

## **Silibinin Suppresses Growth of Human Prostate Carcinoma PC-3 Orthotopic Xenograft via Activation of Extracellular Signal-Regulated Kinase 1/2 and Inhibition of Signal Transducers and Activators of Transcription Signaling**

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**Abstract Purpose:** Silibinin is currently under phase II clinical trial in prostate cancer patients; however, its antitumor effects and mechanisms are not completely understood. Herein, we studied the efficacy and associated mechanisms of silibinin against orthotopically growing advanced human prostate carcinoma PC-3 tumors.

**Experimental Design:** Athymic male mice were orthotopically implanted with PC-3 cells in prostate and 1 week later after surgical recovery were gavaged daily with silibinin (100 mg/kg body weight) for 7 weeks.

**Results:** Silibinin treatment reduced the lower urogenital weight (including tumor, prostate, and seminal vesicle) by 40% ( $P < 0.05$ ) without any toxicity in mice. Silibinin decreased proliferating cell nuclear antigen expression and proliferating cells ( $P < 0.001$ ) but increased cleaved caspase-3-positive cells ( $P < 0.01$ ) and apoptotic cells ( $P < 0.001$ ) and suppressed tumor microvessel density ( $P < 0.001$ ) and vascular endothelial growth factor expression ( $P = 0.02$ ). Decreased levels of cyclin-dependent kinases 2, 4, and 6, CDC2, and cyclins D1, D3, E, and A were observed, indicating an inhibitory effect of silibinin on cell cycle progression. Silibinin showed a tremendous increase in extracellular signal-regulated kinase 1/2 phosphorylation but decreased c-Jun NH<sub>2</sub>-terminal kinase 1/2 and p38 mitogen-activated protein kinase phosphorylation. A moderate decrease in phosphorylated and total levels of Akt was also noted. A marked inhibitory effect of silibinin on signal transducers and activators of transcription (STAT) 1 (Tyr<sup>701</sup>), STAT1 (Ser<sup>727</sup>), STAT3 (Tyr<sup>705</sup>), STAT3 (Ser<sup>727</sup>), and STAT5 (Tyr<sup>794</sup>) phosphorylation together with a decrease in their total levels was also observed.

**Conclusions:** These findings provide evidence for antitumor efficacy of silibinin against orthotopically growing prostate tumor in mice with multitargeted mechanistic insights and support its clinical investigation in prostate cancer.

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in American men that accounts for 29% of total cases of cancer and 9% of deaths from cancer (1). When prostate cancer is diagnosed at the androgen-dependent stage, hormone ablation approaches are frequently employed for prostate cancer treat-

ment that often lead to hormone-refractory stage of malignancy, which also acquires the chemoresistance and radioresistance (2, 3). At present, there is no effective therapy for this advanced-stage prostate cancer, which is the cause of the observed mortality in prostate cancer patients. In such a scenario, chemopreventive approaches are being suggested to inhibit, suppress, and control various types of cancer including prostate cancer (4–6). For the success of this approach, it is desired that the agent should be nontoxic, cost-effective, and physiologically available with significant antitumor efficacy. In this regard, silibinin fulfills all the desired qualities of a chemopreventive agent along with its different pharmacologic properties and antitumor efficacy in various animal carcinogenesis models (6, 7), justifying its further investigation with molecular details in relevant animal tumorigenesis model as done in this study.

The pattern and the kinetics of human tumor growth are usually studied in athymic nude mice model. Most of the studies are done by the ectopic (subcutaneous) implantation of tumor cells in which tumor growth can be easily monitored by the noninvasive methods using digital calipers. However, the more ideal experimental model of tumor growth is when the tumor grows in its tissue microenvironment, which is

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### Translational Relevance

Silibinin is currently under phase II clinical trial in prostate cancer patients; however, its antitumor effects and mechanisms are not entirely known. Present study uses a relevant preclinical animal model of prostate cancer with orthotopically growing advanced human prostate carcinoma PC-3 tumors in athymic mice. This study reports the down-regulation of STAT, JNK1/2, p38 MAPK, and Akt signaling and an up-regulation of ERK1/2 signaling in tumors by silibinin and suggests that these molecular changes could account for antiproliferative, proapoptotic, and antiangiogenic activities of silibinin observed in this model. Together, these findings suggest the multitargeting effects of silibinin with mechanistic insight and support its clinical investigation in prostate cancer.

achieved by surgically operating the animal and orthotopically implanting the cancer cells in the target organ (8). This orthotopic xenograft model is regarded as comparatively better than ectopic xenograft to study the tumor growth and its intervention by a potential anticancer agent, as the tumor grows *in situ* as in humans (8, 9). Present study uses human PC-3 orthotopic xenograft model to study the effect of oral silibinin on tumor growth and associated molecular changes.

Lifestyle and dietary habit may account for the geographic differences in prostate cancer incidences as observed between Western and Asian countries (10–12). Silibinin is a major biologically active compound present in milk thistle and artichoke, which are consumed in various preparations (7). Silymarin, a crude mixture from milk thistle extract, contains silibinin as major bioactive compound and is commonly used as dietary supplement for treating hepatic ailments including liver cirrhosis (7). Remarkably, this compound is largely nontoxic and even very high doses are tolerated by animals (2 g/kg dose by oral gavage in mice) and human (4.7 g/d orally given as silybin-phytosome; refs. 13, 14). Our wide-ranging studies with silibinin and prostate cancer cell lines suggest for its anti-prostate cancer activity (6, 15–18). Further, we have done studies with silibinin in animal models of prostate cancer wherein we have observed its *in vivo* anti-prostate cancer activity (19–22). Recently, we studied the effect of dietary silibinin on human prostate cancer PC-3 cells xenograft, which was ectopically (subcutaneously) implanted in athymic male nude mice (21). However, its antitumor effects and associated molecular mechanisms in complex prostate microenvironment settings are largely unknown. In the present study, we employed more realistic model of PC-3 tumor growth in which these cells were surgically implanted in mouse prostate; therefore, the tumor grew within prostate tissue as observed in clinical cases. We investigated the antitumor effects of oral silibinin and *in vivo* tumor biomarkers and associated molecular alterations in tumor tissues. The observed molecular alterations indicate the multimolecular targeting effects of silibinin, in which it altered mitogen-activated protein kinase (MAPK) and signal transducers and activators of transcription (STAT) signaling as well as cell cycle regulators and proangiogenic factor for its *in vivo* antitumor efficacy against orthotopically growing human PC-3 tumors in athymic mice.

### Materials and Methods

**Tumor xenograft study.** Exponentially growing PC-3 cells (American Type Culture Collection) were detached by trypsinization, washed, and resuspended in serum-free RPMI 1640. Six-week-old athymic *nu/nu* male mice (National Cancer Institute-Frederick) were abdominally operated under sterile condition. Each mouse was injected with  $2 \times 10^6$  PC-3 cells suspended in 20  $\mu$ L serum-free medium in dorsal prostate to initiate orthotopic tumor growth. The success of the injection was confirmed by the bulge at the site of injection. Mice were kept 1 week on regular diet and water for recovery from operation. The healthy animals were maintained on AIN-93M diet (Dyets) and water *ad libitum* and randomly divided in to two groups after 1 week of surgery. One group ( $n = 8$  mice) was gavaged with 200  $\mu$ L/d saline per mouse, whereas the other group ( $n = 7$  mice) was gavaged with 100 mg/kg silibinin in 200  $\mu$ L/d saline per mouse for 5 days a week. Animals were monitored for tumor growth (by abdominal palpation), general health, body weight, and diet consumption twice weekly throughout the study, which was terminated after 7 weeks of the treatment. At necropsy, tumors were excised along with lower urogenital tract, including prostate and seminal vesicle, and weighed; part of which was fixed in buffered formalin and the rest was stored at  $-80^\circ\text{C}$  until further analysis. Animal care was in accordance with the approved protocol and institutional guidelines.

**Immunohistochemical staining for proliferating cell nuclear antigen, CD31, cleaved caspase-3, and vascular endothelial growth factor.** Tumor samples were fixed in 10% buffered formalin for 12 h and processed conventionally to prepare paraffin-embedded block. Tumor sections (5  $\mu$ m thick) were obtained by microtomy and deparaffinized using xylene and rehydrated in a graded series of ethanol and finally in distilled water. Antigen retrieval was done in 10 mmol/L citrate buffer (pH 6.0) in microwave at closer to boiling stage followed by quenching of endogenous peroxidase activity with 3.0%  $\text{H}_2\text{O}_2$  in methanol (v/v). Sections were incubated with specific primary antibodies, including mouse monoclonal anti-proliferating cell nuclear antigen (PCNA; 1:250 dilutions; DAKO), goat polyclonal anti-CD31 (1:100 dilutions; Santa Cruz Biotechnology), rabbit polyclonal anti-cleaved caspase-3 (Asp<sup>175</sup>; 1:100 dilutions; Cell Signaling Technology), and rabbit polyclonal anti-vascular endothelial growth factor (VEGF; 1:200 dilutions; Santa Cruz Biotechnology) for 1 h at  $37^\circ\text{C}$  and then overnight at  $4^\circ\text{C}$  in a humidity chamber. Negative controls were incubated only with universal negative control antibodies (DAKO) under identical conditions. Sections were then incubated with appropriate biotinylated secondary antibody (1:200 dilutions) followed with conjugated horseradish peroxidase streptavidin (DAKO) and 3,3'-diaminobenzidine (Sigma) working solution and counterstained with hematoxylin (20, 21).

**Quantification of PCNA, cleaved caspase-3, CD31, and VEGF immunostaining.** PCNA-positive (brown) cells together with total number of cells at 5 arbitrarily selected fields were counted at  $\times 400$  magnification for the quantification of proliferating cells. The proliferation index was determined as number of PCNA-positive cells  $\times 100$ /total number of cells. Similarly, cleaved caspase-3 staining was quantified as number of positive (brown) cells  $\times 100$ /total number of cells in 5 random microscopic ( $\times 400$ ) fields from each tumor. Tumor microvessel density was quantified by counting the CD31<sup>+</sup> (brown) cells in 5 randomly selected fields at  $\times 400$  magnification from each tumor, and data are presented as number of CD31<sup>+</sup> microvessels per  $\times 400$  microscopic field for each group. Immunoreactivity of VEGF was quantified as 0, 1+, 2+, 3+, and 4+ representing nil, weak, moderate, strong, and very strong staining, respectively, and data are presented as mean  $\pm$  SE score of five randomly selected microscopic ( $\times 400$ ) fields from each tumor from all samples in each group (21).

**In situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining.** Paraffin-embedded tumor sections were used to identify apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining by *In situ*

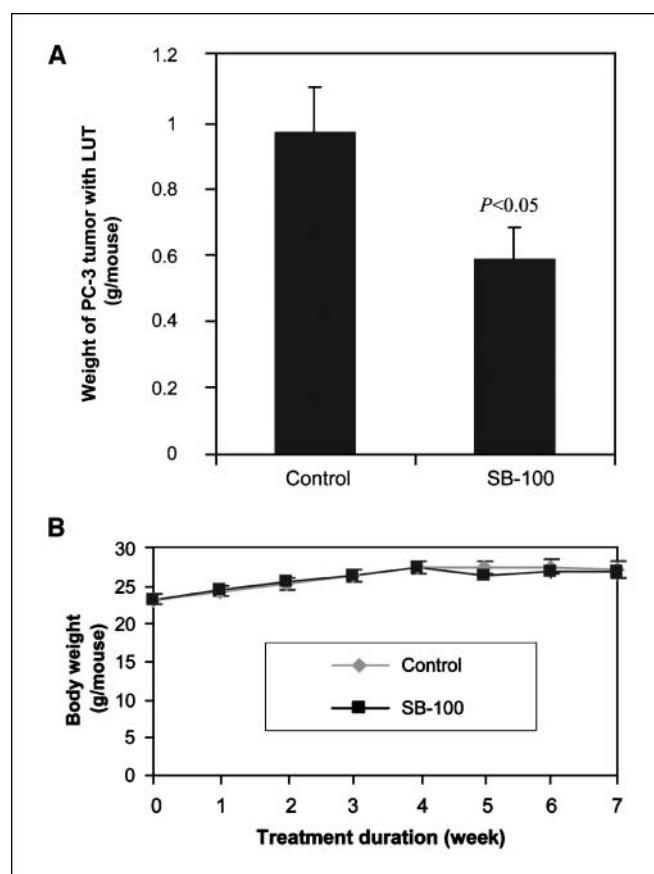
Apoptosis Detection Kit (Promega) following vendor's protocol. A section was incubated with nuclease to generate DNA strand breaks for positive control. Endogenous peroxidase activity was quenched by 5%  $\text{H}_2\text{O}_2$  (in methanol, v/v) and sections were incubated with terminal deoxynucleotidyl transferase labeling buffer followed with terminal deoxynucleotidyl transferase enzyme and biotinylated nucleotides (for negative control, labeling buffer was used instead of terminal deoxynucleotidyl transferase enzyme). Sections were incubated with streptavidin-conjugated horseradish peroxidase followed with 3,3'-diaminobenzidine solution (Sigma) and counterstained with diluted hematoxylin. The apoptosis was evaluated by counting the TUNEL-positive (brown) cells together with total number of cells at 5 randomly selected fields at  $\times 400$  magnifications in each tumor, and data are presented as percent TUNEL-positive (apoptotic) cells.

**Tumor lysate preparation and Western blot analysis.** Individually and randomly selected frozen tumor samples, four from each group, were homogenized and lysates were prepared as reported recently (21), and 60 to 80  $\mu\text{g}$  protein per lysate was denatured with  $2\times$  sample buffer and resolved on 8%, 12%, or 16% Tris-glycine gels by electrophoresis. Separated proteins were transferred onto nitrocellulose membrane by Western blotting, and membrane was blocked for 1 h in blocking buffer and then incubated with specific primary antibodies including anti-cyclin-dependent kinases (CDK) 2, 4, and 6, anti-CDC2, anti-cyclin D1, D3, E, and A, anti-VEGF (Santa Cruz Biotechnology), anti-PCNA (DAKO), anti-phosphorylated and total extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH<sub>2</sub>-terminal kinase 1/2 (JNK1/2), Akt, STAT1, STAT3, and STAT5 (Cell Signaling Technology), anti-phosphorylated p38 MAPK (BD Transduction Laboratory), and anti-total p38 MAPK (Cell Signaling Technology) followed by peroxidase-conjugated appropriate secondary antibody. Finally, proteins were visualized by enhanced chemiluminescence detection and exposure to X-ray film. To confirm equal protein loading, membranes were stripped and reprobed with mouse monoclonal anti- $\beta$ -actin primary antibody (Sigma) in each case.

**Immunohistochemical and statistical analyses.** Stained tumor sections were analyzed by a Zeiss Axioscop 2 microscope (Carl Zeiss). Colored microscopic images were captured by the AxioCam MrC5 camera at  $\times 400$  magnifications and processed by AxioVision software documentation system (Carl Zeiss). Statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific). Quantitative data are presented as mean  $\pm$  SE. Control and silibinin-fed groups were compared by Student's *t* test for tumor weight and immunohistochemical data, and  $P < 0.05$  was considered statistically significant.

## Results

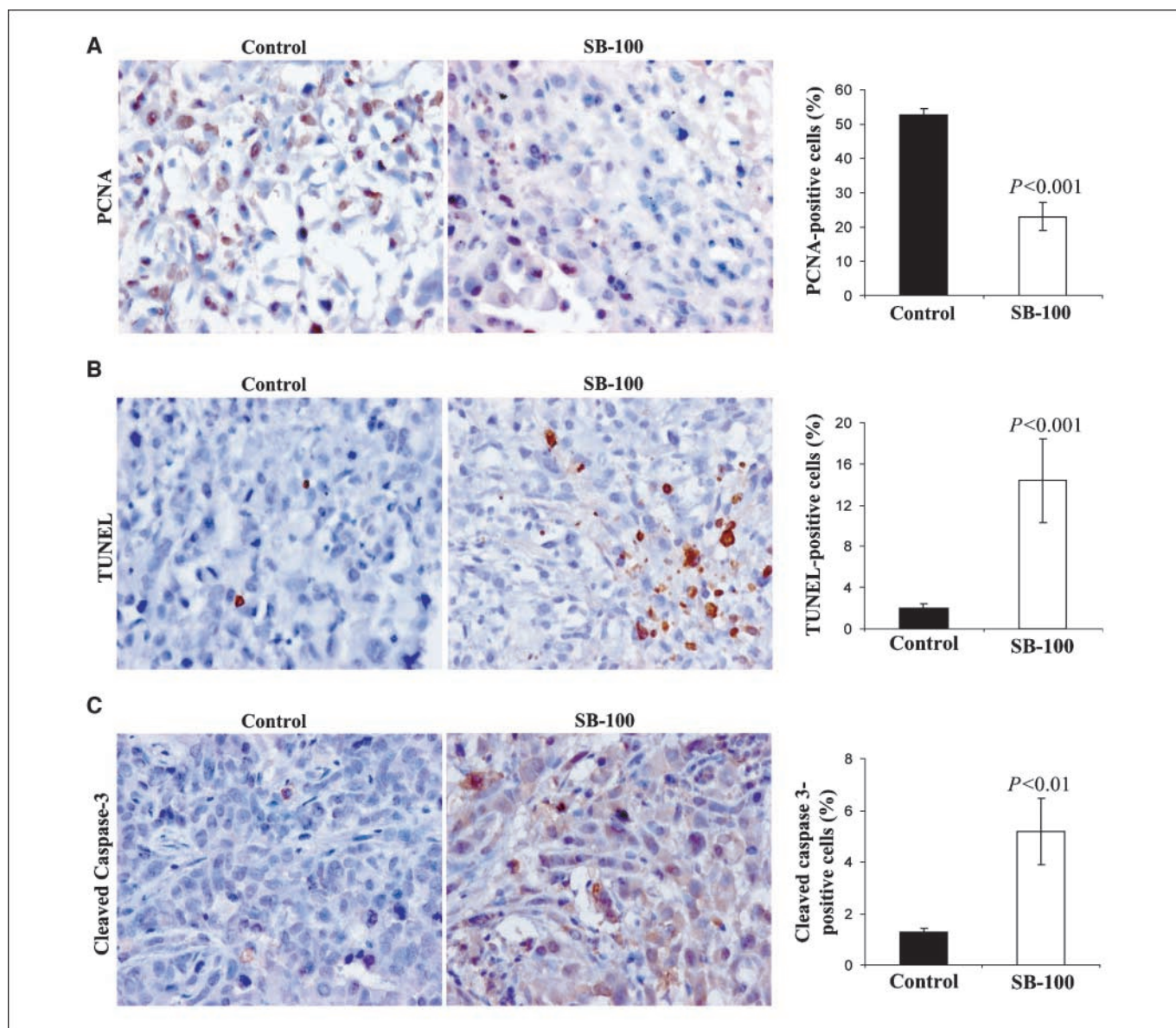
**Oral silibinin inhibits orthotopically implanted PC-3 tumor xenograft growth in athymic nude mice.** When silibinin treatment started after 1 week of tumor cell implantation, all mice had the presence of abdominally palpated tumor. Gavage feeding of 100 mg/kg silibinin in saline suspension for 7 weeks showed an apparent time-dependent inhibition of tumor growth by abdominal palpation. At the time of necropsy, the lower urogenital tract weight including tumor, prostate, and seminal vesicle was  $0.96 \pm 0.14$  g/mouse in control group, which was reduced to  $0.58 \pm 0.10$  g/mouse in silibinin-fed group (Fig. 1A). This accounted for a 40% ( $P < 0.05$ ) decrease in lower urogenital tract weight that included tumor (Fig. 1A). Oral silibinin did not show any gross sign of toxicity as monitored by body weight and diet consumption during the study, as there was no considerable change in body weight gain (Fig. 1B) and diet intake (data not shown) patterns between control and silibinin-fed groups. These results suggest the *in vivo* antitumor efficacy of oral silibinin against orthotopically growing human prostate tumor xenograft in athymic mice



**Fig. 1.** Oral silibinin inhibits orthotopically growing human prostate carcinoma PC-3 tumors in athymic male nude mice. Athymic male mice were orthotopically implanted with  $2 \times 10^6$  PC-3 cells as detailed in Materials and Methods. After 1 wk of surgical recovery, mice were orally gavaged with saline (control;  $n = 8$ ) or 100 mg/kg silibinin (SB-100;  $n = 7$ ) 5 d a week for 7 wk. **A**, weight of PC-3 tumor with lower urogenital tract (g) per mouse at the end of the study. **B**, body weight was monitored throughout the study and presented as a function of time (week). Mean  $\pm$  SE.

without any toxicity. Next, we examined the potential biomarkers of antitumor effect of silibinin by immunohistochemical analysis of tumors.

**Silibinin inhibits cell proliferation, induces apoptosis, and suppresses angiogenesis in orthotopically growing PC-3 tumors.** Proliferation, apoptosis, and angiogenesis are the three extensively used biomarkers, which have been employed for diagnosis and measuring the aggressiveness of solid tumors as well as for prognosis and measuring the efficacy of a given anticancer agent against solid tumors including prostate cancer (23–25). Accordingly, we examined PC-3 tumors by immunohistochemical methods for the possible antiproliferative, proapoptotic, and antiangiogenic effects of silibinin that could have mediated its overall antitumor efficacy. The microscopic examination of PCNA staining of tumors showed weak PCNA immunoreactivity in silibinin-fed group compared with control group (Fig. 2A). The quantification of PCNA staining showed  $53 \pm 2\%$  PCNA-positive cells in control group versus  $23 \pm 4\%$  in silibinin-fed group that accounted for 57% ( $P < 0.001$ ) decrease in proliferation index by silibinin (Fig. 2A). Silibinin showed an increase in TUNEL-positive cells in tumors (Fig. 2B), the quantification of which showed a 7-fold ( $P < 0.001$ ) increase in apoptotic index compared with the control group of



**Fig. 2.** Silibinin inhibits cell proliferation and apoptosis in orthotopic PC-3 tumors. At the end of the study detailed in Fig. 1, tumors were excised and processed for immunohistochemical staining for (A) PCNA, (B) TUNEL, and (C) cleaved caspase-3. Immunohistochemical staining and analysis were done as detailed in Materials and Methods. A representative picture has been shown for both groups in each case at  $\times 400$  magnifications. PCNA-positive, TUNEL-positive, and cleaved caspase-3-positive cells were counted under  $\times 400$  magnifications in five randomly selected areas in each tumor sample. Mean  $\pm$  SE of 7 to 8 tumor samples from individual mouse in each group.

tumors (Fig. 2B). Further, tumors were analyzed for cleaved caspase-3 immunostaining (Fig. 2C), in which silibinin-fed group showed 4-fold ( $P < 0.01$ ) increase in cleaved caspase-3-positive cells over that of control group (Fig. 2C). These results confirmed the apoptotic effect of silibinin in tumors, which could have been mediated by the caspase pathway.

The antiangiogenic effect of silibinin was assessed by the analysis of tumors for CD31 staining (an endothelial cell specific marker), which is a reliable biomarker for the tumor microvessel. The microscopic examination of tumors showed fewer CD31<sup>+</sup> cells in silibinin-fed group compared with the control group (Fig. 3A). The quantification for tumor microvessels showed  $14 \pm 0.9$  and  $10 \pm 1.1$  CD31<sup>+</sup> microvessels in control and silibinin-fed groups, respectively, which accounted for a 29% ( $P < 0.001$ ) decrease in tumor microvessel density

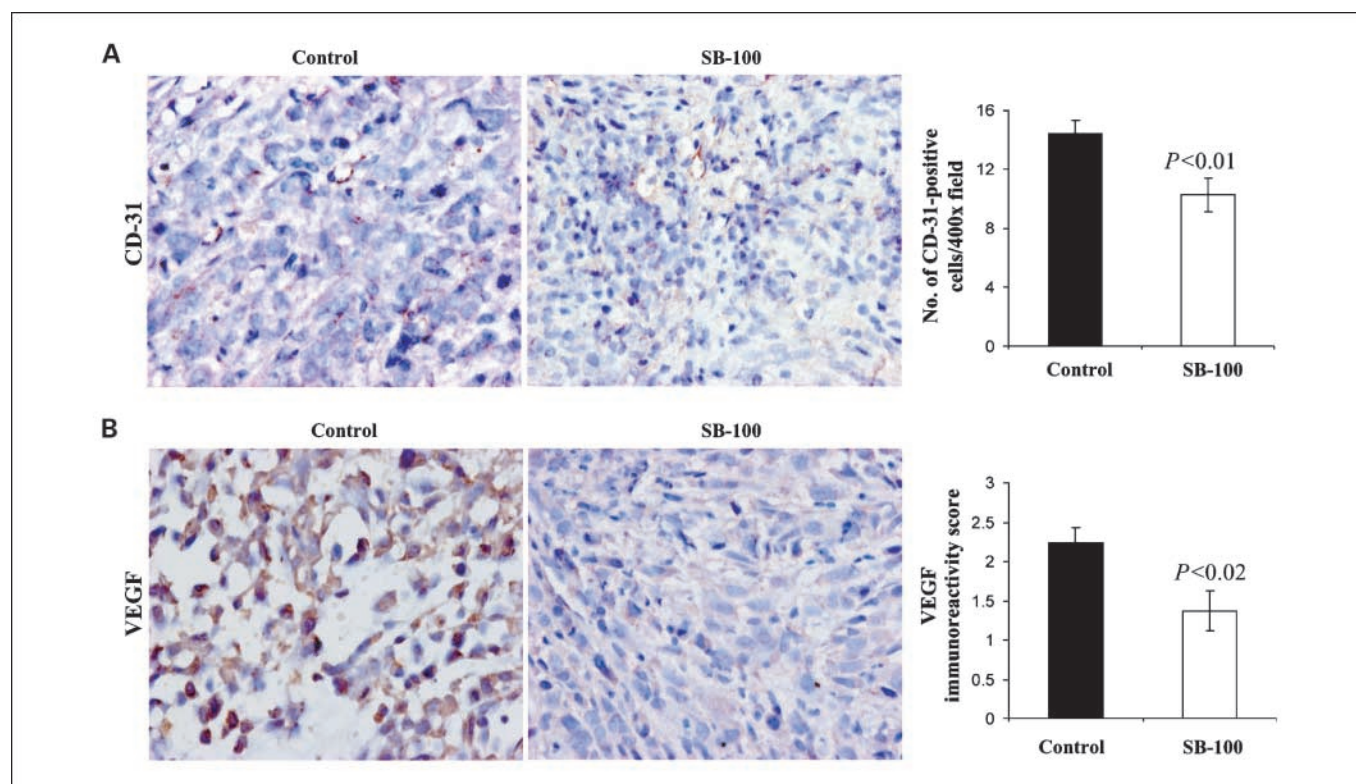
(Fig. 3A). Next, we examined the tumors for the expression level of VEGF, the most potent angiogenic mitogen for endothelial cells. The immunoreactivity of VEGF was reduced by silibinin consistent with the tumor microvessel data (Fig. 3B). The quantification for the intensity of VEGF immunoreactivity showed 39% ( $P < 0.02$ ) decrease by silibinin over that of the control group tumors (Fig. 3B). Consistent with our immunohistochemical data, we also observed decreased expression level of VEGF by Western blotting. Together, these results indicated that silibinin may target VEGF expression for its antiangiogenic activity. Overall, these findings provided an evidence for a multitargeting effect of silibinin for its antitumor efficacy in orthotopically growing PC-3 prostate tumors, which could involve antiproliferative, proapoptotic, and antiangiogenic mechanisms.

**Silibinin decreases the expression of CDKs and cyclins in orthotopically growing PC-3 tumors.** Aberrant cell cycle regulation is a characteristic of almost all types of cancers including prostate cancer (26, 27). The overexpression of CDKs and cyclins has been observed in many solid tumors including prostate, which leads to the unchecked progression through cell cycle checkpoints; thus, their down-regulation could elicit growth-inhibitory effect in cancer cells. In the present study, we observed marked inhibitory effect of silibinin on critical CDKs and cyclins that regulate G<sub>1</sub>-S and G<sub>2</sub>-M transitions. Silibinin decreased the expression levels of CDK4, CDK6, and CDK2 and their regulatory subunits cyclins D1, D3, and E, which regulate G<sub>1</sub>-S transition (Fig. 4). Silibinin also decreased cyclin A level that regulates the progression through S phase, in association with CDK2. CDC2 level was also decreased by silibinin, which regulates G<sub>2</sub>-M transition (Fig. 4). Consistent with our immunohistochemical data, we also observed decreased expression level of PCNA by Western blotting, which possibly indicates the inhibitory effect of silibinin on cell cycle progression in tumor cells that is now clearly evident from the results described above.

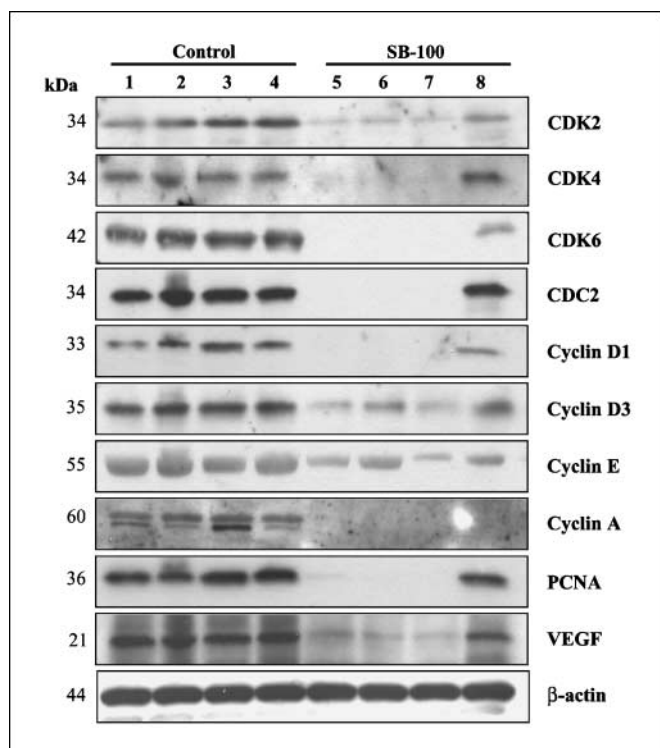
**Silibinin modulates MAPK and Akt phosphorylation in orthotopically growing PC-3 tumors.** The activation of ERK1/2, JNK1/2, and p38 MAPK signaling pathways is mostly linked with its proliferating and survival activities (28); however, sustained ERK1/2 and/or JNK1/2 activation may also lead to apoptotic cell death (29). We recently observed that silibinin-induced sustained ERK1/2 activation in ectopically growing

PC-3 xenograft and chronic ultraviolet B-induced skin tumors was associated with increased level of tumor cell apoptosis (21, 30). Consistent with these results, in the present study as well, silibinin treatment showed a strong increase in phosphorylated ERK1/2 levels without any change in the total ERK1/2 levels in the tumors (Fig. 5). Interestingly, silibinin treatment strongly inhibited the phosphorylation of MEK1/2, which is an upstream regulator of ERK1/2, without significantly affecting the total MEK1/2 levels in the tumor tissue (Fig. 5). This result suggests that silibinin-mediated ERK1/2 activation is independent of MEK1/2 and needs further investigation in future studies. In contrast to ERK1/2 activation, silibinin treatment decreased the phosphorylated JNK1/2 without any change in its total protein levels but decreased the levels of both phosphorylated and total p38 MAPK in the tumors (Fig. 5). A moderate decrease in phosphorylated (Ser<sup>473</sup>) and total Akt levels was also observed by silibinin treatment in the tumors. These results suggest that antiproliferative and proapoptotic effects of silibinin in orthotopically growing PC-3 tumors could be, in part, mediated by sustained activation of ERK1/2 and inhibition of JNK1/2, p38 MAPK, and Akt activities by silibinin.

**Silibinin inhibits STAT signaling in orthotopically growing PC-3 tumors.** STAT signaling, which is mediated by the tyrosine and serine phosphorylation of different STATs by growth factors and cytokines, is known to induce mitogenic, survival, and angiogenic responses (31, 32). Many studies have reported an up-regulation of STAT signaling in prostate cancer, specifically STAT3, which has been suggested as a critical target



**Fig. 3.** Silibinin inhibits angiogenesis in orthotopic PC-3 tumors. At the end of the study detailed in Fig. 1, tumors were excised and processed for immunohistochemical staining for (A) CD31, a marker for endothelial cells, and (B) VEGF. Immunohistochemical staining, analysis, and quantification of the staining were done as detailed in Materials and Methods. A representative picture has been shown for both groups in each case at  $\times 400$  magnifications. Quantitative data for CD31<sup>+</sup> microvessels and levels of VEGF immunoreactivity. Mean  $\pm$  SE of 7 to 8 tumor samples from individual mouse in each group.



**Fig. 4.** Silibinin decreases the expression of CDKs, cyclins, PCNA, and VEGF in orthotopic PC-3 tumors. Four randomly selected individual tumor samples from each group (1-4, control; 5-8, 100 mg/kg silibinin), from the study detailed in Fig. 1, were homogenized and lysates were subjected to Western blot analysis as described in Materials and Methods. Membranes were probed for desired molecules. Same membranes were stripped and reprobed for  $\beta$ -actin as loading control and a representative blot is shown.

for controlling the growth and progression of prostate cancer (32, 33). Accordingly, we also analyzed tumor lysates for both phosphorylated and total protein levels of STAT1, STAT3, and STAT5 by Western immunoblotting, where the tumor samples from the control group showed the detectable levels of both phosphorylated and total protein levels of these three STATs (Fig. 6). Comparatively, tumors from silibinin-treated group showed decreased levels of total protein of these STATs. Consistently, compared with control, silibinin also decreased the phosphorylation levels of STAT1 (Tyr<sup>701</sup>), STAT1 (Ser<sup>727</sup>), STAT3 (Tyr<sup>705</sup>), STAT3 (Ser<sup>727</sup>), and STAT5 (Tyr<sup>794</sup>), among which its strong effect on STAT3 (Ser<sup>727</sup>) phosphorylation could be noted (Fig. 6). Overall, these results suggest a marked inhibitory effect of silibinin on STAT signaling, which could in part account for its observed antitumor effects.

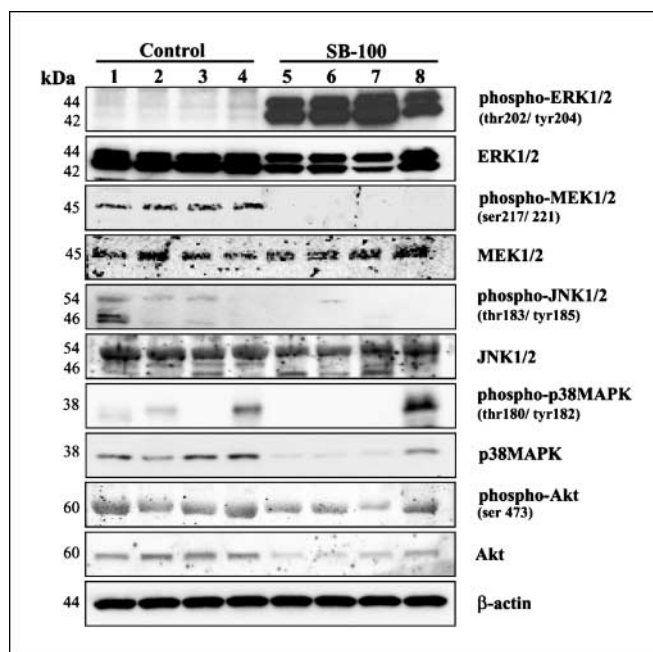
## Discussion

The noteworthy findings in the present study are that a nontoxic dose of oral silibinin inhibits the growth of orthotopically implanted human prostate carcinoma PC-3 xenograft in athymic male nude mice. Silibinin showed antiproliferative, proapoptotic, and antiangiogenic activities in the tumors. Furthermore, these activities of silibinin were in line with a decrease in CDK-cyclin expression, a differential effect on MAPK signaling showing sustained activation of ERK1/2 but inhibition of JNK1/2 and p38 MAPK, and a

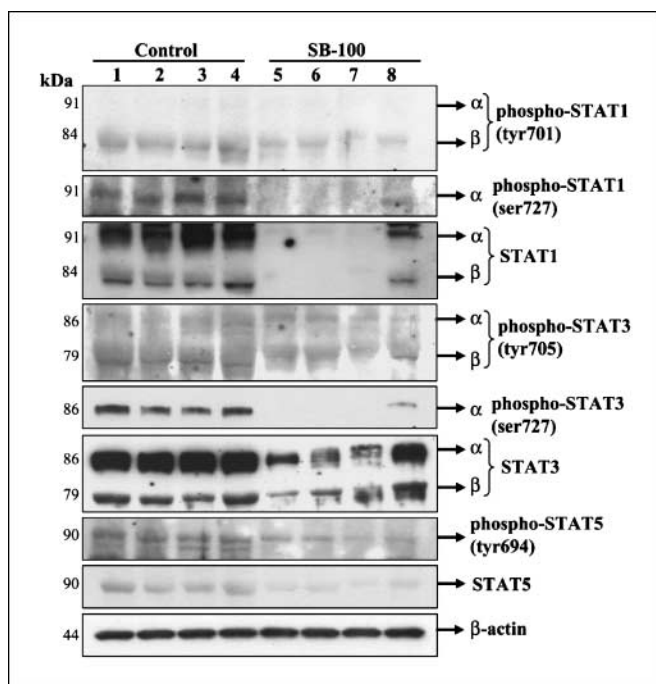
moderate inhibition of Akt signaling together with a marked down-regulation of STAT signaling. Together, these molecular alterations support an overall antitumor effect of silibinin against orthotopic PC-3 tumors.

The management of advanced prostate cancer, which usually arises after the failure of antiandrogen therapies or diagnosed at later stages, poses a critical challenge for its management (2, 3). In such cases, the treatment options of radiotherapy and/or chemotherapy are explored, which are associated with serious side effects. Additionally, if the hormone-refractory prostate cancer is spread in the body, the option of radiotherapy is no longer a better choice of treatment. In the present study, we have used PC-3 human prostate cancer cell line, which has advanced aggressive, invasive, and metastatic characteristics of prostate cancer. As a cancer chemopreventive agent, we have used silibinin, which is a nontoxic naturally occurring flavanolignan (4, 7). Silibinin has been shown to inhibit PC-3 cell growth in cell culture studies (17). Herein, we used a more relevant animal model of human prostate cancer growth, as the tumor cells were implanted orthotopically in mouse prostate rather than its subcutaneous implantation. The 7 weeks of oral silibinin treatment revealed significant antitumor effect on orthotopically growing PC-3 tumors without any adverse health effects in mice.

Aberrant cell proliferation and resistance to apoptosis are peculiar features of cancer cells, including prostate cancer. In this regard, the *in vivo* biomarkers of cell proliferation and apoptosis are commonly used for examining the antitumor efficacy of a given chemopreventive agent (33). Cell proliferation is usually assessed by PCNA expression, which correlates



**Fig. 5.** Silibinin modulates MAPK and Akt signaling in orthotopic PC-3 tumors. Four randomly selected individual tumor samples from each group (1-4, control; 5-8, 100 mg/kg silibinin), from the study detailed in Fig. 1, were homogenized and lysates were subjected to Western blot analysis as described in Materials and Methods. Membranes were probed for phosphorylated and total levels of ERK1/2, MEK1/2, JNK1/2, p38 MAPK, and Akt. Same membranes were stripped and reprobed for  $\beta$ -actin as loading control and a representative blot is shown.



**Fig. 6.** Silibinin inhibits STAT signaling in orthotopic PC-3 tumors. Four randomly selected individual tumor samples from each group (1-4, control; 5-8, 100 mg/kg silibinin), from the study detailed in Fig. 1, were homogenized and lysates were subjected to Western blot analysis as described in Materials and Methods. Membranes were probed for phosphorylated and total levels of STAT1, STAT3, and STAT5. Same membranes were stripped and reprobed for  $\beta$ -actin as loading control and a representative blot is shown.

with the level of DNA synthesis, whereas apoptosis is measured by TUNEL assay, which identifies the cells having fragmented DNA (20). In the present study, the analysis for these both markers revealed strong antiproliferative and proapoptotic effects of silibinin in growing PC-3 tumors. Further, we also observed a decrease in cyclin D1 expression and an increase in cleaved caspase-3-positive cells, suggesting that silibinin-caused antiproliferative effect could be via inhibiting the cell cycle progression, whereas apoptosis could have been mediated by the activation of caspase pathway in tumor cells.

In addition to cell proliferation and apoptosis, tumor angiogenesis is another prognostic biomarker, which has been suggested to be an attractive target to assess the growth of solid tumors, including prostate cancer (33, 34). CD31 is an established marker for endothelial cells and is used for determining the tumor microvessel density (20, 21). Tumor cells produce VEGF, the potent angiogenic growth factor for endothelial cells, and via paracrine mechanism induce tumor vascularization to support their much needed supply of oxygen and nutrients and to remove metabolic wastes. Silibinin-fed mice showed reduction in PC-3 tumor microvessel density and thus revealed another antitumor activity of silibinin in orthotopically growing PC-3 tumor model. Additionally, silibinin also decreased VEGF expression in tumors, suggesting a potential mechanism for its antiangiogenic effect; silibinin could also have its direct effect on endothelial cells, which has been observed in an endothelial cell culture study (35). These observations are supported by our previous studies in which silibinin decreased tumor microvessel density as well as VEGF

expression in ectopically growing prostate cancer xenografts (20, 21).

CDK-cyclin complex positively regulates cell cycle progression. In many tumor cells, these molecules are expressed aberrantly at high levels and thus make the normal control at G<sub>1</sub>-S or G<sub>2</sub>-M transition point ineffective; therefore, they could be targeted to inhibit cell proliferation (26, 27, 36). The overexpression of cyclin D1 is usually considered as an oncogenic change (37). While further examining the molecular alterations associated with antiproliferative effect of silibinin, we observed its modulatory effect on molecules associated with both G<sub>1</sub>-S and G<sub>2</sub>-M cell cycle checkpoints. Silibinin decreased the expression levels of CDK2, CDK4, and CDK6 together with cyclins D1, D3, and E in tumors, which normally regulate the progression through G<sub>1</sub>-S checkpoint. CDK2-cyclin A complex can promote the progression through S phase (27), and silibinin reduced the levels of both these molecules. Further, it also decreased the expression levels of CDC2 that regulates G<sub>2</sub>-M transition (27, 38). These findings clearly suggest that silibinin could target deregulated cell cycle progression by down-regulating the expression of both CDKs and cyclin to inhibit cell proliferation in orthotopically growing PC-3 tumors.

Mitogenic and survival signaling mediated through MAPK and Akt pathways constitute among major mechanisms in cancer cells, including prostate cancer (28, 39). The inhibition of these signaling is regarded as an effective strategy to inhibit the growth of cancer cells. Because we observed both antiproliferative and proapoptotic effects of silibinin in tumors, we anticipated that silibinin could inhibit these signaling mechanisms. Surprisingly, analysis of MAPK molecules in tumors showed a strong increase in phosphorylated ERK1/2 by silibinin treatment but decreased the phosphorylated levels of JNK1/2 and p38 MAPK. There are reports suggesting that persistent activation of ERK1/2 signaling is associated with apoptosis induction (29, 30), which is also supported by our recent study in which we observed the activating effect of silibinin on ERK1/2 signaling and increased apoptosis in ectopic PC-3 xenograft (21). Interestingly, our results also showed that silibinin strongly inhibits the activation of MEK1/2 in tumor tissue, suggesting the role of other signaling events in silibinin-mediated ERK1/2 activation, which needs to be examined in future studies. In other analysis, silibinin moderately decreased the level of phosphorylated (Ser<sup>473</sup>) Akt in tumors. Together, these observations suggest that inhibition of JNK1/2, p38 MAPK, and Akt and activation of ERK1/2 signaling by silibinin could be associated with its antiproliferative and proapoptotic effects in orthotopic PC-3 tumors. However, further studies are needed in future to investigate the differential effects of silibinin on MAPK activation and its downstream targets in these tumors.

STAT transduces both proliferative and survival signals of many growth factors and cytokines via tyrosine and serine phosphorylation that causes its dimerization, which is necessary for its nuclear translocation and transcriptional activity (31, 32). STAT signaling is under tight and transient regulation in normal cells but becomes aberrant in many cancers, including prostate cancer (32). Among seven STAT proteins, STAT3 is persistently activated in prostate cancer and most frequently in hormone-resistant stage of the disease (32). Studies have shown that disruption of STAT3 activation or

expression results in growth inhibition and apoptotic death of prostate cancer cells (32, 40, 41). Further, STAT3-responsive elements have been identified in many genes that influence cell cycle progression, such as cyclin D1, and angiogenesis, such as VEGF (32). Together, these reports suggest STAT as a potential target for the intervention of prostate cancer including hormone-resistant phenotype of the disease. In the present study, silibinin decreased tyrosine and/or serine phosphorylation as well as the total levels of STAT1, STAT3, and STAT5 and also decreased the expression of cyclin D1 and VEGF in PC-3 tumors. In a recently completed *in vitro* study, we have observed that silibinin inhibits constitutively active STAT3 in human prostate cancer DU145 cells (42). Therefore, it is likely that inhibition of STAT signaling, by silibinin, has a role in mediating its *in vivo* antiproliferative, proapoptotic, and antiangiogenic effects in prostate tumors.

It is important to compare the results of present orthotopic study with the earlier published results of silibinin in ectopic model (21). In both these studies, silibinin treatment strongly inhibited the proliferation and angiogenesis but induced apoptosis in tumor tissues; silibinin treatment also resulted in ERK1/2 activation in tumor tissue in both studies. These results suggest that silibinin might have similar underlying mechanism of action in both these models. However, the present study also showed the effect of silibinin on repertoire of signaling molecules, including cell cycle regulators (cyclins

and CDKs), mitogenic signaling (JNK1/2 and p38 MAPK), cell survival signaling (Akt activation) and STAT signaling (STAT1, STAT3, and STAT5). These mechanistic details as well as the fact that efficacy of silibinin was investigated and shown for the first time against prostate cancer cells growing in its microenvironment, which is more relevant clinically, further support the significance of the orthotopic model for the present study.

In summary, our study suggests the *in vivo* antitumor efficacy of oral silibinin against orthotopically growing PC-3 tumors without any adverse health effect in mice. Silibinin inhibited cell proliferation, induced apoptosis, and suppressed angiogenesis in tumors, which were accompanied by down-regulation of CDK-cyclin expression, differential effects on MAPK signaling, and inhibition of Akt and STAT signaling. Largely, our extensive *in vitro* and *in vivo* prostate cancer studies with silibinin indicate its multitargeting effects against tumor growth. Phase I study with silibinin in prostate cancer patients has been successfully completed (14). The findings in the present study could be relevant for examining the biomarkers and associated molecular changes in ongoing phase II pilot study in prostate cancer patients.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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