Silibinin suppresses *in vivo* growth of human prostate carcinoma PC-3 tumor xenograft

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Chemoprevention is an upcoming approach to control cancer including prostate cancer (PCa). Here, we studied the efficacy and associated mechanisms of a chemopreventive agent silibinin against ectopically growing and established advanced human prostate carcinoma PC-3 tumor xenografts in athymic nude mice. Dietary silibinin (0.5%, w/w) did not show any adverse health effect in mice. In first protocol, silibinin started 1 week prior to xenograft implantation and continued for 60 additional days, whereas in the second protocol, silibinin treatment was started after 25 days of established tumors for 4, 8 and 16 days. Silibinin inhibited tumor growth rate in both protocols showing up to 35% ($P = 0.010$) and 18–56% ($P = 0.002$ to <0.001) decrease in tumor volume per mouse and 27% ($P < 0.01$) and 44% ($P = 0.014$) decrease in tumor weight per mouse, respectively. In first protocol, silibinin decreased ($P < 0.001$) tumor cell proliferation and microvessel density but increased ($P < 0.001$) apoptosis. An increase in insulin-like growth factor-binding protein-3 (IGFBP-3) expression with a concomitant decrease in vascular endothelial growth factor (VEGF) expression was noted. Silibinin strongly increased phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), Cip1/p21 and Kip1/p27 (cyclin-dependent kinase inhibitors) levels but moderately decreased Bcl-2 and survivin levels. In established tumors, similar biomarkers and molecular changes were observed due to silibinin corresponding to its antitumor efficacy. These findings identified *in vivo* antitumor efficacy of silibinin against PC-3 human PCa in both intervention protocols accompanied with its anti-proliferative, pro-apoptotic and anti-angiogenic activities. At molecular level, silibinin increased IGFBP-3, Cip1/p21, Kip1/p27 levels and ERK1/2 activation and decreased Bcl-2, survivin and VEGF levels in tumors.

Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer-related deaths in American men (1). The estimated cancer statistics for 2007 suggest 218 890 new cases of PCa accounting for 29% of total cases of cancer and 27 050 deaths due to PCa that is equivalent to 9% of total estimated deaths from cancer (1). Life style and dietary habit may account for the difference in PCa incidence between Western and Asian countries (2–4). Hormone ablation therapy is usually employed for the first line of PCa treatment which often leads to an androgen-independent stage of PCa that does not respond to this therapy and also develops resistance to

Abbreviations: CDKI, cyclin-dependent kinase inhibitor; ERK1/2, extracellular signal-regulated kinase 1/2; IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor-binding protein-3; PCa, rostate cancer; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling; VEGF, vascular endothelial growth factor.

chemotherapy as well as radiotherapy (5,6). The use of chemopreventive strategies is being put forward to control various types of cancer including PCa (7,8). In this regard, non-toxic, cost-effective and physiologically available phytochemicals, such as silibinin, with diversified pharmacological properties have shown promising activities for the prevention and/or intervention of PCa (8,9).

Silibinin is a major biologically active compound present in the Compositae family of plants with abundance in milk thistle (*Silybum marianum*) and artichoke (*Cynara scolymus*). Milk thistle extract is widely used as dietary supplement for hepatoprotective effect, and silymarin, the crude polyphenolic component containing silibinin, is clinically used to treat liver diseases or toxicity including that of amanita poisoning and liver cirrhosis (9). Our extensive studies with silibinin suggest that it has anti-PCa activity in cell culture as well as in animal models (8,10–12) and that it is non-toxic to mice up to 2 g/kg dose by oral gavage (13). Silibinin is observed to inhibit both *in vitro* and *in vivo* growth of advanced human prostate carcinoma DU145 cells which represents a brain metastatic cell line (12). Further, we have observed that silibinin inhibits the *in vitro* growth of human prostate carcinoma PC-3 cells (14,15); however, the *in vivo* effect of silibinin on PC-3 cells is yet to be studied. Therefore, in the present study, we used PC-3 xenograft model in athymic male nude mice to study the effect of dietary silibinin on tumor growth when silibinin administration was started (i) before the xenograft implantation and (ii) after the establishment of the xenograft. Additionally, we investigated the potential *in vivo* biomarkers of silibinin efficacy and associated molecular alterations in prostate tumor xenografts. Mostly, the molecular alterations observed *in vivo* showed a translational relevance of our earlier *in vitro* findings in PC-3 cell culture with silibinin (14,15).

Materials and methods

Tumor xenograft study

Exponentially growing PC-3 cells were detached by trypsinization, washed and re-suspended in serum-free RPMI 1640 medium. Six-week-old athymic nu/nu male mice (NCI-Frederick, Bethesda, MD) were subcutaneously injected with 2×10^6 PC-3 cells mixed with matrigel (1:1), in right flank of each mouse to initiate tumor growth. There were two different dietary silibinin treatment protocols, and diets were commercially prepared by Dyets (Bethlehem, PA). In first protocol, mice were exposed to control (AIN-93M) or silibinin (0.5% w/w, in AIN-93M) diets ($n = 9$ mice per group) and water *ad libitum* 1 week before tumor cell inoculation and continued for a total of 67 days. In second protocol, xenograft was allowed to grow for 25 days with mice on control diet. Thereafter, two mice were euthanized (day 0) and remaining divided into two groups each having approximately equal tumor burden, one group continued on the control diet while other switched to the 0.5% silibinin diet. Five mice from each group were euthanized after 4, 8 and 16 days of silibinin treatment. In both protocols, body weight and diet consumption were recorded twice weekly throughout the study. After xenografts started growing, their sizes were measured twice weekly. Tumor volume was calculated by '0.5236 $L_1(L_2)^2$, where L_1 is long axis and L_2 is short axis of the tumor' (12). At euthanasia, tumors were excised, weighed and one part was fixed in buffered formalin and remaining stored at $-80°C$ until further analysis. Animal care was in accordance with the approved protocol and institutional guidelines.

Immunohistochemical staining for PCNA, CD31, cleaved caspase-3, IGFBP-3 and VEGF

Tumor samples were fixed in 10% buffered formalin for 12 h and processed conventionally. The paraffin-embedded tumor sections (5 μm thick) were heat immobilized and deparaffinized using xylene and rehydrated in a graded series of ethanol with a final wash in distilled water. Antigen retrieval was done in 10 mM citrate buffer (pH 6.0) in microwave followed by quenching of endogenous peroxidase activity with 3.0% H_2O_2 in methanol (vol/vol). Sections were then incubated with specific primary antibodies, including mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (1:400 dilutions) (Dako, Carpinteria, CA), goat polyclonal anti-CD31 (1:200 dilutions) (Santa Cruz Biotechnology,

Santa Cruz, CA), rabbit polyclonal anti-cleaved caspase-3 (Asp175) (1:100 dilutions) (Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-vascular endothelial growth factor (VEGF) (1:200 dilutions) (Santa Cruz Biotechnology) and goat polyclonal anti-insulin-like growth factor-binding protein-3 (IGFBP-3) (1:200 dilutions) (Santa Cruz Biotechnology) for 1 h at 37°C followed by overnight at 4°C in humidity chamber. Negative controls were incubated only with universal negative control antibodies under identical conditions. Sections were then incubated with appropriate biotinylated secondary antibody (1:200–400 dilutions) followed with conjugated horseradish peroxidase–streptavidin (Dako) and 3,3′-diaminobenzidine (Sigma Chemical Co., St Louis, MO) working solution and counterstained with hematoxylin (16).

Quantification of PCNA, CD31, cleaved caspase-3, IGFBP-3 and VEGF immunostaining

Proliferating cells were quantified by counting the PCNA-positive cells and the total number of cells in five arbitrarily selected fields at ×400 magnification. The proliferation index was determined as number of PCNA-positive (brown) cells × 100/total number of cells. Tumor microvessel density was quantified by counting the CD31-positive cells in five randomly selected fields at ×400 magnification from each tumor, and the data are presented as number of CD31-positive microvessels/×400 microscopic field for each group. Similarly, cleaved caspase-3 staining was quantified as number of positive cells × 100/total number of cells in 10 random microscopic (×400) fields from each tumor. Immunoreactivities of IGFBP-3 and VEGF were quantified as 0, 1+, 2+, 3+ and 4+ representing nil, weak, moderate, strong and very strong staining, respectively, and data are presented from all samples in each group as mean score ± SEM of five randomly selected microscopic (×400) fields from each tumor (17).

In situ TUNEL staining

Tumor sections were used to identify apoptotic cells by terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL) staining by Tumor TACS *in situ* Apoptosis Detection Kit (R & D Systems, Minneapolis, MN) following manufacturer's protocol. For positive control, section was incubated with TACS-nuclease to generated DNA strand breaks. Endogenous peroxidase activity was quenched using 5% H_2O_2 (in methanol, vol/vol) 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, St. Louis, MO) and counterstained in 1% methyl green (16). The apoptosis was evaluated by counting the TUNEL-positive (brown) cells as well as the total number of cells in five arbitrarily selected fields at ×400 magnifications in each tumor, and data are presented as percent TUNEL-positive (apoptotic) cells.

Tumor lysate preparation and western blot analysis

Tumor sample lysates were prepared as reported recently (17), and 60–80 µg protein per lysate was denatured with 2× sample buffer and resolved on 12 or 16% Tris–glycine gels by sodium dodecyl sulfate–polyacrylamide gel 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 for phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), ERK1/2 (Cell Signaling Biotechnology, Beverly, MA), Cip1/p21 (Upstate, Lake Placid, NY), Kip1/p27 (Neomarkers, Fremont, CA), Bcl-2 (Upstate USA, Charlottesville, VA) or survivin (Novus Biologicals, Littleton, CO) followed by peroxidase-conjugated appropriate secondary antibody (17). Finally, proteins were visualized by enhanced chemiluminescence detection and exposure to X-ray film. To confirm equal protein loading, membranes were stripped and re-probed with mouse monoclonal anti-β-actin primary antibody (Sigma).

Immunohistochemical and statistical analyses

Microscopic immunohistochemical analyses were done with a Zeiss Axioscop 2 microscope (Carl Zeiss, Jena, Germany). Microscopic images were taken by AxioCam MrC5 camera at ×400 magnification and processed by AxioVision software documentation system (Carl Zeiss). All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific, San Rafael, CA). Quantitative data are presented as mean and SEM. Control and respective silibinin-fed groups were compared for tumor volume and tumor weight by Paired *t*-test in the first protocol and one-way ANOVA followed by Bonferroni *t*-test for all pairwise multiple comparison in the second protocol. Immunohistochemical data were compared by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Dietary silibinin suppresses ectopically implanted and established PC-3 tumor xenograft growth

Dietary feeding of silibinin (0.5% w/w) for a total of 67 days starting 1 week prior to PC-3 xenograft implantation showed a time-dependent inhibition of tumor growth. After 5 weeks of xenograft implantation, a significant difference ($P = 0.041$) in tumor volume was noted by silibinin feeding (Figure 1A). At the end of the study, silibinin decreased tumor volume from 2652.2 ± 684.6 mm^3 per mouse in control group to 1726.5 ± 419.3 mm^3 per mouse, corresponding to a 35% ($P = 0.010$) reduction in tumor volume (Figure 1A). Likewise, tumor weight in silibinin-fed group was also decreased by 27% ($P = 0.008$) when compared with the control group (Figure 1B). The second protocol was designed to study the therapeutic effect of silibinin on established tumors (∼250 mm^3 in volume after 25 days of PC-3 cells injection). We did not observe any regression in established tumors by silibinin; however, it slowed the growth rate of tumors as compared with the control group of tumors. Mice were killed after 4, 8 and 16 days of silibinin feeding in order to study its effect on the time kinetics of biomarkers and molecular alterations and their association with its efficacy. We did not observe any significant difference in the tumor burden after 4 and 8 days of silibinin treatment; however, 16 days of silibinin treatment showed both statistically and clinically relevant decrease in tumor burden accounting for 56% ($P < 0.001$) decrease in tumor volume per mouse (Figure 1C) and 44% ($P = 0.014$) reduction in tumor weight per mouse as compared with the control group (Figure 1D).

Dietary silibinin did not show any gross sign of toxicity as monitored by body weight and diet consumption, as there was no considerable change in body weight gain and diet intake profiles between control and silibinin-fed groups (data not shown). These results suggest the *in vivo* antitumor efficacy of oral silibinin when mice were exposed to it before prostate tumor xenograft implantation as well as when mice had established growing tumors, without any toxicity. Further, we investigated the potential biomarkers of silibinin efficacy in tumors.

Silibinin inhibits cell proliferation and induces apoptosis in PC-3 tumors

An anomalous cell proliferation and resistance to apoptosis are peculiar features of almost every type of cancer, including PCa (18). Therefore, we analyzed PC-3 tumor xenografts for potential antiproliferative and apoptotic effects of silibinin that may have played a role in its overall antitumor efficacy. In the first protocol, microscopic analysis of PCNA staining of tumors showed a moderate decrease in PCNA immunoreactivity in silibinin group as compared with control (Figure 2A and B). Quantification of PCNA staining showed 19% ($P < 0.001$) decrease in proliferation index by silibinin from that of control group (Figure 2C). An increase in TUNEL-positive cells was observed in silibinin-fed group of tumors (Figure 2D and E) which accounted for a 2.2-fold ($P < 0.001$) increase in apoptotic index as compared with control group of tumors (Figure 2F). Similar trends in the effects of silibinin for proliferation and apoptosis were observed in the second protocol in which dietary silibinin was started when mice had established PC-3 tumors (immunostaining data not shown). Silibinin did not show any considerable change in proliferation index after 4 and 8 days of the treatments; however, a statistically significant decrease of 39% ($P < 0.001$) in PCNA-positive cells (26.3 ± 0.7 in control group versus 16.1 ± 1.5 in silibinin group) was observed after 16 days of silibinin treatment (Figure 3A). In these established tumors, silibinin also showed apoptotic cell death which was moderate at 4 and 8 days of silibinin treatment but became significant after 16 days of silibinin treatment that accounted for a 2-fold ($P = 0.02$) increase over the control group (Figure 3B). The TUNEL-staining data were also confirmed with cleaved caspase-3 immunostaining of tumors (data not shown). These findings suggested that antitumor efficacy of silibinin against *in vivo* prostate tumor growth involved both inhibition of cell proliferation as well as an induction of apoptosis.
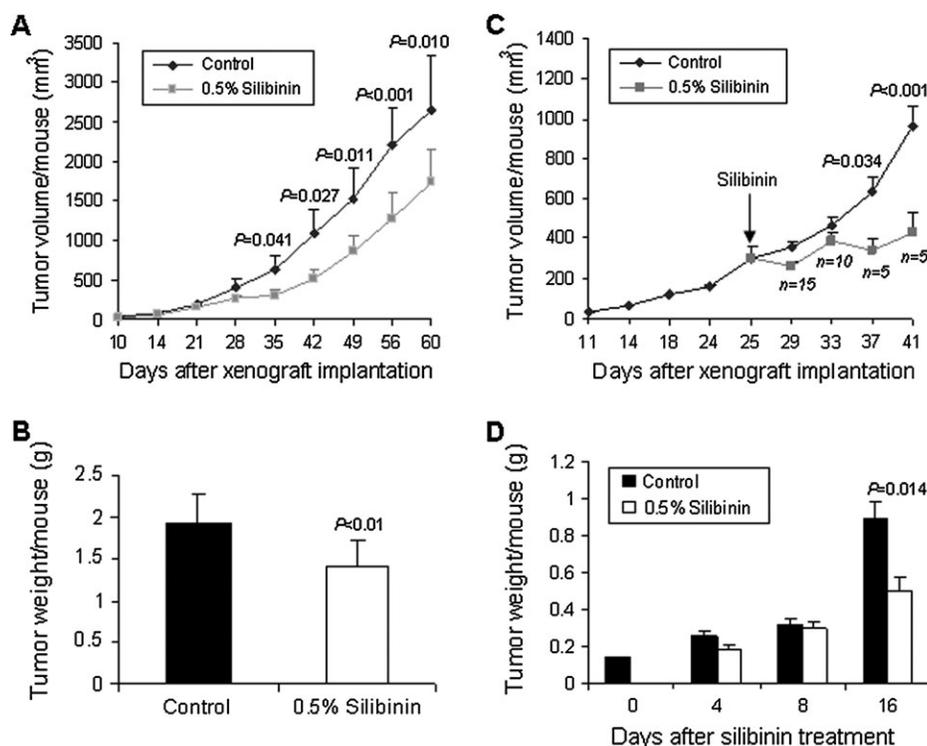
Fig. 1. Effect of dietary feeding of silibinin on human prostate carcinoma PC-3 tumor xenograft growth in athymic male nude mice. Athymic male mice were subcutaneously injected with 2×10^6 PC-3 cells mixed with matrigel. (**A**) In the first protocol, mice were exposed to control (AIN-93M) or silibinin (0.5% w/w) diet ($n = 9$ mice per group) 1 week before xenograft implantation and continued for a total of 67 days. Tumor growth was monitored and presented as tumor volume per mouse as a function of time. (**B**) Tumor weight per mouse at the end of the study from the first protocol ($n = 9$ mice per group). (**C** and **D**) In the second protocol, xenograft was allowed to grow for 25 days ($n = 32$ mice), and mice were fed with control or 0.5% silibinin diet and killed after 0 ($n = 2$ mice), 4 ($n = 5$ mice per group), 8 ($n = 5$ mice per group) and 16 ($n = 5$ mice per group) days of silibinin treatment. (**C**) Tumor growth was monitored and presented as tumor volume per mouse as a function of time from the remaining number of mice from each group at the time of killing. (**D**) Tumor weight per mouse is from two tumor samples on day 0 and from 5 tumor samples on days 4, 8 and 16 of silibinin treatment from individual mouse in each group. In each case, data are presented as mean ± SEM.

Silibinin inhibits angiogenesis in PC-3 tumors

Tumor angiogenesis is suggested as an attractive target to control the growth of solid tumors and also a prognostic biomarker in cancer treatment, including that of PCa (19,20). Therefore, we analyzed the tumors for CD31 staining (an endothelial cell-specific marker) to assess the tumor microvessel density. The microscopic examination of tumors showed decreased CD31-positive (brown) cells in silibinin-treated group as compared with the control group (Figure 2G and H), which accounted for a 58% ($P < 0.001$) decrease in microvessel density (Figure 2I). A similar effect of silibinin on tumor microvessel density was observed in the second protocol in which dietary silibinin was started when mice had established PC-3 tumors (immunostaining data not shown). Silibinin decreased microvessel density, though it was not significant after 4 and 8 days of the treatments; however, it became significant after 16 days of silibinin treatment showing 47% ($P < 0.001$) decrease as compared with the control group (Figure 3C). These results suggested the anti-angiogenic effect of silibinin in its antitumor affect against prostate PC-3 tumors.

Together, the immunohistochemical studies provided an evidence for pleiotropic mechanisms, including anti-proliferative, pro-apoptotic and anti-angiogenic mechanisms, in antitumor efficacy of silibinin against *in vivo* PC-3 prostate tumor xenograft in nude mice.

Silibinin induces IGFBP-3 protein expression in PC-3 tumors

In our earlier study, we have observed that silibinin up-regulates IGFBP-3 expression as one of its anti-proliferative mechanisms in PC-3 cells in culture (14). Therefore, in order to assess the *in vivo* effect of silibinin feeding on IGFBP-3 protein levels in PC-3 prostate tumors, paraffin-embedded sections of the tumor samples were analyzed by immunohistochemical staining for IGFBP-3 using specific antibody. In the first protocol, microscopic examination of IGFBP-3-stained tumor sections showed a strong increase in IGFBP-3-positive (brown) staining in silibinin-fed group of tumors when compared with control group of tumors (Figure 4A and B). The quantification of the staining showed 3.1-fold ($P = 0.013$) increase in IGFBP-3 immunoreactivity by silibinin as compared with the control group (Figure 4C). In the second protocol, we also observed an increase in IGFBP-3 immunoreactivity in silibinin-fed groups of tumors compared with their respective control groups (immunostaining data not shown). The 4 and 8 days of silibinin treatments although showed increased levels of IGFBP-3 but were not significant from their respective control; however, 16 days of silibinin treatment showed 3.2-fold ($P < 0.001$) increase in IGFBP-3 levels which was mainly due to a decrease in IGFBP-3 level in the control group of tumors with the time of their growth/progression (Figure 4G). Together, these findings suggested that up-regulation of IGFBP-3 by silibinin could be a potential *in vivo* mechanism to induce growth inhibition and/or apoptosis in PC-3 tumors growing in nude mice.

Silibinin inhibits VEGF expression in PC-3 tumors

Tumor cells produce and secrete VEGF which is a potent and strong angiogenic factor needed for the tumor vascularization. Since we observed that silibinin decreases tumor microvessel density in PC-3 tumors (Figures 2H, I and 3C), we anticipated that it might also modulate VEGF expression level of tumors as an anti-angiogenic mechanism. In order to assess the *in vivo* effect of silibinin feeding on VEGF protein levels in PC-3 prostate tumor xenograft, tumor sections were analyzed by immunohistochemical staining for VEGF.
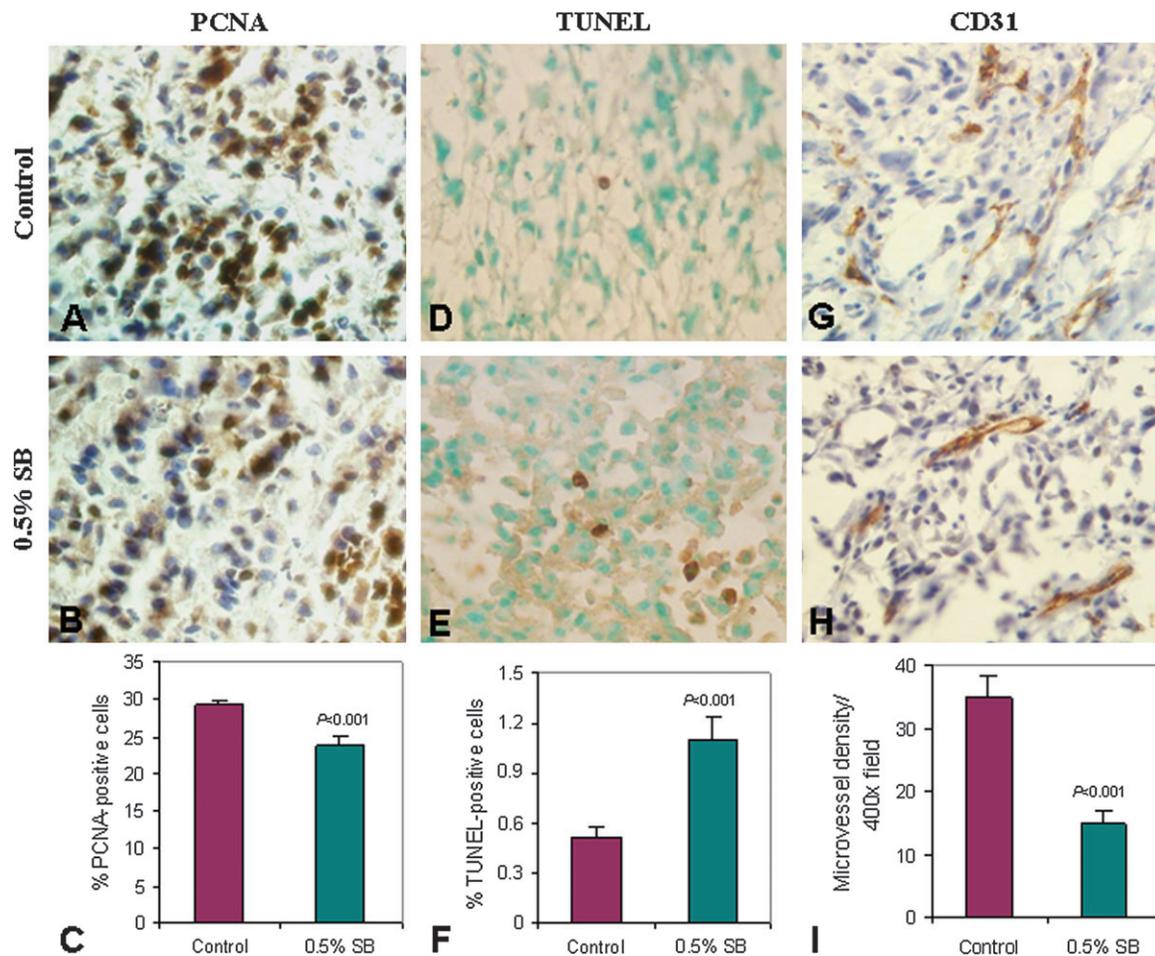
Fig. 2. *In vivo* anti-proliferative, pro-apoptotic and anti-angiogenic effects of dietary feeding of silibinin on PC-3 xenograft. At the end of the xenograft study detailed in Figure 1A, tumors were excised and processed for immunohistochemical staining for (**A** and **B**) PCNA, (**D** and **E**) TUNEL and (**G** and **H**) CD31, an endothelial cell-specific marker. Immunohistochemical staining and analysis were done as detailed in 'Materials and Methods'. A representative picture has been shown for both the groups in each case. (**C**) PCNA-positive cells, (**F**) TUNEL-positive and (**I**) CD31-positive microvessel density were calculated by number of positive (brown) cells × 100/total number of cells counted under ×400 magnifications in five randomly selected areas in each tumor sample. The quantitative data shown are mean ± SEM of nine tumor samples from individual mouse in each group. SB, silibinin.

In the first protocol, microscopic examination of VEGF-stained tumor sections showed a decrease in the intensity of VEGF-positive (brown) staining in silibinin-fed group of tumors as compared with control group of tumors (Figure 4D and E), which accounted for a 30% decrease ($P = 0.039$) in VEGF immunoreactivity (Figure 4C). In the second protocol, we also observed a decrease in VEGF immunoreactivity in all the three time points of silibinin-fed groups of tumors compared with their respective control groups (immunostaining data not shown). The control group of tumors showed a steady increase in VEGF expression as a function of time. The 4 days of silibinin treatment although did not show any significant effect on VEGF level, 8 and 16 days of silibinin treatments showed 50% ($P = 0.007$) and 78% ($P = 0.018$) decrease in VEGF levels with their respective control groups (Figure 4H). These findings suggested that down-regulation of VEGF by silibinin could be a potential *in vivo* mechanism to inhibit tumor angiogenesis in PC-3 tumors.

Silibinin causes ERK1/2 activation in PC-3 tumors

The activation of mitogen activated protein kinase/ERK1/2-signaling pathway is mostly linked with its proliferating and survival activities (21); however, it has also been suggested that sustained ERK1/2 activation may also lead to cellular apoptosis (22). We have recently observed a silibinin-induced ERK1/2 activation in chronic ultraviolet B-induced skin tumorigenesis which was associated with increased

apoptosis (23). Consistent with these reports, in the present study, silibinin treatment starting 1 week before PC-3 xenograft implantation for 67 days showed a strong increase in phospho-ERK1/2 levels without any change in the total ERK1/2 levels (Figure 5A). In the second (established tumor) protocol, control tumors had low levels of phospho-ERK1/2 and silibinin treatment for 4 days did not show any increase in ERK1/2 phosphorylation; however, 8 days as well as 16 days of silibinin treatment showed consistently high levels of phosphorylated ERK1/2 (Figure 5B). We did not observe any considerable change in Akt activation in tumors by silibinin treatment in both the protocols (data not shown). Since silibinin treatment showed anti-proliferative and pro-apoptotic activities in PC-3 tumors, the enhanced and sustained activation of ERK1/2 might be associated with pro-apoptotic effect of silibinin in tumors.

Silibinin induces cyclin-dependent kinase inhibitor expression but moderately suppresses Bcl-2 and survivin expression in PC-3 tumors

The up-regulation of Cip1/p21 and Kip1/p27 by silibinin has been identified as a potential mechanism for anticancer activity in PC-3 cells in cell culture study (15). Therefore, for its *in vivo* significance in the present study, we analyzed PC-3 tumor lysates for Cip1/p21 and Kip1/p27 protein levels by immunoblot analysis. Similar to cell culture study, silibinin treatment in the first protocol showed high levels of Cip1/p21 and Kip1/p27 protein expression (Figure 6A). In the
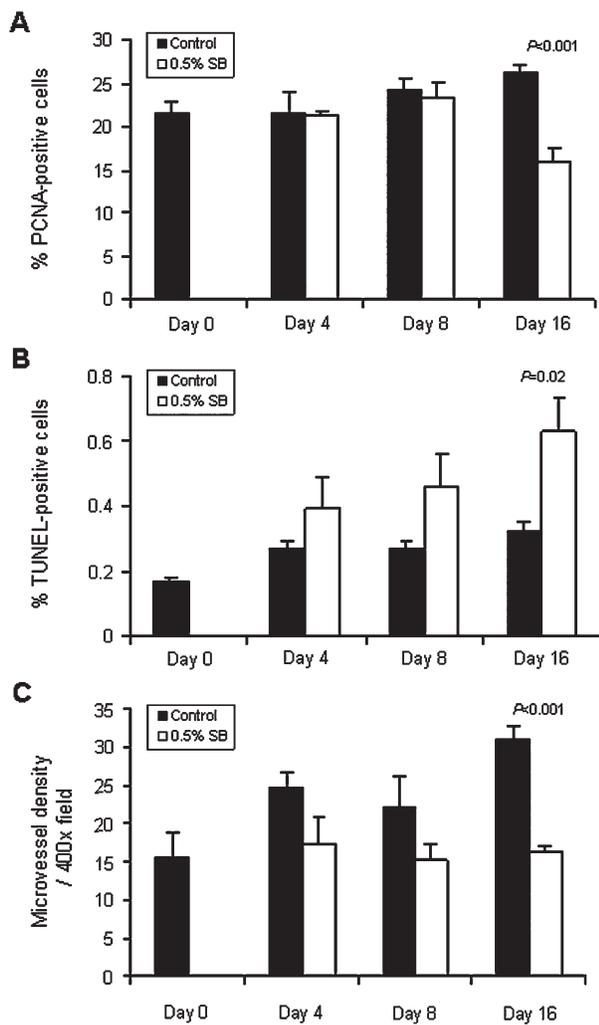
Fig. 3. *In vivo* anti-proliferative, pro-apoptotic and anti-angiogenic effects of silibinin on established PC-3 xenograft. At the end of the xenograft study detailed in Figure 1B, tumors were excised and processed for immunohistochemical staining for PCNA, TUNEL and CD31, an endothelial cell specific marker. Immunohistochemical staining (data not shown) and analysis were done as detailed in 'Materials and Methods'. (**A**) Percent PCNA-positive cells, (**B**) percent TUNEL-positive cells and (**C**) CD31-positive microvessel density were calculated by number of positive (brown) cells × 100/total number of cells counted under ×400 magnifications in five randomly selected areas in each tumor sample. The quantitative data shown are mean ± SEM of two tumor samples on day 0 and from five tumor samples on days 4, 8 and 16 of silibinin treatment from individual mouse in each group. SB, silibinin.

second protocol, only 16 days of silibinin treatment showed a consistent increase in Cip1/p21 protein level with a slight effect on Kip1/p27 expression (Figure 6B). Overall, these findings suggest Cip1/p21 and Kip1/p27 as a potential targets for silibinin efficacy against PCa in both treatment protocols of tumor studies.

Silibinin also showed a moderate decrease in Bcl-2 protein expression in the first treatment protocol, and this effect was clearly evident after 4 days of silibinin treatment in the second protocol (Figure 6A and B). After 8 and 16 days of silibinin treatments, second protocol showed only a slight and moderate decrease Bcl-2 protein level, respectively. In case of survivin, only a slight decrease in the protein level was observed in the first protocol, whereas in the second protocol, this effect was evident only after 16 days of silibinin treatment (Figure 6A and B). These effects of silibinin on Bcl-2 and survivin levels may, in part, account for its pro-apoptotic and antitumor effects in PC-3 tumors.

Discussion

The significant findings in the present study are that dietary feeding of silibinin either started before xenograft implantation or after the establishment of xenograft inhibits the growth of human advanced prostate carcinoma PC-3 tumors in athymic nude mice without any toxicity. The *in vivo* antitumor efficacy of silibinin was accompanied by its anti-proliferative, pro-apoptotic and anti-angiogenic activities. These biological effects of silibinin were associated with the increased protein levels of IGFBP-3, Cip1/p21 and Kip1/p27 and decreased protein levels of VEGF, Bcl-2 and survivin in PC-3 tumors. Interestingly, a persistent activation of ERK1/2 signaling by silibinin was also observed in PC-3 tumors. These observations indicate a pleiotropic mechanism of silibinin for its anti-PCa efficacy.

The control and management of advanced PCa in human is a matter of concern, which usually arises from the first line of PCa treatments including anti-androgen strategies. The hormone-refractory PCa also becomes resistant to chemotherapy and shows increased invasive and metastatic behavior (5,6). PC-3 cell line used in the present study represents advanced bone metastatic phenotype of PCa. Therefore, the inhibitory effect of dietary silibinin on PC-3 tumor growth in athymic nude mice suggests likely efficacy against advanced human PCa. This finding is also supported by our previous study in which dietary silibinin suppressed the growth of advanced human PCa DU145 xenograft in athymic nude mice (12). Further, this finding is an *in vivo* translation of the *in vitro* anticancer efficacy of silibinin observed in prostate carcinoma PC-3 cell line in culture (14,15). Additionally, consistent with our many previous studies, we did not observe any adverse health effect of dietary feeding of silibinin in mice (12,13,23). We have used silibinin doses at 0.01–1% w/w in diet and 0.1–2 g/kg body wt by oral gavage in different animal studies (8,12,13,17). Therefore, the dose used in the present study is practical that showed antitumor activity against PC-3 xenograft growth without any toxicity in mice. We observed that silibinin administration 7 days before cell inoculation has lower efficacy as compared with that when it was administered to mice with established tumors. One of the likely reasons could be that the growing tumors in silibinin environment from beginning may have relatively better adaptive response and more number of silibinin-refractory cells for the continued growth as compared with those tumors which were exposed to silibinin during their exponential growth phase.

The study of biomarkers is an important consideration for the evaluation of cancer chemopreventive efficacy of a test agent. In this regard, the most commonly used *in vivo* biomarkers are proliferation, apoptosis and angiogenesis (16). The molecular biomarkers to examine these biological processes are PCNA (cofactor for DNA polymerase), DNA fragmentation (assayed by TUNEL analysis) and CD31 (specifically expressed on endothelial cells), respectively (16). Silibinin decreased tumor cell proliferation and angiogenesis but increased apoptosis in its antitumor efficacy against PC-3 xenograft in both the silibinin treatment protocols. The findings in the second protocol suggest that a 16-day of silibinin treatment regimen is sufficient to significantly inhibit PC-3 tumor growth as well as significantly alter the three biomarkers discussed above for the anti-PCa efficacy.

Recent studies suggest a close association between PCa risk and plasma level of insulin-like growth factor-1 (IGF-1):IGFBP-3 ratio (24). Some studies demonstrate that lower level of IGFBP-3 and increased level of free IGF-1 are positively correlated with PCa growth and progression in humans (24,25). IGFBP-3 sequesters IGF-1 to prevent its mitogenic and survival action. A transgenic mice study has also shown the significance of IGF-receptor signaling and IGFBPs in aberrant cellular growth of PCa (26). These studies suggest that mitogenic and survival role of IGF-1/IGF-1R pathway leading to advanced stage of PCa could be suppressed by IGFBP-3. Consistent with these reports, we have observed that silibinin inhibits proliferation of PC-3 cells via inducing IGFBP-3 levels in cell culture (14). Some reports also suggest that IGFBP-3 can impart antitumorigenic effect via induction of apoptosis independent of its effect on IGF-1/IGF-1R signaling (27). In the present study, we assessed whether
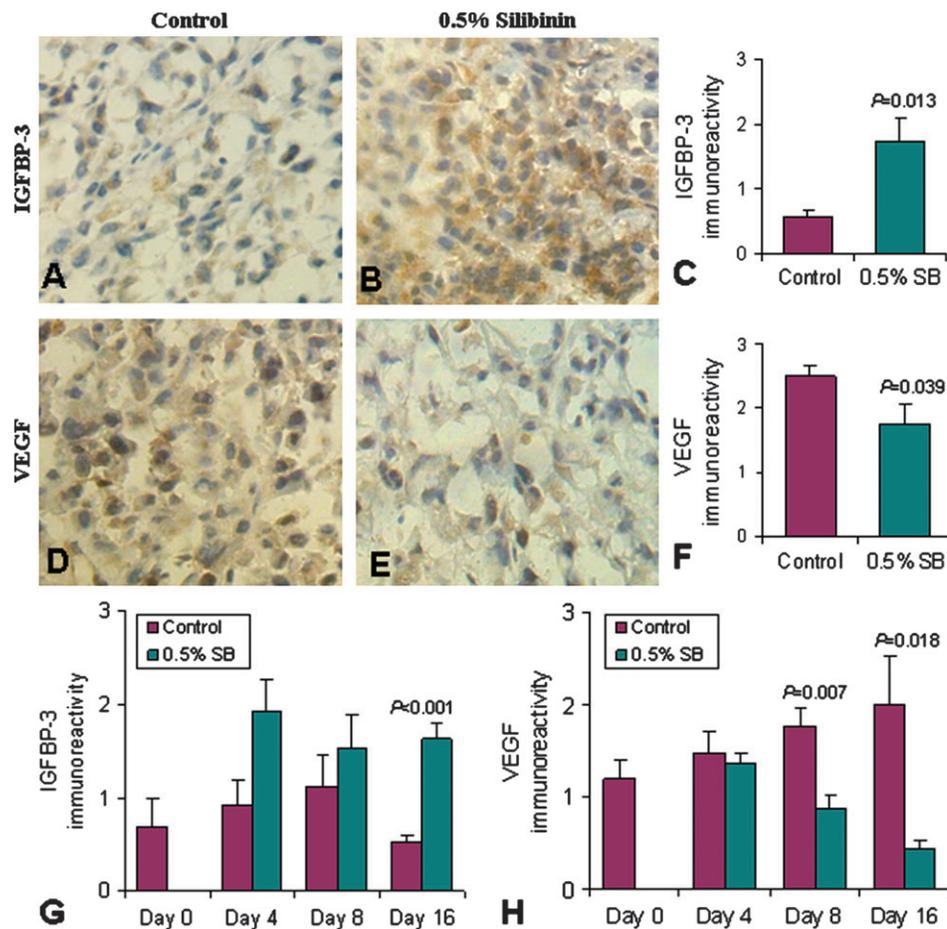
Fig. 4. *In vivo* effects of silibinin on IGFBP-3 and VEGF expression in PC-3 xenograft. PC-3 tumors from the first protocol were processed for (**A** and **B**) IGFBP-3 and (**D** and **E**) VEGF immunohistochemical staining and quantified (**C** and **F**) as mean ± SE of nine samples, each analyzed in five randomly selected areas at an arbitrary scale 0, 1+, 2+, 3+ and 4+ representing nil, weak, moderate, strong and very strong immunoreactivity, respectively. The pictures shown are at ×400 magnifications. Similarly, tumors from the second protocol were immunohistochemically stained and quantified for (**G**) IGFBP-3 and (**H**) VEGF in five randomly selected areas at the same arbitrary scale. The quantitative data shown are mean ± SEM of immunoreactivity from two tumor samples on day 0 and from five tumor samples on days 4, 8 and 16 of silibinin treatment from individual mouse in each group. SB, silibinin.

inhibition of PC-3 xenograft growth by silibinin, which also exhibits decreased cell proliferation and enhanced apoptosis, is associated with an increased expression of IGFBP-3 in tumors. In accord with our cell culture finding, silibinin treatment showed increased protein levels of IGFBP-3 in both the experimental protocols. These findings suggest that IGFBP-3 may serve as an *in vivo* potential target for antitumor activity of silibinin.

Tumor microvessel density has been suggested as a useful prognostic biomarker for a wide range of cancers (19,28). Therefore, quantification of tumor microvessel density is employed to assess tumor growth potential and associated metabolic burden. Silibinin-fed mice in both protocols showed reduction in prostate tumor microvessel density and revealed another antitumor property of silibinin in addition to its anti-proliferative and pro-apoptotic effects. The *in vivo* anti-angiogenic effect of silibinin is further supported by its inhibitory effect on VEGF expression in tumor xenograft. Further, these observations are supported by our previous study in which silibinin is reported to decrease tumor microvessel density as well as VEGF expression (16). We have also observed that silibinin directly inhibits growth, survival, invasion, migration and tubular differentiation of human umbilical vein endothelial cells (29). These findings could be sufficient to support the anti-angiogenic effect of silibinin against prostate tumor growth.

Since we observed anti-proliferative effect of silibinin in PC-3 tumors, we anticipated its inhibitory effect on mitogenic signaling

mediated via ERK1/2. To our surprise, silibinin treatment in first protocol (60 days after xenograft implantation) showed increased levels of phospho-ERK1/2 in tumors as compared with the control group of tumors. This effect was not due to any change in the total level of ERK1/2. Then we analyzed the tumors from the second protocol, in which short term (4 days) of silibinin feeding did not show any effect on ERK1/2 phosphorylation; however, longer (8 and 16 days) silibinin treatments showed increased level of ERK1/2 phosphorylation consistent with the finding in the first protocol. Although majority of the reports suggest the mitogenic action of ERK1/2 signaling, in some studies its persistent activation is associated with apoptosis in cancer cells (22,23), as observed in the present study. However, more studies are needed to investigate the role of persistent *in vivo* activation of ERK1/2 signaling by silibinin in PC-3 tumors.

CDK and cyclin positively regulate cell cycle progression and proliferation which can be impeded by the action of cyclin-dependent kinase inhibitor (CDKI) (30). CDKI binds with the CDK–cyclin complex and inhibits its kinase activity. In this regard, many chemopreventive agents have been shown to induce CDKI level and inhibit CDK–cyclin kinase activity (31). The CDKI Cip1/p21 is a tumor suppressor protein that negatively regulates G_1–S and G_2–M phases of cell cycle transitions. Another important CDKI is Kip1/p27 that preferably and negatively regulates G_1–S phase transition (32). In the present study, silibinin showed a marked enhancing effect on Cip1/p21 protein level of tumors in both the protocols; however, in the second
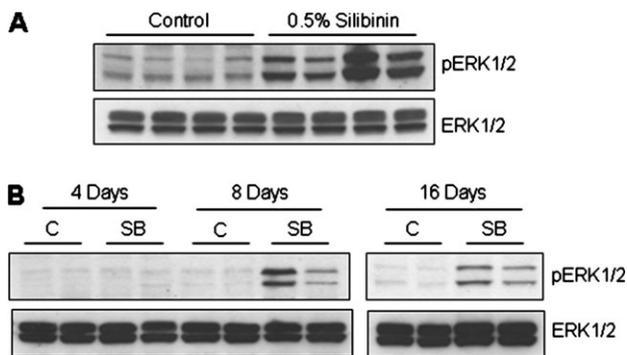
Fig. 5. Silibinin enhances ERK1/2 phosphorylation in PC-3 tumors. Four and two randomly selected tumor samples from the (**A**) first and (**B**) second protocols, respectively, from the study detailed in Figure 1, were homogenized and lysate was subjected to western blot analysis as described in 'Materials and Methods'. (**A** and **B**) Membranes were probed for phospho-ERK1/2 level and stripped and re-probed for the total ERK1/2 protein level. C, control; SB, silibinin.
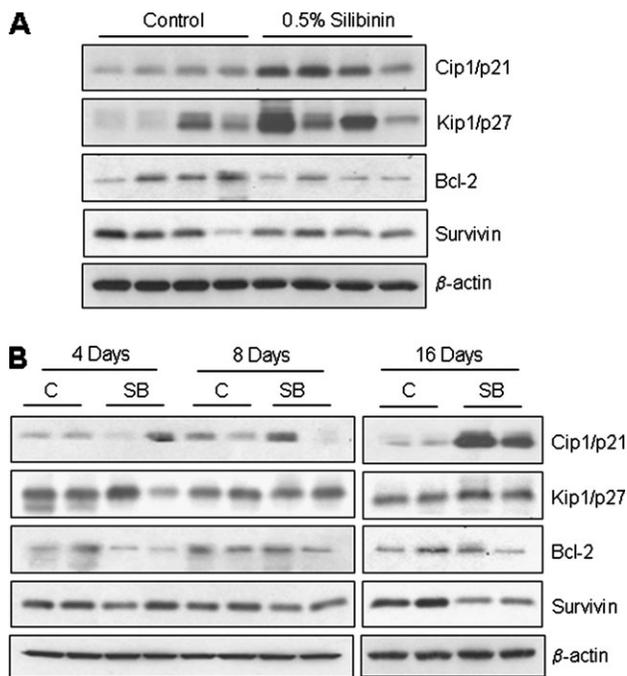
Fig. 6. Effect of silibinin on CDKI, Bcl-2 and survivin in PC-3 tumors. Four and two randomly selected tumor samples from the (**A**) first and (**B**) second protocols, respectively, from the study detailed in Figure 1, were homogenized and lysate was subjected to western blot analysis as described in 'Materials and Methods'. (**A** and **B**) Membranes were probed for Cip1/p21, Kip1/p27, Bcl-2 and survivin protein levels and stripped and re-probed for beta-actin to check the equal protein loading. C, control; SB, silibinin.

protocol, it was evident after 16 days of silibinin treatment. Silibinin also showed a moderate increase in Kip1/p27 level; however, it was less evident in the second protocol. We did not observe any considerable change in CDK and cyclin protein levels (data not shown). In PC-3 cell culture study, we have recently reported that silibinin induces CDKI protein levels and inhibits CDK–cyclin kinase activity (15). Therefore, it is likely that silibinin-induced CDKI might interfere with CDK–cyclin kinase activity to inhibit cell cycle progression and proliferation in PC-3 tumors. Additionally, we also observed a moderate inhibitory effect of silibinin on Bcl-2 and survivin only in some silibinin treatment groups, which may in part account for its

pro-apoptotic effect on tumors. Overall, the *in vivo* up-regulation of CDKI by silibinin in PC-3 tumors could be clinically relevant as the low level of CDKIs shows poor disease-free survival and reduced response to radiotherapy in PCa patients (33,34).

In summary, our study supports the *in vivo* anti-PCa activity of silibinin without any adverse health effect in mice, which has efficacy to inhibit prostate tumor cell proliferation and angiogenesis and to induce apoptosis. Silibinin was observed to alter the expression level of many molecules, including an increase in IGFBP-3, Cip1/p21 and Kip1/p27 and a decrease in VEGF, Bcl-2 and survivin in PC-3 tumors. A persistent activation of ERK1/2 signaling by silibinin was also observed that might be associated with its anti-apoptotic effect; however, more studies are needed to establish the biological significance of this observation. Overall, based on our extensive studies with silibinin and PCa in cell culture, animal models and phase I trial in PCa patients (35), the clinical application of silibinin in PCa chemoprevention may be expected in near future.

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