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<b>14. ABSTRACT</b> The traditional surgery and therapy has not been successful in the management of prostate cancer (CaP). Therefore, the search for novel agents and approaches for the treatment of CaP continues. Natural plant-based products have shown promise as anticancer agents. Sanguinarine, a benzophenanthridine alkaloid derived from the root of Sanguinaria Canadensis, has been shown to possess anti-microbial, antioxidant and anti-inflammatory properties. Our earlier studies suggested that sanguinarine may be developed as an agent for the management of prostate cancer. Based on this rationale, funded by the DOD (Award - W81XWH-04-1-0220), we initiated a study to investigate the hypothesis that sanguinarine will impart antiproliferative effects against prostate cancer via a modulation in NF-kB-pathway-mediated apoptosis. In the current reporting period (01/16/2005 – 01/15/2006) we have made significant advancement. The key accomplishments for this reporting period are as follows. We have demonstrated that sanguinarine possesses chemopreventive/anti-proliferative effects against prostate cancer in an athymic nude mice xenograft model. Further, our data suggested that sanguinarine-caused effects are mediated via modulations in NF-kB pathway and cyclin kinase inhibitor-cyclin-cyclin dependent kinase machinery. In addition, our pilot study has shown that transgenic TRAMP model, which mimics human disease, is suitable for chemoprevention/intervention studies with sanguinarine.						
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## **Introduction:**

In American men, **Cancer of the Prostate (CaP)**, continues to be one of the most frequently occurring malignancies, representing ~29% of all new cancer cases (1). The traditional surgery and therapy has not been successful in the management of CaP. Therefore, the search for novel agents and approaches for the treatment of CaP continues. Natural plant-based products have shown promise as anticancer agents. Ideally, the anti-cancer drugs should specifically target the neoplastic cells with minimal "collateral damage" to normal cells. Thus, the agents, which can eliminate the cancerous cells without affecting the normal cells, may have therapeutic advantage for the elimination of cancer cells. Sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium), derived from the root of *Sanguinaria Canadensis* and other poppy-fumaria species, is a benzophenanthridine alkaloid and a structural homologue of chelerythrine and has been shown to possess anti-microbial, antioxidant and anti-inflammatory properties (2-4). Our published and preliminary studies suggested that sanguinarine may be developed as an agent for the management of prostate cancer (5-6). Based on this rationale, funded by the Department of Defense (DOD; Idea Development Award - W81XWH-04-1-0220), we initiated a study to investigate the hypothesis that *sanguinarine will impart antiproliferative effects against prostate cancer via a modulation in NF- $\kappa$ B-pathway-mediated apoptosis.*

Based on our preliminary studies and the progress so far, we expect that a successful completion of this proposal may define i) the potential of sanguinarine against CaP, and ii) molecular mechanism(s) of the biological effects of sanguinarine. This may pave the way for the development of novel strategies for the management of CaP.

## **Main Body of the Progress Report:**

In the 'Idea Development Award' selected for funding by the 'US Army Medical Research and Material Command', we proposed to test the hypothesis that *a plant-derived alkaloid sanguinarine will impart antiproliferative effects against prostate cancer via a modulation in NF- $\kappa$ B-pathway-mediated apoptosis.* In the current reporting period (January 16, 2005 – January 15, 2006) we have made significant advancement with respect to our proposed hypothesis. A brief description of the progress is presented in the following pages.

### **Evaluation of anti-proliferative effects of sanguinarine against prostate cancer in nude mice xenografts.**

Continuing with our ongoing work, we conducted detailed studies to determine the efficacy of sanguinarine against prostate cancer in athymic nude mice implanted with human prostate cancer cells. Further, we conducted experiments to determine the molecular mechanism associated with the observed anti-proliferative effects of sanguinarine. A brief account of this study is given below.

#### ***Study Design:***

To determine the chemopreventive and therapeutic potential of sanguinarine against CaP in vivo, we employed the athymic nude mice xenografts model. For this experiment, the athymic (nu/nu) male nude mice (obtained from NxGen Biosciences, San Diego, CA) were randomly divided into different groups of 10 animals each and CW22Rv1 cells ( $1 \times 10^6$  cells in 50  $\mu$ l RPMI + 50  $\mu$ l Matrigel) were implanted in athymic nude mice by a sub-cutaneous injection on left and right sides, below the shoulders (2 tumors/mouse). The rationale for the choice of CWR22Rv1 cells is based on the fact that

our major goal was to determine the chemopreventive effects of sanguinarine in early stages of CaP development, when the disease is androgen-dependent. Another reason for employing CWR22Rv1 cells was that they make PSA, which is arguably considered a *gold standard* for monitoring the CaP in humans. The animals were treated with sanguinarine (1 or 5 mg/kg body weight in 0.2 ml PBS, five days a week) by intra-peritoneal injection either one week post cell implantation to establish the preventive potential or after the development of a sizable tumor (200 mm<sup>3</sup>) to examine the therapeutic potential. Thus, two different protocols were employed.

The detail of **Protocol-1** is as follows:

**Group I: Control** – cells were implanted (at the start of the experiment; day 0), no further treatment was given;

**Group II: Sanguinarine (1 mg/kg)** – cells were implanted on day 0 and the mice were injected with sanguinarine (1mg/kg body weight; *i. p.*) 5 days/ week (Monday – Friday);

**Group III: Sanguinarine (5 mg/kg)** – cells were implanted on day 0 and the mice were injected with sanguinarine (5mg/kg body weight; *i. p.*) 5 days/ week (Monday – Friday)

The detail of **Protocol-2** is as follows:

**Group 1: Control** – cells were implanted (at the start of the experiment), no further treatment was given;

**Group 2: Sanguinarine (1 mg/kg)** – cells were implanted and the tumors were allowed to grow and achieve a volume of 200 mm<sup>3</sup>, when the treatment with sanguinarine (1mg/kg body weight; *i. p.*; 5 days/ week – Monday – Friday) was started and continued until the termination of experiment;

**Group 3: Sanguinarine (5 mg/kg)** – cells were implanted and the tumors were allowed to grow and achieve a volume of 200 mm<sup>3</sup>, when the treatment with sanguinarine (5mg/kg body weight; *i. p.*; 5 days/ week – Monday – Friday) was started and continued until the termination of experiment;

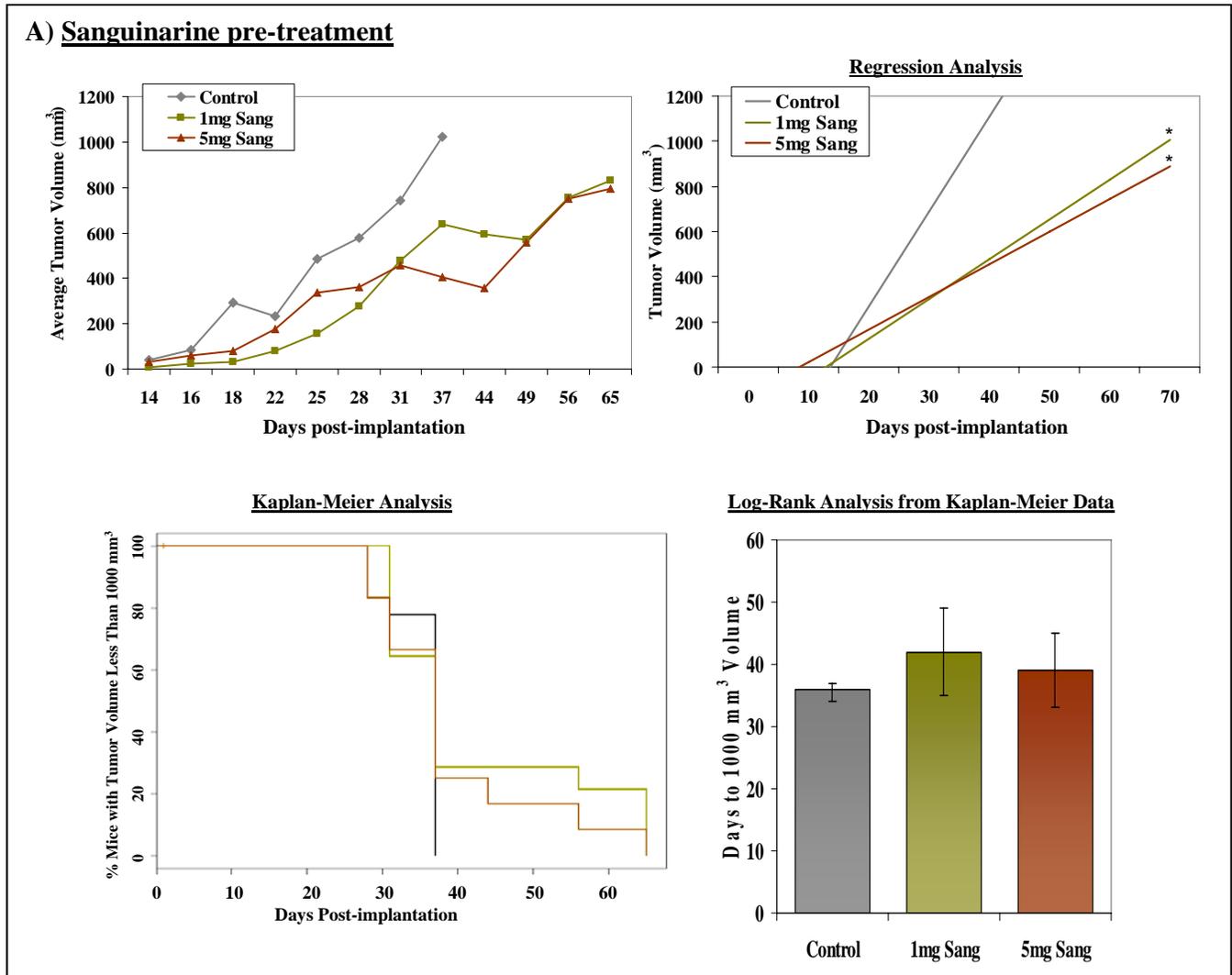
In both the protocols, the control animals received vehicle only. The treatment schedule was continued until the tumors reached a volume of 1000 mm<sup>3</sup>. At this point, the animals were withdrawn from the study and euthanized. Throughout the experiment the animals were housed under standard housing conditions and had free access to autoclaved laboratory chow diet. In this protocol, to assess the possibility of treatment-toxicity, the effect of treatments on food/water consumption and body weight was monitored twice weekly throughout the study. Further, blood was withdrawn periodically to determine the effects of treatments on PSA levels in serum. The effect of sanguinarine treatment was determined on the growth of implanted tumors and the serum levels of prostate specific antigen (PSA).

At the termination of experiment, the tumors were harvested for further studies to evaluate the mechanism of the antiproliferative effects of sanguinarine against prostate cancer.

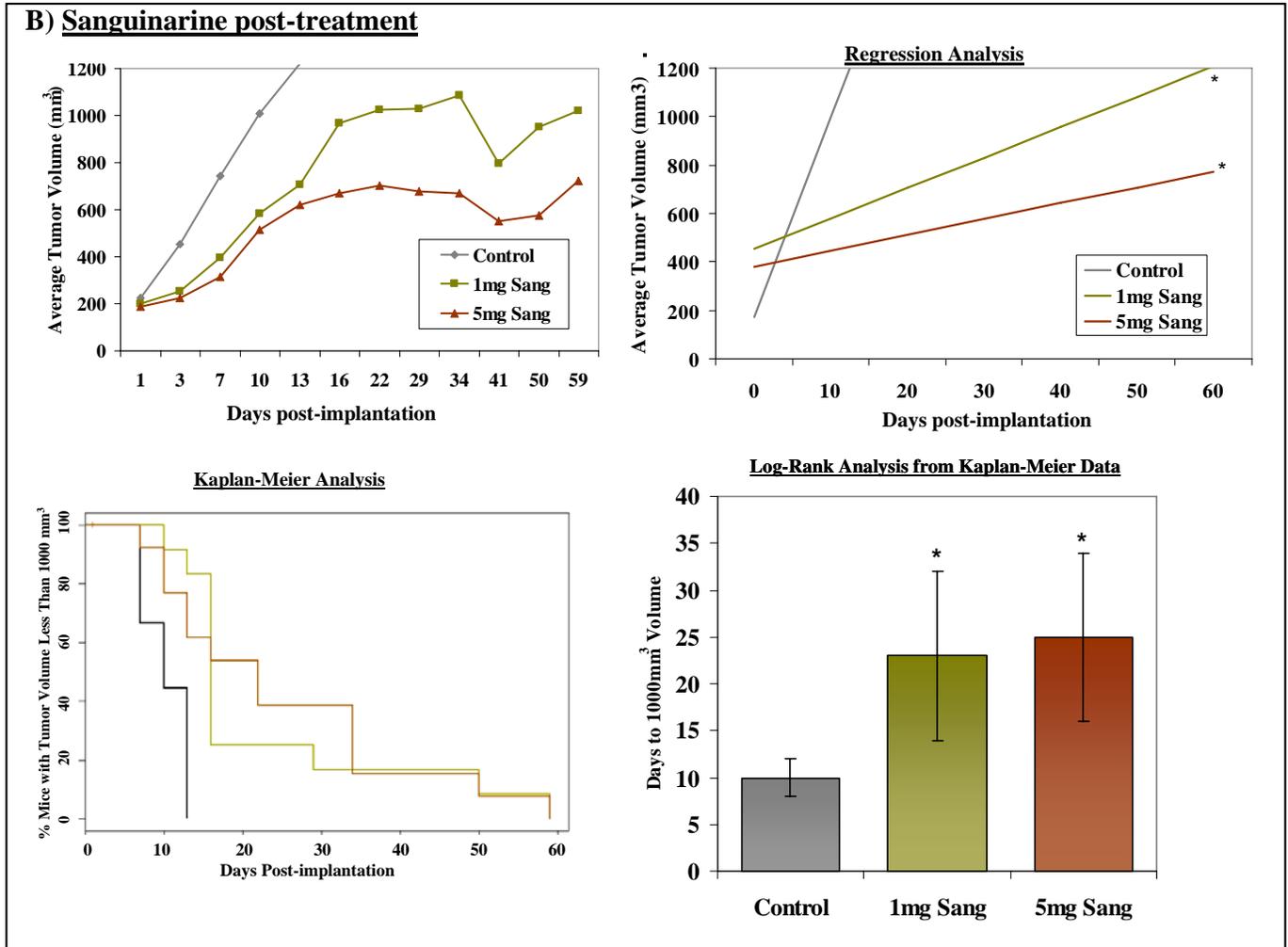
### **Results & Conclusion:**

**Anti-proliferative Effects of Sanguinarine Against CaP:** As shown in on the next page (Figures 1 & 2), our data demonstrated that sanguinarine (both pre- and post- treatments) resulted in a highly

significant inhibition in the rate of tumor growth as assessed by a regression analysis. Further, the Kaplan-Meier Analysis demonstrated that in sanguinarine treated animals (post-treatment), the rate of tumor growth (to reach to a 1000 mm<sup>3</sup> target volume) was significantly delayed. Furthermore, treatment of mice with sanguinarine (both pre- and post- tumor) resulted in a significant reduction in serum levels of prostate-specific antigen (PSA) in nude mice implanted with CWR22Rv1 cells.

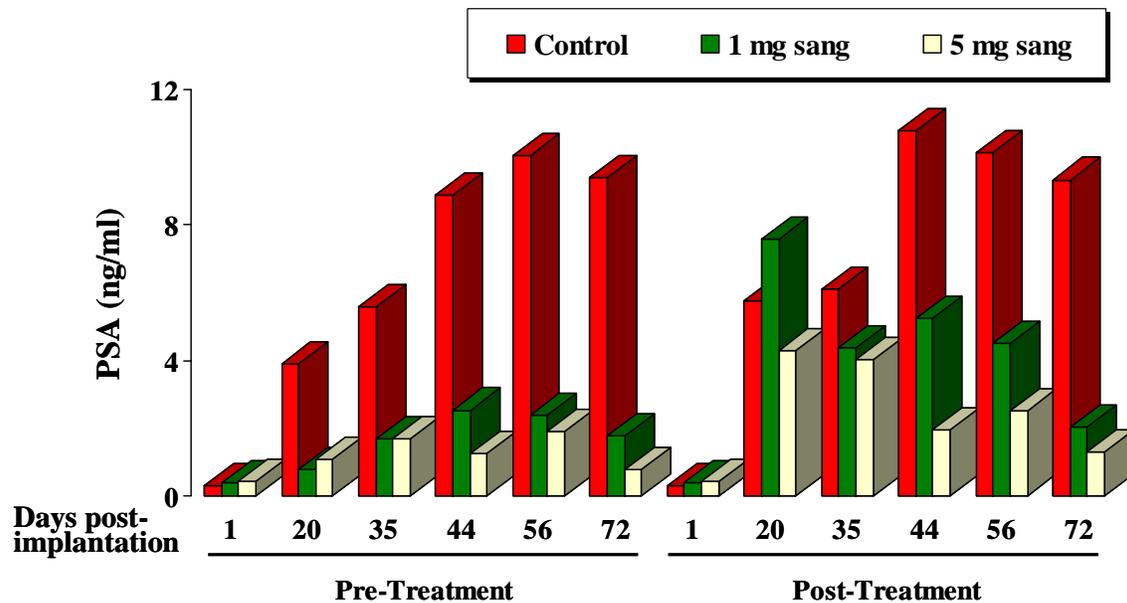


**Fig. 1: Effects of sanguinarine treatment on the growth of CWR22Rv1 cell-implanted prostate tumors in athymic nude mice.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. The effect of sanguinarine pre-treatment was measured in terms of average tumor volume of as a function of time. Further, the rate of tumor growth was assessed by linear regression analysis. Tumor-free survival was assessed by Kaplan-Meier plot and the average time to reach 1000 mm<sup>3</sup> tumor volumes was assessed by Log-Rank analysis of Kaplan-Meier data. \*p<0.05 was considered significant.



**Fig. 2: Effects of sanguinarine post-treatment on the growth of CWR22Rv1 cell-implanted prostate tumors in athymic nude mice.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. The effect of sanguinarine treatment on the growth of established tumors was measured in terms of average tumor volume of as a function of time. Further, the rate of tumor growth was assessed by linear regression analysis. Tumor-free survival was assessed by Kaplan-Meier plot and the average time to reach 1000 mm<sup>3</sup> tumor volumes was assessed by Log-Rank analysis of Kaplan-Meier data. \*p<0.05 was considered significant.

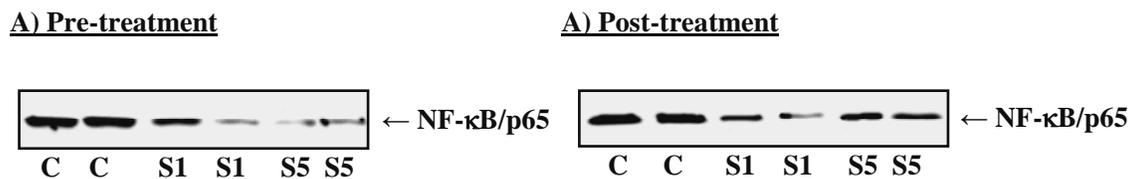
**Effect of Sanguinarine on Serum PSA:** Further, as described earlier, for this study, we used CWR22Rv1 cell because these cells are known to secrete PSA. As shown in figure 3, our data clearly demonstrated that treatment of mice with sanguinarine (both pre- and post- tumor) resulted in an appreciable reduction in serum levels of prostate-specific antigen (PSA) in nude mice implanted with CWR22Rv1 cells. This is an important observation because serum PSA is considered to be an important marker for identifying humans CaP and, several investigators have also reported the usefulness of serum PSA as a follow up marker for local recurrence and/or distant disease in the patients after radical prostatectomy, radiation and hormonal therapy.



**Fig. 3: Effects of sanguinarine treatment on the levels of serum PSA in athymic nude mice implanted with CWR22Rv1 cells.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. For determining PSA levels in the serum, following the treatments of animals with sanguinarine, at different times post-tumor cell inoculation, blood was collected by ‘mandibular bleed’ and serum was separated. The levels of PSA were determined by using a quantitative Human PSA enzyme linked immunosorbent assay (ELISA) kit (Anogen, Ontario, Canada) as per the manufacturer’s protocol.

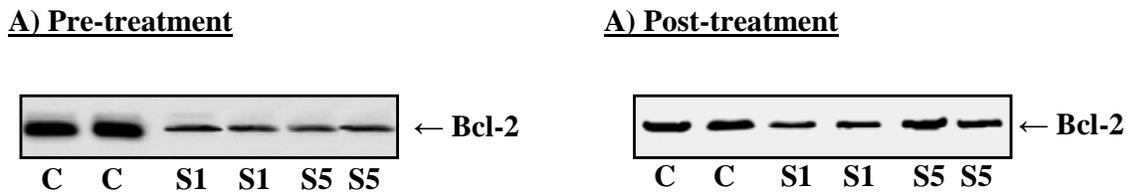
Thus, this study, for the first time, demonstrated the chemopreventive and therapeutic effects of sanguinarine against PCa development under in vivo situations. Based on our data, we suggest that sanguinarine is a promising candidate for chemoprevention and/or intervention against PCa.

**Effect of Sanguinarine on NF-κB Pathway:** Further, we also determined the mechanism of growth inhibitory effects of sanguinarine against CaP in nude mice implanted with CWR22Rv1 tumors. As shown below in figure 4, we found that sanguinarine treatments (in both the protocols) resulted in an appreciable down-modulation in the protein levels of NF-κB/p65 (in the nucleus) suggesting that the observed effects of sanguinarine may be mediated via inhibition of NF-κB pathway.



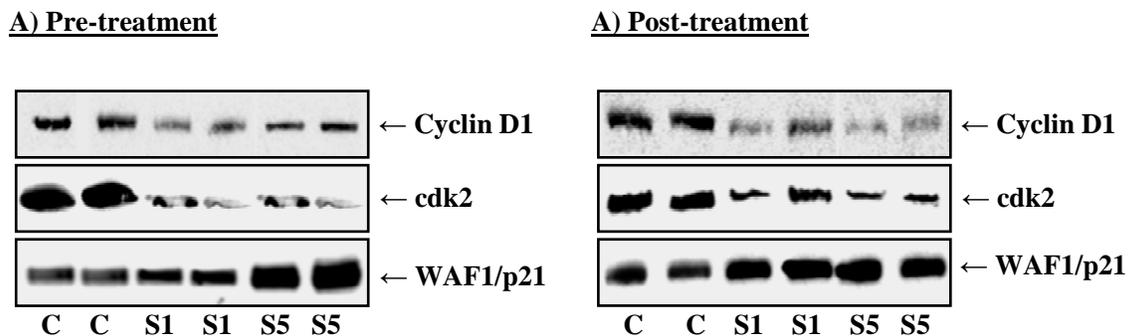
**Fig. 4: Effect of sanguinarine on nuclear levels of NF-κB/p65 in nude mice implanted with CWR22Rv1 tumors.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and tumors were surgically removed and the levels of nuclear NF-κB/p65 were examined by the western blot analysis using the appropriate primary and secondary antibodies. The data shown here are representative of three independent immunoblots with similar results. **C = control; S1 = sanguinarine 1mg/ml; S5 = sanguinarine 5mg/ml.**

In addition, as shown below in figure 5, our data also demonstrated that sanguinarine treatment resulted in an appreciable decrease in the protein levels of anti-apoptotic Bcl-2 that is an established downstream target of NF- $\kappa$ B.



**Fig. 4: Effect of sanguinarine on Bcl-2 protein levels in nude mice implanted with CWR22Rv1 tumors.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and tumors were surgically removed and the levels of Bcl-2 proteins were examined in total cell lysate with western blot analysis using the appropriate primary and secondary antibodies. The data shown here are representative of three independent immunoblots with similar results. **C = control; S1 = sanguinarine 1mg/ml; S5 = sanguinarine 5mg/ml.**

Further, as shown in figure 5, our data also demonstrated that a down-modulation of cyclin D1 and cdk2 and an upregulation of WAF1/p21 during sanguinarine-mediated growth inhibition of CWR22Rv1-implanted prostate tumors in nude mice.



**Fig. 4: Effect of sanguinarine on cell cycle regulatory proteins in nude mice implanted with CWR22Rv1 tumors.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and tumors were surgically removed and the levels of proteins were examined in total cell lysate with western blot analysis using the appropriate primary and secondary antibodies. The data shown here are representative of three independent immunoblots with similar results. **C = control; S1 = sanguinarine 1mg/ml; S5 = sanguinarine 5mg/ml.**

These studies are currently ongoing; and we are performing immunohistochemical- Real-Time PCR- and Western Blot- analyses to unravel the effect of sanguinarine on NF- $\kappa$ B pathway, Bcl-2 family protein and *cyclin kinase inhibitor-cyclin-cyclin dependent kinase network* in athymic nude mice implanted with CWR22Rv1 tumors.

**Evaluation of anti-proliferative effects of sanguinarine against prostate cancer in transgenic adenocarcinoma mouse prostate (TRAMP) model.**

**Study Design:**

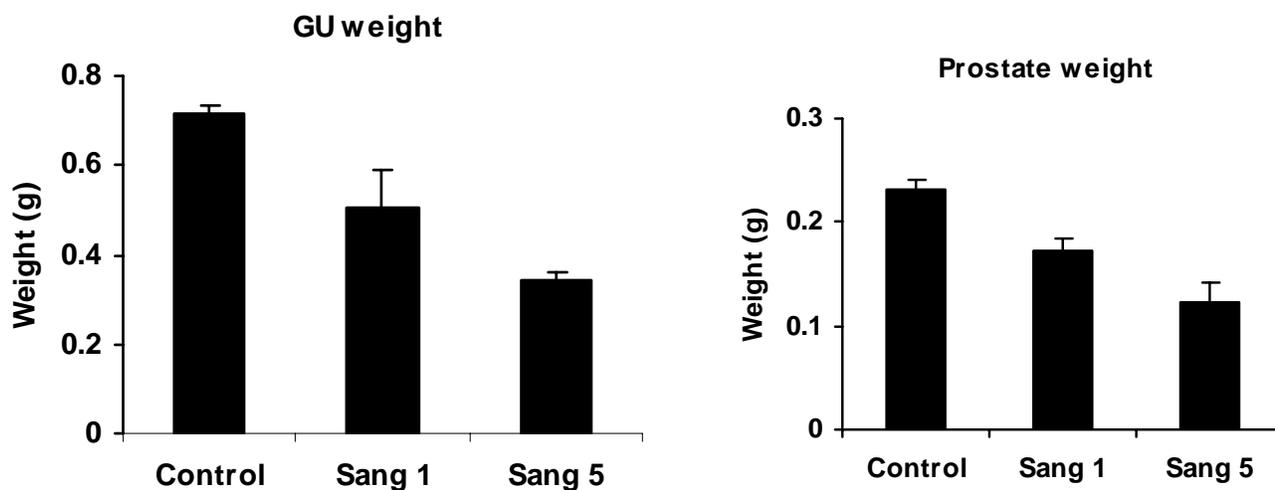
In order to assess the feasibility of our plan to determine the chemopreventive/therapeutic effects of sanguinarine against CaP in transgenic TRAMP mice that mimic the features of human disease, we first conducted a preliminary experiment with limited number of animals. In our preliminary studies, 12 male heterozygous C57BL/TGN TRAMP mice, line PB Tag 8247NG (12-14 weeks old; obtained from our breeding colony at the Animal Care Facility, School of Medicine, University of Wisconsin) were divided into three groups and subjected to sanguinarine treatments as indicated below:

- Group 1:** Control (PBS alone; *intraperitoneal injection*; 5 days/week)
- Group 2:** Sanguinarine (1 mg/kg in PBS; *intraperitoneal injection*; 5 days/week)
- Group 3:** Sanguinarine (5 mg/kg in PBS; *intraperitoneal injection*; 5 days/week)

This treatment was given to the animals 5 days per week for 6 weeks. Throughout each experiment, the animals had free access to laboratory chow and water *ad libitum*. At the end of six weeks, the experiment was terminated and the animals from both experimental and control groups were sacrificed. At the time of sacrifice, the lower GU tract, including the bladder, testes, seminal vesicles, and prostate, was removed en bloc. The GU wet weight was recorded followed by surgical dissection of the prostate gland. The weight of whole prostate gland was also recorded. Protein lysates were prepared from the prostate glands by homogenization in lysis buffer followed by the immunoblot analysis.

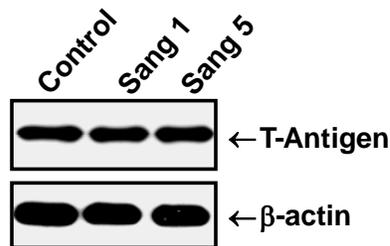
**Results & Conclusion:**

As shown in figure 5, we found that compared to control, sanguinarine treatments resulted in an appreciable decrease in GU weight and prostate weight. Further, the treatments were not found to have any evident toxic effects (body weight, food/fluid consumption) on the TRAMP mice.



**Fig. 5: Effect of Sanguinarine on GU and prostate weights in TRAMP mice.** The mice were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and GU and prostate weights were recorded. The data showed here represent mean  $\pm$  SEM from four animals per group.

Further, we also assessed whether or not sanguinarine treatment affects the levels of transgene in TRAMP mice. As shown by the immunoblot analysis (Figure 6), treatment of mice with sanguinarine did not result in any significant change in the levels of transgene protein expression. This data clearly suggested that TRAMP mice are suitable model for our proposed pre-clinical trial.



**Fig. 6: Effect of sanguinarine on protein levels of T-antigen in the prostate of TRAMP mice.** The effect of sanguinarine on the protein levels of T-antigen was assessed by immunoblot analyses using anti SV40TagAg antibody obtained from Santa Cruz Biotechnology., Inc. Equal loading was confirmed by reprobng the blot with  $\beta$ -actin. The data shown here are representative of three independent immunoblots with similar results.

However, these are very preliminary short-term studies conducted with very limited number of animals. Thus, while these studies provide a rationale and support our working hypothesis, any conclusion can not be drawn from these data.

#### **Future Plan:**

The nude mice studies are currently ongoing; and we are performing histological-, immunohistochemical- Real-Time PCR- and Western Blot- analyses to unravel the effect of sanguinarine on apoptosis, proliferation, NF- $\kappa$ B pathway, Bcl-2 family protein and *cyclin kinase inhibitor-cyclin-cyclin dependent kinase network* in athymic nude mice implanted with CWR22Rv1 tumors.

We are currently breeding the TRAM mice and should be able to obtain sufficient number of animals to start the detailed trial in the next month.

In addition, we are also conducting detailed experiments to delineate the involvement of NF- $\kappa$ B pathway during anti-proliferative effects of sanguinarine. For these studies, we plan to use human prostate carcinoma cells viz. LNCaP (androgen-responsive), DU145 (androgen-unresponsive), PC-3 (androgen-unresponsive), 22Rv1 (androgen-responsive) and normal human prostate epithelial PrEC cells. We plan to specifically investigate the effect of sanguinarine on  $\alpha$ -dihydrotestosterone-mediated activation of NF- $\kappa$ B. These experiments should not be started because, at present, we do not have an access to normal PrEC cells. The cells were purchased twice but failed to grow. The vendor (Cambrex Inc.) is replacing the cells and we should be able to start the experiments shortly.

#### **Key Research Accomplishments:**

Based on our progress in the last 12 months, the key research accomplishments are itemized below.

1. *We have demonstrated that sanguinarine possesses chemopreventive/anti-proliferative effects against prostate cancer in an athymic nude mice xenograft model. Further, our data suggested*

*that sanguinarine-caused effects are mediated via modulations in NF- $\kappa$ B pathway and cyclin kinase inhibitor-cyclin-cyclin dependent kinase machinery.*

2. *Our pilot study has shown that transgenic TRAMP model, which mimics human disease, is suitable for chemoprevention/intervention studies with sanguinarine*

### **Reportable Outcome:**

The following publications are associated with the funding from the DOD.

1. Adhami VM, Aziz MH, Reagan-Shaw SR, Nihal M, Mukhtar H, Ahmad N. Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery. *Mol Cancer Ther.* 2004 Aug;3(8):933-40.
2. Aziz MH, Siddiqui IA, Ahsan H, Reagan-Shaw SR, Ahmad N. Preclinical evaluation of plant alkaloid sanguinarine against prostate cancer development in a nude mice xenograft model. *Proc Amer Assoc Cancer Res* 46: 1012-1013, 2005.
3. Reagan-Shaw S and Ahmad N: Silencing of polo like kinase (Plk) 1 via siRNA causes induction of apoptosis and impairment of mitosis-machinery in human prostate cancer cells: Implications for the treatment of prostate cancer. *FASEB J* 19: 611-3, 2005. A detailed version of this paper is available at <http://www.fasebj.org/cgi/reprint/04-2910fjev1?ijkey=g/sVJ.EQHQP7w&keytype=ref&siteid=fasebj>.
4. Reagan-Shaw S, Breur J and Ahmad N: Enhancement of UVB radiation-mediated apoptosis by sanguinarine in HaCaT human immortalized keratinocytes. *Mol Cancer Ther* (In press).
5. Aziz MH, Fu VX, Jarrard DF and Ahmad N: Resveratrol-caused apoptosis of human prostate carcinoma LNCaP cells is mediated via modulation of PI3K/Akt pathway and Bcl-2 family proteins. *Mol Cancer Ther* (Acceptable pending revision).

### **Conclusions:**

In conclusion, we have made significant progress in the last 12 months of funding of this proposal by the Prostate Cancer Research Program of the 'US Army Medical Research and Material Command'. Further, we are confident that our ongoing *in vitro* work in cultured human PCa cells and *in vivo* work in athymic nude mice implanted with human PCa cells and in the transgenic TRAMP mice (which spontaneously develop metastatic-CaP that closely mimics human disease) will provide useful information regarding the anti-prostate cancer effects of this promising plant-based agent. Further, in the third year of funding we will start detailed experiments employing TRAMP mice to study the effect of sanguinarine against prostate cancer under *in vivo* situations. We will also evaluate the effect of sanguinarine on i) induction of apoptosis, ii) cellular proliferation, and iii) modulations in NF- $\kappa$ B-pathway, during sanguinarine-mediated inhibition of prostate tumorigenesis in TRAMP mice.

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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  $\mu\text{mol/L}$ ) 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<sub>0</sub>-G<sub>1</sub> 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

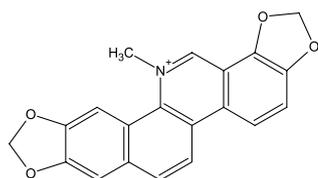
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**Figure 1.** Chemical structure of sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5]phenanthridinium).

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 anti-proliferative 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<sub>2</sub> 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  $2 \times 10^5$  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 MgCl<sub>2</sub>, 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, Foto-dyne Inc., Hartland, WI).

#### Quantitation of Apoptosis by Flow Cytometry

The cells were grown at a density of 1 × 10<sup>6</sup> 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 re-suspended 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<sub>3</sub>VO<sub>4</sub>, 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

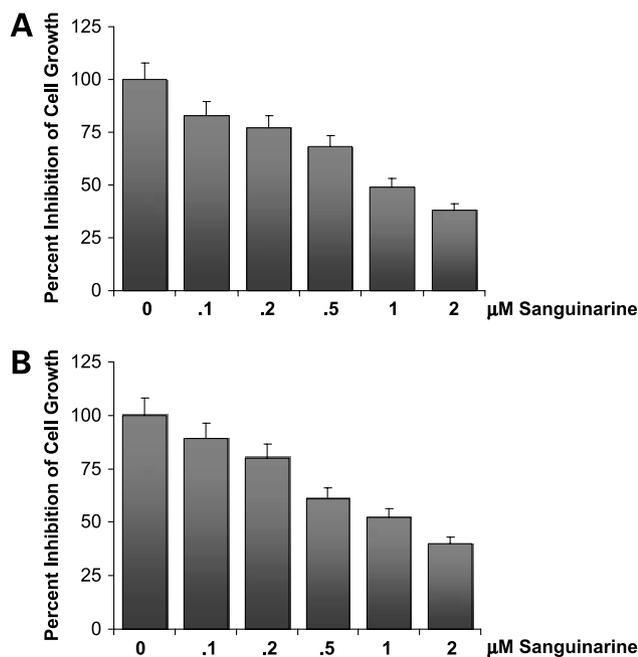
Results were analyzed using a two-tailed Student's *t* test to assess statistical significance. Values of *P* < 0.05 were considered statistically significant.

## Results and Discussion

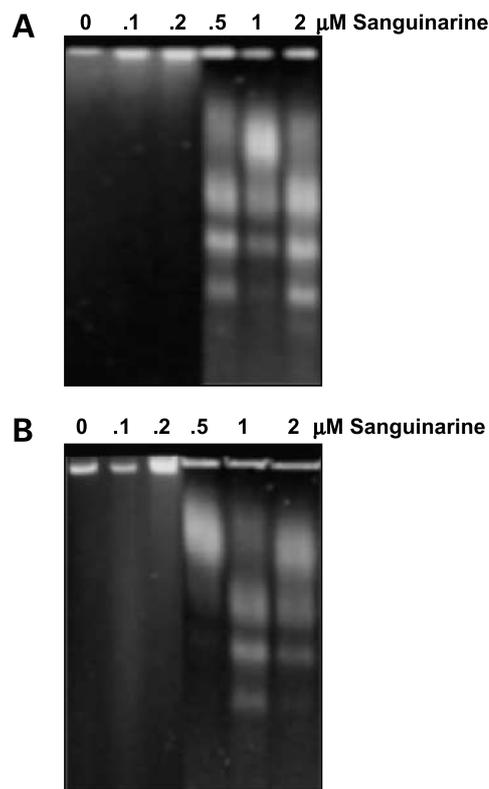
Prostate cancer in humans progresses from an androgen-responsive to an androgen-unresponsive state, and at the time of clinical diagnosis, most prostate cancers represent a mixture of androgen-responsive and androgen-unresponsive cells (29). Whereas androgen-responsive cells undergo rapid apoptosis on 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 cell 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  $\mu\text{mol/L}$ ) treatment of DU145 and LNCaP cells resulted in dose-dependent decrease in the growth of both cell types (Fig. 2). Interestingly, an  $\text{IC}_{50}$  of  $\sim 1 \mu\text{mol/L}$  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  $\mu\text{mol/L}$ ), 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  $\mu\text{mol/L}$ ) 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

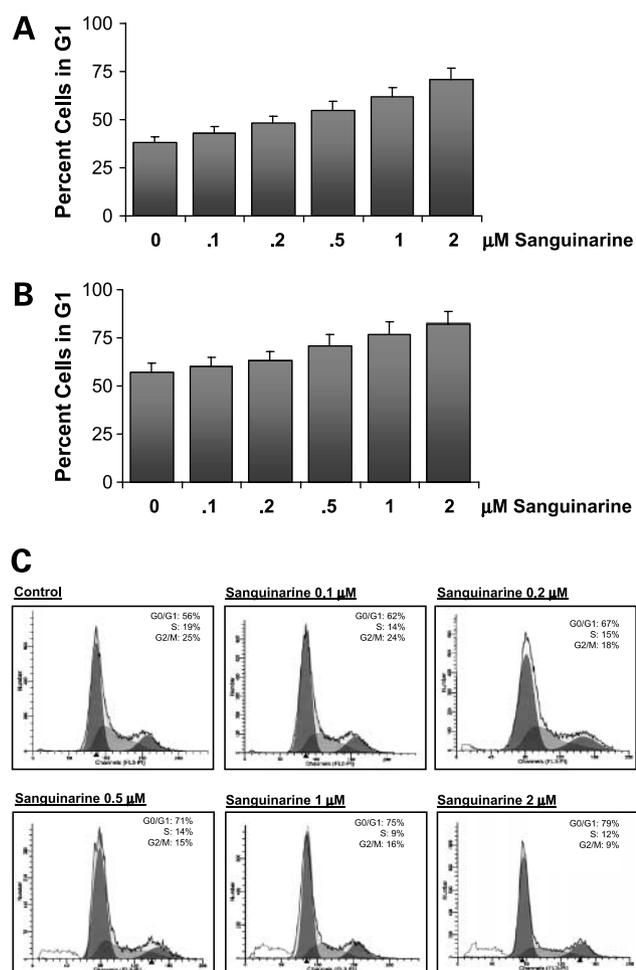
Treatment	% Apoptosis	
	LNCaP Cells	DU145 Cells
Control	2.7 $\pm$ 0.03	3.1 $\pm$ 0.02
Sanguinarine ( $\mu\text{mol/L}$ )		
0.1	4.4 $\pm$ 0.25	5.3 $\pm$ 0.36
0.2	6.1 $\pm$ 0.08	5.9 $\pm$ 0.08
0.5	12.2 $\pm$ 0.38*	13.2 $\pm$ 0.68*
1.0	19.0 $\pm$ 2.34 <sup>†</sup>	18.6 $\pm$ 3.11 <sup>†</sup>
2.0	41.1 $\pm$ 2.47 <sup>‡</sup>	43.7 $\pm$ 4.13 <sup>‡</sup>

Cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2  $\mu\text{mol/L}$ ) 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  $\pm$  SE of three experiments done in triplicate.

\* $P < 0.05$ .

<sup>†</sup> $P < 0.01$ .

<sup>‡</sup> $P < 0.001$ .



**Figure 4.** Effect of sanguinarine on cell cycle in prostate cancer cells. The growing cells ( $\sim 60\%$  confluent) were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2  $\mu\text{mol/L}$ ) 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.

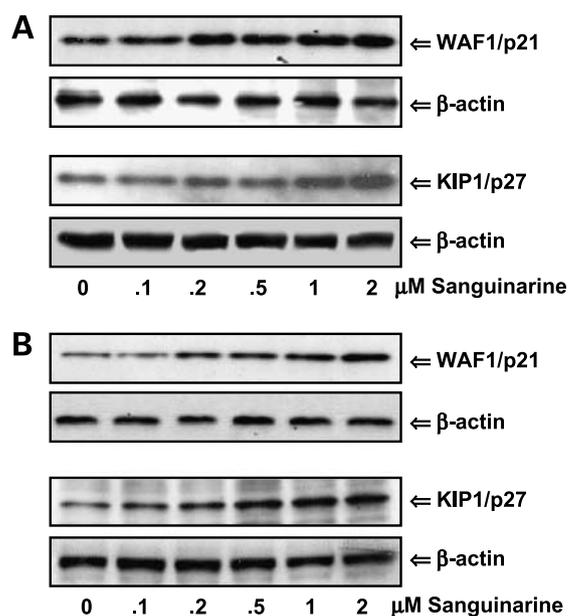
treatment of both androgen-responsive LNCaP cells and androgen-unresponsive DU145 cells resulted in the formation of DNA ladder, a hallmark of apoptosis. These results were further verified by terminal deoxynucleotidyl transferase-mediated nick end labeling assay.

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

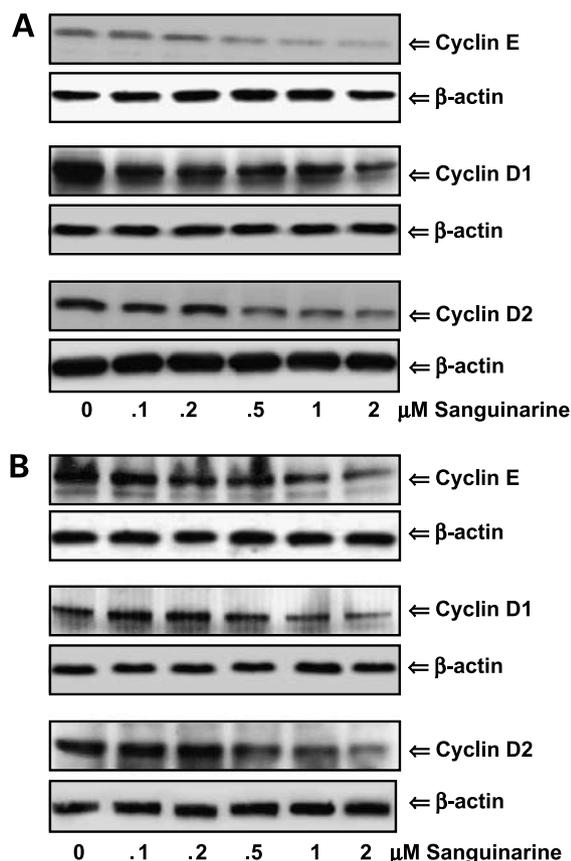
damage (31) and represents a distinct form of cell death that differs from necrotic cell death (32). Hence, agents that can modulate apoptosis may be useful in the management and therapy of cancer (33, 34).

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<sub>1</sub> 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<sub>1</sub>-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



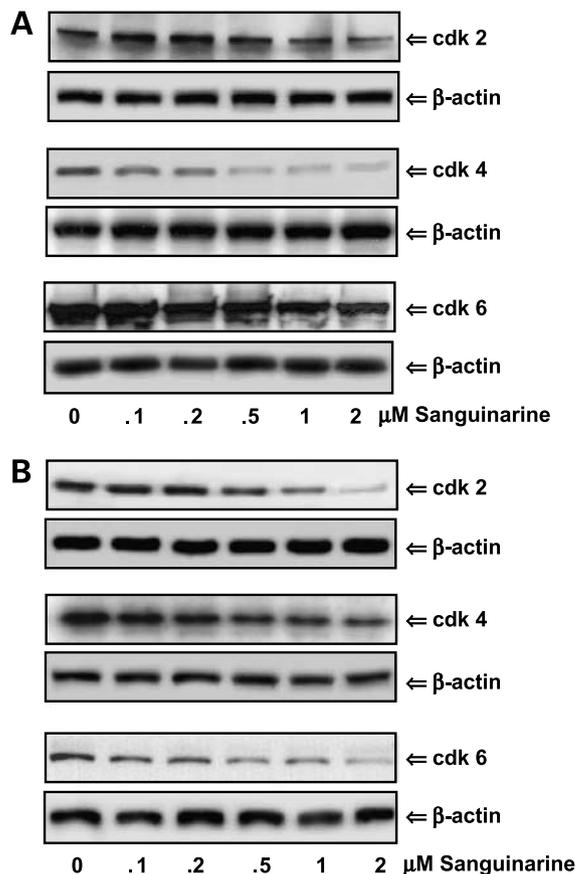
**Figure 5.** Effect of sanguinarine on the protein expression of p21/WAF1 and p27/KIP1 in prostate cancer cells LNCaP (**A**) and DU145 (**B**). The cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2  $\mu\text{mol/L}$ ) and harvested at 24 hours following the treatment. Total cell lysates were prepared and protein (50  $\mu\text{g}$ ) 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  $\beta$ -actin. Data are representative of a typical experiment repeated three times with similar results.



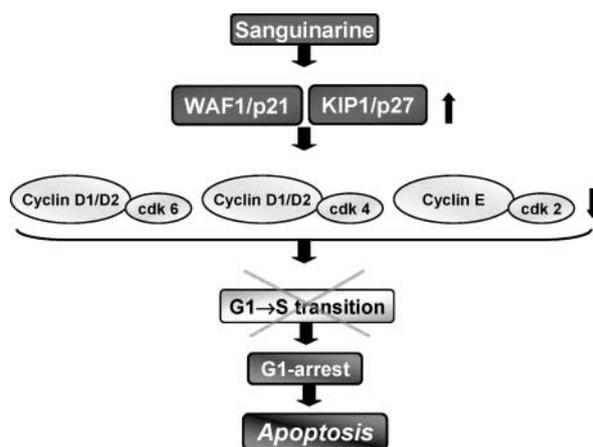
**Figure 6.** Effect of sanguinarine on the protein expression of cyclin E, D1, and D2 in prostate cancer cells LNCaP (A) and DU145 (B). The cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2  $\mu\text{mol/L}$ ) and harvested at 24 hours following the treatment. Total cell lysates were prepared and protein (20  $\mu\text{g}$ ) 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  $\beta$ -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 cdk (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  $\mu\text{mol/L}$  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 cdk (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  $\mu\text{mol/L}$ ) and harvested at 24 hours following the treatment. Total cell lysates were prepared and protein (20  $\mu\text{g}$ ) 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  $\beta$ -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 cdk 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  $\mu\text{mol/L}$  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  $\mu\text{mol/L}$ ) 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 cdk 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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Appendix-2  
**96th Annual Meeting**  
**April 16-20, 2005**  
**Anaheim/Orange County, CA**



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<b>Abstract Number:</b>	4286
<b>Presentation Title:</b>	Preclinical evaluation of plant alkaloid sanguinarine against prostate cancer development in a nude mice xenograft model.
<b>Presentation Start/End Time:</b>	Tuesday, Apr 19, 2005, 8:00 AM -12:00 PM
<b>Board Number:</b>	Board #11
<b>Author Block:</b>	<i>Moammir H. Aziz, Imtiaz A. Siddiqui, Haseeb Ahsan, Shannon R. Reagan-Shaw, <u>Nihal Ahmad</u>.</i> University of Wisconsin, Madison, WI

Prostate Cancer (PCa) is one of the most common malignancies of men in the USA and many other countries in world. Each year ~543,000 new cases are reported worldwide and the disease kills 200,000 (mostly older men) in developed countries. The traditional surgery and therapy has not been successful in the management of PCa. Therefore, the search for novel agents and approaches for the treatment of PCa continues. Chemopreventive strategies, especially with naturally occurring plant-based agents, have shown promise for prevention as well as treatment of PCa. We recently demonstrated that sanguinarine(13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium), derived from the root of *Sanguinaria Canadensis* and other poppy-fumaria species, causes cell cycle arrest and apoptotic death of human prostate carcinoma cells (*Mol Cancer Ther* 3: 933-940, 2004). Sanguinarine is a benzophenanthridine alkaloid and a structural homologue of chelerythrine and has been shown to possess anti-microbial, antioxidant and anti-inflammatory properties. In this study, we determined the chemopreventive and therapeutic potential of sanguinarine against prostate cancer *in vivo* in athymic nude mice implanted with androgen responsive human prostate carcinoma CW22Rv1 cells. For this purpose, CW22Rv1 cells ( $1 \times 10^6$  cells in 50  $\mu$ l RPMI + 50  $\mu$ l Matrigel) were implanted in athymic nude mice by a sub-cutaneous injection on left and right sides, below the shoulders (2 tumors/mouse). The animals were treated with sanguinarine (1 or 5 mg/kg body weight in 0.2 ml PBS, five days a week) by intra-peritoneal injection either one week post cell implantation to establish the preventive potential or after the development of a sizable tumor (200 mm<sup>3</sup>) to examine the therapeutic potential. The control animals received vehicle only. Our data demonstrated that sanguinarine (both pre- and post- treatments) resulted in a highly significant inhibition in the rate of tumor growth as assessed by a regression analysis. Further, the Kaplan-Meier Analysis demonstrated that in sanguinarine treated animals (post-treatment), the rate of tumor growth (to reach to a 1000 mm<sup>3</sup> target volume) was significantly delayed. Furthermore, treatment of mice with sanguinarine (both pre- and post- tumor) resulted in a significant reduction in serum levels of prostate-specific antigen (PSA) in nude mice implanted with CWR22Rv1 cells. This study, for the first time, demonstrated the chemopreventive and therapeutic effects of sanguinarine against PCa development under *in vivo* situations. Based on our data, we suggest that sanguinarine is a promising candidate for chemoprevention and/or intervention against PCa.

**96th Annual Meeting**  
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## **Silencing of polo-like kinase (Plk) 1 via siRNA causes induction of apoptosis and impairment of mitosis machinery in human prostate cancer cells: implications for the treatment of prostate cancer**

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### **ABSTRACT**

Prostate cancer (PCa) is one of the most common cancers in men. Each year ~543,000 new cases are reported worldwide, and the disease kills 200,000 (mostly older men) in developed countries. The existing treatment approaches and surgical intervention have not been able to effectively manage this dreaded cancer and, therefore, continuing efforts are ongoing to explore novel targets and strategies for the management of PCa. The activity of polo-like kinase 1 (Plk1) is elevated in tissues and cells with a high mitotic index, including cancer cells. An increasing body of evidence suggests that the level of Plk1 expression has prognostic value for predicting outcomes in patients with some cancers. A close correlation between Plk1 expression and carcinogenesis has been documented. However, the role of Plk1 in PCa is not known. We propagated a hypothesis that Plk1 inhibition will result in elimination of human PCa cells via a mitotic arrest followed by apoptosis (1). To define the role of Plk1 in PCa, we used the technique of RNA silencing via small interfering RNA (siRNA). First, using a series of human prostate carcinoma cells and normal human prostate epithelial (PrEC) cells, we assessed Plk1 levels in PCa. Immunoblot analyses clearly showed a significant expression of Plk1 in LNCaP, DU145, and PC3 human PCa cells. Interestingly, Plk1 was not detectable in normal PrEC cells. Next, we transfected the PCa cells with Plk 1 siRNA, which resulted in a significant inhibition in Plk1 protein in all PCa cells. Plk1 depletion resulted in a decrease in cell viability and induction of apoptosis in PCa cells but had no appreciable effect in normal PrEC cells. Our data also demonstrated that Plk1 siRNA transfection of PCa cells resulted in 1) a mitotic cell cycle arrest, 2) failure of cytokinesis, and 3) defects in centrosome integrity and maturation. Thus, our study suggested that 1) Plk1 plays a critical role in the process of PCa development and 2) gene therapeutic approaches aimed at Plk1 or the pharmacological inhibitors of Plk1 may be developed for the management of PCa.

Key words: cell cycle • cytokinesis • centrosome

**P**rostate cancer (PCa) is a major public health concern and a leading cause of cancer-related deaths among males in the United States (2). According to estimates from the American Cancer Society, 230,110 new cases of PCa and 29,900 PCa-related deaths are predicted for the year 2004, in the United States alone (2). The available therapeutic approaches and surgery options have been proven to be inadequate for the management of PCa. At present, Americans are living longer; therefore, more cases of PCa are being diagnosed. It is believed that as the baby boomers continue to age, the numbers of new PCa cases and the associated deaths will continue to increase. It is predicted that by 2010, the number of annual cases will skyrocket to 330,000 (<http://www.pcacoalition.org>). Thus, there is an urgent need to intensify our efforts to better understand this disease and develop novel approaches and strategies for the management of PCa. A clear understanding of genetic controls of cellular proliferation and cell division may provide the basis for the rational design of specific targets and therapeutic strategies for the management of PCa. This study was designed to investigate the hypothesis that polo-like kinase (Plk) 1 plays a critical role in the development of prostate cancer, and the silencing of Plk1 will result in elimination of human PCa cells via inactivation of cyclin-dependent kinase 1 (Cdc2)/cyclin B1-mediated mitotic arrest followed by apoptosis (1).

Plks belong to a family of serine/threonine kinases and are the human counterpart of polo in *Drosophila melanogaster* and of CDC5 in *Saccharomyces cerevisiae* (3–5). Plk1 has been shown to be intimately involved in spindle formation and chromosome segregation during mitosis and, therefore, in the regulation of cell cycle (6–9). The activity of Plk1 has been found to be elevated in tissues and cells with a high mitotic index, including cancer cells, and Plk1 expression levels are believed to have prognostic value for predicting outcomes in patients with some cancers (10–14). Studies have suggested that Plk1 is an important regulator of many processes involved in cell division and cell cycle progression (3, 15). Studies have also shown that depletion of Plk1 results in an induction of apoptosis of certain cancer cells (7, 16). The role of Plk1 in the development or progression of PCa is not well understood. Here, to study the role of Plk1 in PCa, we used small interfering RNA (siRNA) to specifically deplete Plk1 in human prostate cell lines. A targeted siRNA depletion of Plk1 resulted in 1) a significant decrease in cell viability, 2) induction of apoptosis, 3) defects in important mitosis processes (failure of cytokinesis, and defects in centrosome integrity and maturation), and 4) G2/M phase arrest in several human PCa cell lines representing different stages of disease progression.

## **MATERIALS AND METHODS**

### **Cell culture**

Three human prostate carcinoma cell lines differing in androgen association as well as p53 status were used in this study. The cell lines, namely LNCaP (wild-type p53, androgen-responsive), DU145 (mutant p53, androgen-unresponsive), and PC-3 (p53 null, androgen-unresponsive), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS). Normal human prostate epithelial (PrEC) cells obtained from Clonetics (Walkersville, MD) were cultured in prostate epithelial cell medium (Clonetics). All the cells were maintained at standard cell culture conditions (37°C, 5% CO<sub>2</sub> in a humidified incubator).

## **Depletion of Plk1 by siRNA**

The siRNA SMARTpool used in this study contained four pooled siRNA duplexes with “UU” overhangs and a 5' phosphate on the antisense strand. Thus, a mixture of several siRNAs ensures an effective depletion of the Plk1 gene in the cells. The cells were plated in six-well plates ( $3.5 \times 10^5$ ) or two-well slides ( $1.5 \times 10^5$ ). Twenty-four hours later, the cells were transfected with 100 nM Plk1 siRNA SMARTpool or nonsense siRNA (Dharmacon, Lafayette, CO) using oligofectamine reagent (Invitrogen) as described by the manufacturer.

Briefly, both the siRNA and the oligofectamine reagent were diluted with serum-free media in two-thirds the transfection volume for 10 min. Then, the diluted oligofectamine was added to the diluted siRNA and incubated at room temperature for 20 min. The culture medium was aspirated from the cells, the cells were washed with serum-free media, and the siRNA-oligofectamine complex was added drop-wise to the cells and incubated at 37°C for 4 h. Then, one-third volume media with 30% FBS was added, and the cells were incubated for another 20 h (for a total incubation of 24 h). At this time, the cells were ready for further experiments as described below.

## **Trypan blue exclusion assay**

Trypan blue exclusion assay was used to assess the effects of siRNA transfection on the growth and viability of PCa cells. Briefly, following the transfection of cells with siRNA, as described above, the culture media was collected in a 1.5 mL Eppendorf tube. The cells were trypsinized and collected in the same Eppendorf tube. The cells were pelleted by centrifugation, and the cell pellet was resuspended in phosphate-buffered saline (PBS) (300  $\mu$ L). Trypan blue (0.4% in PBS; 10  $\mu$ L) was added to a smaller aliquot (10  $\mu$ L) of cell suspension, and the number of cells (viable-unstained and nonviable-blue) were counted using a haemocytometer.

## **Western blot analysis**

Following the transfection of cells with Plk1 siRNA, as described above, the medium was aspirated and the cells were washed with ice-cold PBS (10 mM, pH 7.4). Ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% NP-40) with freshly added PMSF (1 mM) and protease inhibitors (10  $\mu$ g/ml; Protease Inhibitor Cocktail Set III, Pierce, Rockford, IL) was added to the plates. The cells were then scraped, and the suspension (cells with lysis buffer) was transferred into a centrifuge tube and was placed on ice for 15 min with occasional inversion to ensure a complete lysis of the cells. The cell suspension was cleared by centrifugation at 14,000g for 15 min at 4°C, and the supernatant (total cell lysate) was either used immediately or stored at -70°C. The protein concentration was determined using the BCA Protein Assay (Bio-Rad Laboratories, Hercules, CA) as per the manufacturer's protocol.

For immunoblot analysis, 30  $\mu$ g protein was subjected to SDS-PAGE (using 10–12% Tris-glycine gel). The protein was transferred onto a nitrocellulose membrane and blocked with TBS-Tween (0.1%) plus 5% dry milk. The membrane was probed with an appropriate primary antibody followed by a secondary horseradish peroxidase (HRP)-conjugated antibody. The membrane was detected by freshly prepared chemiluminescent solution (100 mM Tris-HCl [pH 8.5], 0.018% H<sub>2</sub>O<sub>2</sub> [v/v], 1.25 mM Luminol, 225 nM Coumaric acid). The following antibodies were used: anti-Plk1 (BD Transduction Laboratories, San Diego, CA), anti-Cdc25C (Cell

Signaling Technology, Beverly, MA), anti-cdc2 (Cell Signaling Technology), anti-<sup>Thr161</sup>phospho-cdc2 (Cell Signaling Technology), anti-cyclin B1 (Cell Signaling Technology), and anti- $\beta$ -actin (Sigma Chemical Company, St. Louis, MO). The quantification of proteins was performed by a digital analysis of protein bands (TIFF images) using UN-SCAN-IT software (Silk Scientific, Orem, UT).

### **Apoptosis assay by confocal microscopy**

The effect of Plk1 depletion on apoptosis was assessed by using the Vybrant apoptosis assay kit (Molecular Probes, Eugene, OR) as per the manufacturer's protocol. Briefly, the cells were transfected with Plk1 siRNA in a two-well culture slide as described above. At 24 h posttransfection, the media was aspirated and the cells were washed with PBS. The cells were then incubated with annexin binding buffer (1 $\times$ ), FITC-annexin V, and propidium iodide (PI) for 45 min at room temperature. The cells were washed with annexin binding buffer (1 $\times$ ) followed by PBS. The cells were mounted in PBS using coverslips and visualized with a Bio-Rad MRC1000 scan head mounted transversely to an inverted Nikon Diaphot 200 microscope in the Keck Neural Imaging Lab at the University of Wisconsin.

### **Apoptosis and cell cycle analysis by flow cytometry**

The extent of apoptosis and cell cycle distribution was assessed with the APO-BrdU TUNEL apoptosis assay kit (Molecular Probes) as per the manufacturer's protocol. Briefly, at 24 h posttransfection, the culture media was collected. The cells were gently trypsinized and added to the culture media and pelleted by centrifugation. The pellet was washed with PBS and counted, and the cells ( $1 \times 10^6$ ) were fixed overnight in ethanol (90%). The cells were washed and labeled with UTP-BrdU overnight, washed again with PBS, and incubated with Alexa488 Anti-BrdU antibody followed by counterstaining with PI. Cells were analyzed using a FACScan benchtop cytometer (BD Biosciences, San Jose, CA) in the UWCCC Flow Cytometry Facility in the University of Wisconsin. The analyses were performed using Cell Quest software (BD Biosciences) for apoptosis and ModFit LT software (Verity Software House, Topsham, ME) for cell cycle analysis.

### **Immunocytochemistry**

For immunocytochemical studies, the cells were treated with siRNA in a two-well culture slide as described above. At 24 h posttransfection, the media was aspirated and the cells were fixed with ethanol/methanol (1:1) for 1 min at room temperature. Cells were blocked with 8% BSA in PBS for 1 h at room temperature. The cells were labeled with  $\alpha$ -tubulin or  $\gamma$ -tubulin primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h followed by incubation with a secondary FITC-conjugated antibody for 1.5 h. Nucleic acids were visualized by staining with PI for 30 min. Cells were mounted in Vectashield Hardset Mounting Medium (Vector Labs, Burlingame, CA). Cells were visualized with a Bio-Rad MRC1000 scan head mounted transversely to an inverted Nikon Diaphot 200 microscope in the Keck Neural Imaging Lab at the University of Wisconsin.

## Statistical analysis

The results are expressed as the mean  $\pm$  SE. Statistical analysis of the data between Plk1-depleted cells and untreated cells for each cell line was performed by Student's *t* test.  $P < 0.01$  was considered statistically significant.

## RESULTS AND DISCUSSION

This in vitro study was an effort to investigate whether Plk1 could be exploited as a target for designing novel approaches for the treatment of PCa, which is a growing concern worldwide, more so in developed countries. Plk1 is the most well studied member of the Plk family of conserved serine-threonine kinases. The polo kinase family includes mammalian Plk1, Plk2, Plk3, mouse Snk and Fnk, *Drosophila* polo, *Xenopus laevis* Plx1, and budding yeast Cdc5, which are all key regulators for mitotic progression (3, 4, 17). Plk1 has been shown to be a key regulator of mitotic progression by playing a critical role in several events that are important for cell division (6–9, 15, 18–22).

Recent studies have suggested that Plk1 could be a useful target for antitumor therapies. However, an exact role of Plk1 in PCa is not well-established. Recently, we propagated the hypothesis that Plk1 plays a critical role in the development of PCa, and the silencing of Plk1 will result in elimination of human PCa cells via an inactivation of cyclin-dependent kinase 1 (Cdc2)/cyclin B1-mediated mitotic arrest followed by apoptosis (2). In the first step of our study, using siRNA, we determined the effect of a targeted depletion of the Plk1 gene on the viability of three types of human PCa cells (LNCaP, DU145, and PC-3, differing in multiple respects as detailed in Materials and Methods) and PrEC cells. For an efficient depletion of Plk1, we used a siRNA SMARTpool that is a mixture of four siRNAs targeted at different areas of the Plk1 coding region. Western blot analysis demonstrated that Plk1 protein was abundantly present in PCa cells ([Fig. 1A](#)) but was virtually undetectable in normal PrEC cells (data not shown). This observation is consistent with reported literature showing that Plk1 is differentially expressed (in high detectable levels) in cancer cells vs. normal cells. This observation makes Plk1 a target for development of novel strategies for the management of PCA.

Our data demonstrated that the targeted depletion of Plk1 resulted in a significant decrease in 1) the endogenous levels of Plk1 protein (50–72% decrease; [Fig. 1B](#)), 2) the viability of cells (45–72% decrease; [Fig. 1C](#)), and 3) the growth of cells (19–21% decrease; [Fig. 1C](#)) in all PCa cell lines studied. On the other hand, the similar concentration (100 nM) of Plk1 siRNA had no effect on the viability or growth of normal PrEC cells ([Fig. 1C](#)). These observations suggested that Plk1 depletion, via siRNA, could be useful in controlling the growth of PCa cells without compromising the viability of normal prostate cells.

Because Plk1 depletion has been shown to induce apoptosis in cancer cells (6–8, 16), our next aim was to determine whether or not the loss of cell viability and growth inhibition was mediated by the induction of apoptosis in PCa cells. As shown by confocal microscopy ([Fig. 2A](#)), the transfection of PCa cells with Plk1 siRNA resulted in an appreciable annexin V binding (green fluorescence), indicating an increased externalization of phosphatidylserine on the outer leaflet of the plasma membrane that is an established early sign of apoptosis in the cells. The control cells (not treated with Plk1 siRNA) failed to show fluorescent staining (data not shown). A

higher concentration of Plk1 siRNA (200 nM) also resulted in significant necrosis (red and green fluorescence) along with cell death (red fluorescence) in the PCa cells (data not shown). To quantitate this apoptosis, we used an APO-BrdU assay that quantitatively measures the extent of apoptosis in cells. This assay is based on the principle that when DNA strands are cleaved (apoptosis), a large number of 3'-hydroxyl ends are exposed, which are detected using an Alexa Fluor 488 dye-labeled anti-BrdU monoclonal antibody. This kit also provides PI for determining total cellular DNA content. As shown by the data in [Fig. 2B](#) and [2C](#), we found that Plk1 depletion by siRNA resulted in significant apoptosis (46–57%) in PCa cells (all three cell lines used) when compared with untreated cells (1–3% apoptosis; data not shown). It is important to mention that transfection of PCa cells with nonsense siRNA (100 nM) did not result in any appreciable effect on cell viability, cell growth, or apoptosis (data not shown). The effect of Plk1 depletion on cell cycle distribution was also determined using this assay. As shown by the analysis of data ([Fig. 2D, 2E](#)), the depletion of Plk1 resulted in a significant accumulation of PCa cells in G2/M phase of the cell cycle (4N DNA content).

Studies have demonstrated that microinjection of Plk1-specific antibodies resulted in abnormal distribution of condensed chromatin and monoastrial microtubule arrays that were nucleated from duplicated but unseparated chromosomes (23). To assess the effect of Plk1 depletion on centrosome abnormalities, we immunolabeled the cells with antibodies directed against  $\gamma$ -tubulin. Plk1 depletion was found to cause significant abnormalities in the distribution of centrosomes. The distribution of centrosome was found to be defective in 54–60% of Plk1-depleted cells as compared with 25–28% in control cells ([Fig. 3A–D](#)). Thus, a high percentage of Plk1-depleted cells showed unseparated chromosomes and multiple centrosomes in a single nuclear membrane, indicating abnormal centrosome distribution in interphase cells. By closely observing Plk1-depleted cells using confocal microscopy, we found three different populations of chromatin structures in PCa cells: 1) normal chromatin, 2) dumbbell-like, and 3) fragmented, indicating defects in centrosome integrity. About 17–20% of Plk1-depleted cells showed dumbbell-like chromatin structures, as compared with <2% of control cells ([Fig. 3D](#)). Similarly, 45–51% of Plk1-depleted cells showed fragmented chromatin ([Fig. 3D](#)). This phenotype, in fact, is consistent with the subgenomic DNA population previously observed ([Fig. 2D, 2E](#)). Earlier studies have shown similar results in other cancer cells (6, 7, 9).

The appearance of a dumbbell-like DNA structure in Plk1-depleted PCa cells indicated that sister chromatids had not completely separated. Staining of cells with  $\alpha$ -tubulin confirmed that these connected chromosomes were within one cell, probably in the interphase. Several studies have established the role for Plk1 in the process of cytokinesis (6, 9). It was interesting to observe that the targeted depletion of Plk1 resulted in a failure of cytokinesis in 20–25% of PCa cells, as compared with a very small percentage in untreated cells ([Fig. 3E, 3F](#)). As revealed by  $\alpha$ -tubulin immunofluorescence, these defective cells were found to be connected by a cytoplasmic bridge.

Depletion of Plk1 has been shown to disrupt mitotic cell cycle progression via the Cdc25C and cdc2/cyclin B1 positive feedback loop at the onset of mitosis (6). Therefore, we were interested in defining the role of Cdc25C and cdc2/cyclin B1 in Plk1 depletion-mediated mitotic arrest of PCa cells. As shown by Western blot analysis, the targeted depletion of Plk1 resulted in significant accumulation of Cdc25C, cdc2, phospho-cdc2, and cyclin B1 proteins ([Fig. 4A, 4B](#)). Studies have shown that phosphorylation of the protein phosphatase Cdc25C by Plk1 promotes nuclear localization of Cdc25C during prophase (23). In Plk1-depleted PCa cells, we found an

accumulation of Cdc25C in the interphase, indicating that Cdc25C has not been translocated to the nucleus and thus had not been phosphorylated (Fig. 4A, 4B). Phosphorylated Cdc25C activates the cdc2/cyclin B1 complex by dephosphorylating cdc2, resulting in the initiation of mitotic events (22). Our data clearly demonstrated that Plk1 depletion in PCa cells resulted in a significant up-regulation in the protein levels of total cdc2 and phosphorylated cdc2 (Fig. 4A, 4B). Subsequent phosphorylation of cyclin B1 promotes the rapid nuclear translocation of cdc2/cyclin B1 at the G2/M transition where cyclin B1 is then degraded (22). Our data showing an accumulation of cyclin B1 protein levels in Plk1-depleted cells further supported the hypothesis that the mechanism by which Plk1 depletion induces mitotic arrest is via the Cdc25C/cdc2/cyclin B1 feedback loop (Fig. 4A, 4B).

Taken together, our data support the hypothesis proposed earlier that Plk1 plays a critical role in PCa, and the silencing of Plk1 will result in the elimination of human PCa cells via an inactivation of cdc2/cyclin B1-mediated mitotic arrest followed by apoptosis (Fig. 5). Based on this study, it is conceivable that gene therapeutic approaches aimed at Plk1 or pharmacological inhibitors aimed at Plk1 may be developed for the management of PCa. Further, our data demonstrated that Plk1 depletion results in an effective elimination of all the three PCa cell lines observed. This is an important observation suggesting that Plk1 depletion-mediated mitotic arrest and apoptosis is a general phenomenon for PCa cells, irrespective of 1) stage of PCa progression, 2) status of tumor suppressor p53 gene, and 3) association with androgen receptor. Thus, Plk1 inhibition-based approaches may be useful for the management of early to advanced stages of PCa.

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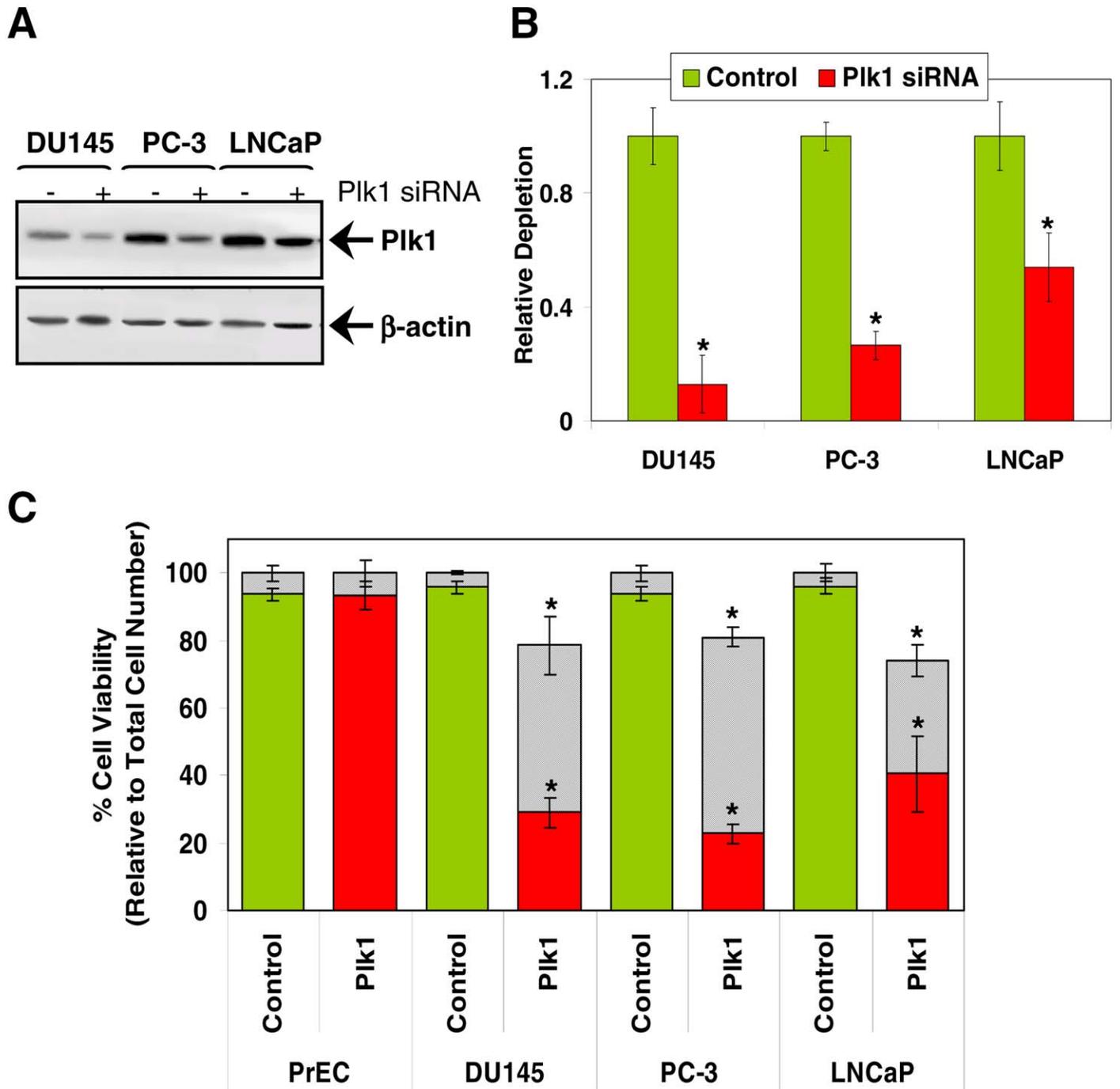
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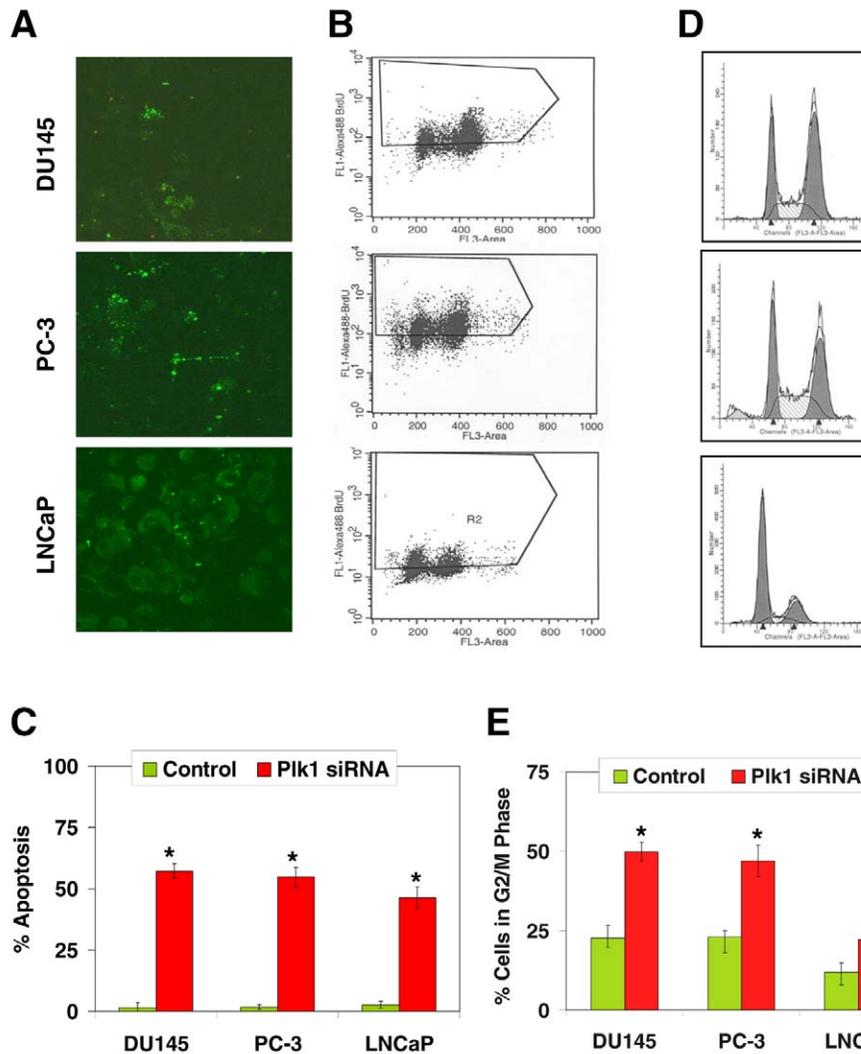
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Fig. 1



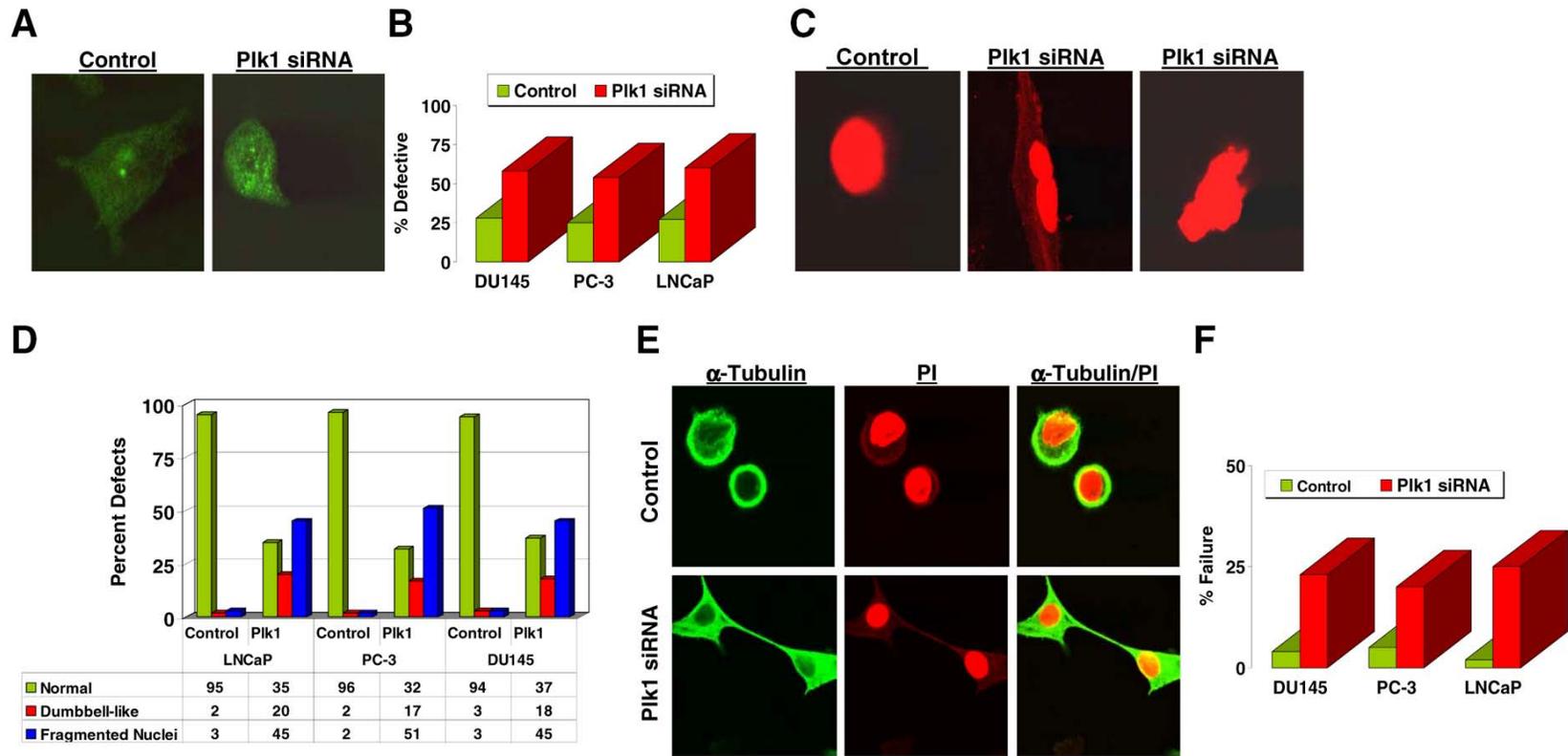
**Figure 1. Targeted depletion of Plk1 by siRNA results in a significant decrease in the viability and growth of PCa cells.** Following transfection of cells (PrEC, DU145, PC-3, and LNCaP) with Plk1 siRNA, the Plk1 protein levels were detected by Western blot analysis (A) and quantitated by densitometric analysis of protein bands (B). Equal loading was confirmed by stripping the blot and reprobing it for  $\beta$ -actin. The bands shown here are from a representative experiment repeated three times with similar results. The data (relative density normalized to  $\beta$ -actin) is expressed as mean  $\pm$  SE of three experiments ( $*P < 0.01$ ). Plk1 protein was undetectable in PrEC cells (data not shown). The effect of Plk1 depletion on cell viability and growth (C) was measured using Trypan Blue Exclusion analysis. Cell viability data are expressed as the percent viable cells (colored bars) out of the total number of cells. Cell growth data are expressed as the percent cells (hatched bars) for the Plk1-treated group relative to the untreated cells. The data are expressed as the mean  $\pm$  SE of three experiments ( $*P < 0.01$ ). Details of the experiments are given in Materials and Methods.

Fig. 2



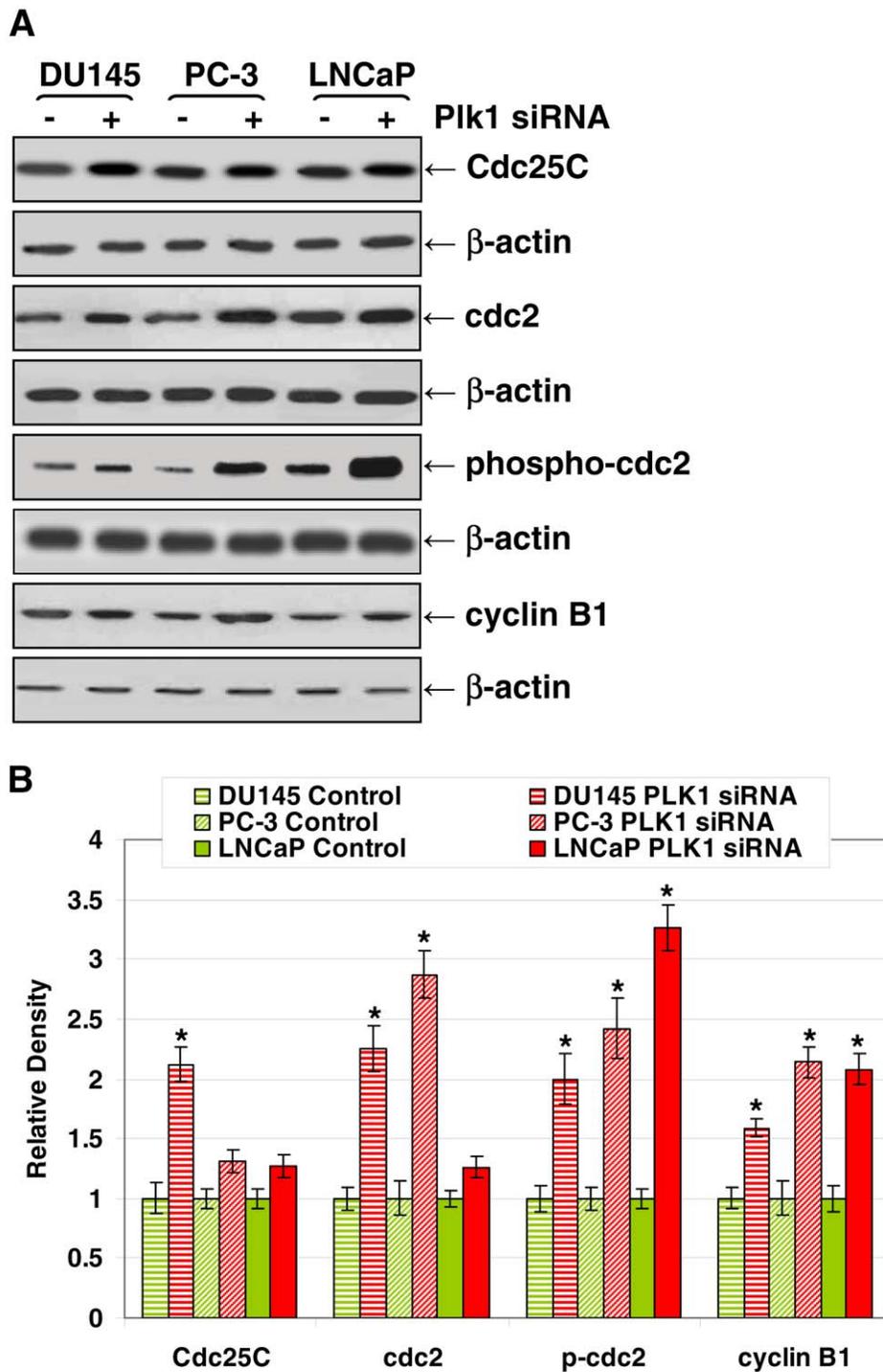
**Figure 2. Plk1 depletion results in a significant induction of apoptosis and a G2/M phase arrest in PCa cells. A)** Determination of apoptosis by confocal microscopy. The cells (DU145, PC-3, and LNCaP) were transfected with Plk1 siRNA followed by staining with FITC Annexin V and PI using Vybrant Apoptosis Assay kit (Molecular Probes, Eugene, OR). Annexin V binds to phosphatidylserine, which appears in the outer leaflet of the plasma membrane as a late sign of apoptosis. Apoptotic cells show green fluorescence, necrotic cells show red and green fluorescence, dead cells show red fluorescence, and live cells show no fluorescence. The results shown are from a representative experiment repeated three times with similar results. The cells not treated with siRNA were not stained (data not shown). **B)** Determination of apoptosis by flow cytometry. Following transfection of cells (DU145, PC-3, and LNCaP) with Plk1 siRNA, the extent of apoptosis was assessed with the APO-BrdU TUNEL Assay kit (Molecular Probes, Eugene, OR). The fragmentation of DNA in apoptotic cells is measured by BrdU incorporation, which is visualized by conjugation to an Alexa Fluor 488 dye-labeled anti-BrdU antibody. The results shown are from a representative experiment repeated three times with similar results. **C)** Quantitation of apoptosis. The extent of apoptosis was quantified by a computational analysis of cells staining positive for BrdU, using Cell Quest software (BD Biosciences, San Jose, CA). The data are expressed as mean  $\pm$  SE of three experiments ( $*P < 0.01$ ). **D)** Determination of cell cycle distribution. Following transfection of cells (DU145, PC-3, and LNCaP) with Plk1 siRNA, the cell cycle distribution was assessed by staining with PI using the APO-BrdU TUNEL Assay kit. The results shown here are from a representative experiment repeated three times with similar results. **E)** Quantification of cell cycle distribution. The quantitation of cell cycle distribution was performed using ModFit LT software (Verity Software House, Topsham, ME), and the number of cells in G2/M phase are shown. The data are expressed as mean  $\pm$  SE of three experiments ( $*P < 0.01$ ). Details of all the experiments are given in Materials and Methods.

**Fig. 3**



**Figure 3. Plk1 depletion causes defects in centrosomal distribution, centrosomal abnormalities, and failure of cytokinesis in PCa cells.** **A)** Determination of centrosome distribution. Following transfection of cells (DU145, PC-3, and LNCaP) with Plk1 siRNA, they were labeled with  $\gamma$ -tubulin antibody and visualized with a Bio-Rad (Hercules, CA) MRC1000 scan head mounted transversely to an inverted Nikon Diaphot 200. An abnormal distribution of centrosomes in cells treated with Plk1 siRNA due to localization of unseparated chromosomes and multiple centrosomes in the same nuclear membrane is shown. Representative pictures from LNCaP cells are shown. **B)** Quantification of cells with defective distribution of centrosomes. The normal and defective cells in multiple fields were counted under microscope, and the data (as percent cells with abnormal centrosomes) is shown as a histogram. **C)** Determination of centrosomal abnormalities. Following transfection of cells (DU145, PC-3, and LNCaP) with Plk1 siRNA, they were labeled with PI and visualized with a Bio-Rad MRC1000 scan head mounted transversely to an inverted Nikon Diaphot 200. Three typical images seen were normal (*left*), dumbbell-like structure (*center*), and fragmented nuclei (*right*). Representative pictures from LNCaP cells are shown. **D)** Quantification of cells with centrosomal abnormalities. The normal and defective cells in multiple fields were counted under microscope, and the data (as percent cells with abnormal centrosomes) is shown as a histogram. **E)** Determination of failure of cytokinesis. Following transfection of cells (DU145, PC-3, and LNCaP) with Plk1 siRNA, they were labeled with  $\alpha$ -tubulin and PI and visualized with a Bio-Rad MRC1000 scan head mounted transversely to an inverted Nikon Diaphot 200. The failure of cytokinesis was clearly seen in the cells where they were found to be connected by a cytoplasmic bridge (as revealed by  $\alpha$ -tubulin immunofluorescence). Representative pictures from LNCaP cells are shown. **F)** Quantification of cells with cytokinesis failure. The normal and defective cells in multiple fields were counted under microscope, and the data (as percent cells with cytokinesis failure) is shown as a histogram. Details of all the experiments are given in Materials and Methods.

Fig. 4



**Figure 4. Plk1 depletion results in significant increases in Cdc25C, cdc2, phospho-cdc2, and cyclin B1 in PCa cells.** Following transfection of cells (DU145, PC-3, and LNCaP) with Plk1 siRNA, the protein levels of Cdc25C, cdc2, phospho-cdc2, and cyclin B1 were assessed by Western blot analysis (A) and quantitated by densitometric analysis of protein bands (B). Equal loading was confirmed by stripping the blot and reprobing it for  $\beta$ -actin. The bands shown here are from a representative experiment repeated three times with similar results. The data (relative density normalized to  $\beta$ -actin) are expressed as mean  $\pm$  SE of three experiments (\* $P$ <0.01). Details of the experiments are given in Materials and Methods.

Fig. 5

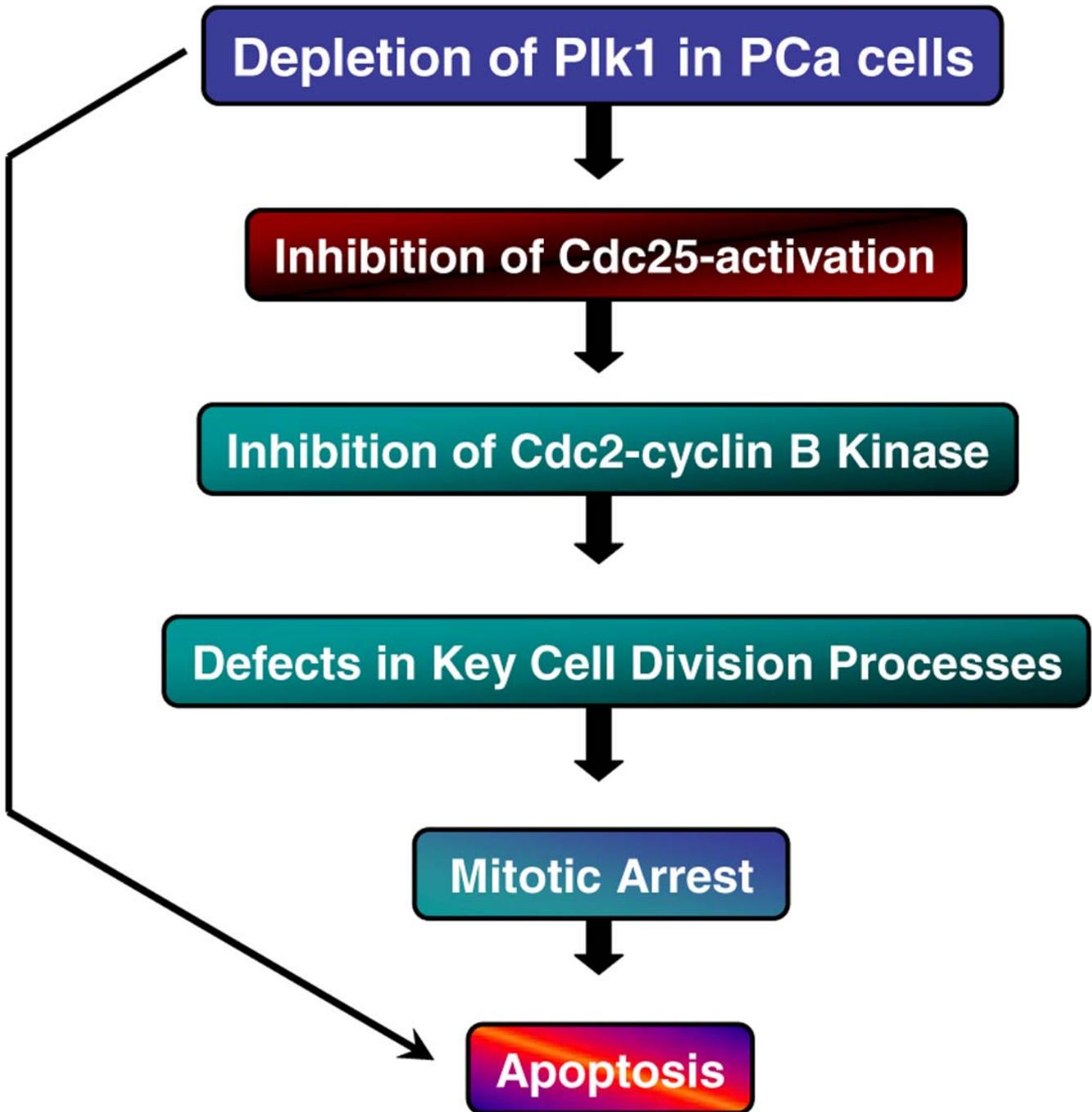


Figure 5. Proposed mechanism of Plk1 depletion-mediated elimination of human PCa cells.