAF1 by sanguinarine seems to be p53 independent in DU145 cells (with mutant p53). Thus, sanguinarine seems to impart its growth inhibitory and cell cycle dysregulatory effects irrespective of p53 status. However, more studies are required to assess a definite association between p53 status in cancer cells and the biological effects of sanguinarine. Recent studies (46, 50) have shown the critical role of p27/KIP1 in apoptosis and cell cycle progression through G_0-G_1 phase. We observed a significant induction of p27/KIP1 by sanguinarine. Thus, sanguinarine imparts cell cycle dysregulation in both androgen-sensitive and androgen-insensitive human prostate carcinoma cells via an up-regulation of ckis involved in G_0-G_1 progression.

Several studies have shown that overexpression of cyclins and cdks is commonly associated with human malignancies (47, 51). Therefore, we next evaluated the effect of sanguinarine treatment on modulations in the levels of the major cyclins operative in G_0-G_1 phase of the cell cycle, viz., cyclin D1, D2, and E. We observed that treatment of LNCaP and DU145 cells with sanguinarine (0.2–2.0 μmol/L for 24 hours) resulted in a dose-dependent decrease in the protein expression of cyclin D1, D2, and E in both cell types (Fig. 6). Similarly, we found that treatment of LNCaP and DU145 cells with sanguinarine (0.2–2.0 μmol/L) for 24 hours resulted in a dose-dependent decrease in cdk 2, 4, and 6 in LNCaP and DU145 cells (Fig. 7). Studies have shown that cdk 2, 4, and 6 are critical for progression of cells through G_1 and entry into the S phase of the cell cycle (47, 52). These results suggest that sanguinarine is capable of restoring proper checkpoint control in both types of human prostate carcinoma cells.

Cell cycle regulatory molecules are the critical regulatory elements, which control the progression of cells in early and late G_1 phases of the cell cycle (46–53). Our data, showing a decrease in the protein levels of the cyclin D1, D2, and E and cdk 2, 4, and 6 by sanguinarine treatment in both cell lines, agree with the fact that the cdks and cyclins operate in association with each other by forming complexes, which may bind to and are inhibited by ckis. This series of events imposes a blockade of G_1-S transition, resulting in G_0-G_1 phase arrest of the cell cycle. Thus, taken together, as shown in the composite scheme in Fig. 8, we suggest the series of events by which sanguinarine results in the blockade of cell cycle via imposing an artificial checkpoint at G_1-S transition. This causes an arrest of cancer cells in the G_1 phase of the cell cycle, which is an irreversible process that ultimately results in an apoptotic cell death. Several other possibilities of cell cycle arrest by sanguinarine cannot be ruled out. It is also possible that the down-regulation of cyclin D/cdk4/cdk6 is the cause for cell cycle arrest, whereas the modulations in the levels of p21/WAF1 and p27/KIP1 by sanguinarine are regulated with completely different mechanisms such as at a transcriptional level via p53-dependent and p53-independent pathways (in case of p21/WAF1) and through post-translational mechanisms such as proteasome-mediated degradation (in case of p27/KIP1). Further studies are needed to access these possibilities. It is also possible that the apoptosis induction by sanguinarine is a process independent from G_1-phase arrest. Further studies are needed to clarify this assumption.

One major finding of this study is that sanguinarine has been shown to cause cell cycle blockade and apoptosis of human prostate cancer cells irrespective of their androgen status. This is an important finding because prostate cancer is known to undergo a transition from an early ''androgen-sensitive'' form of cancer to a late (metastatic) ''androgen-insensitive'' cancer, and at the time of clinical diagnosis, most prostate cancer represent a mixture of androgen-sensitive and androgen-insensitive cells. Therefore, the key to the control of prostate cancer seems to lie in the elimination of both types of prostate cancer cells (without affecting the normal cells) via mechanism-based preventive/therapeutic approaches. To our knowledge, this is the first study showing the involvement of cki-cyclin-cdk machinery during cell cycle arrest and apoptosis of prostate cancer cells by sanguinarine. These results suggest that sanguinarine may be developed as an agent for the management of prostate cancer.

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