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Silibinin inhibits fibronectin induced motility, invasiveness and survival in human prostate carcinoma PC3 cells via targeting integrin signaling

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Abstract

Prostate cancer (PCA) is the 2nd leading cause of cancer-related deaths among men in the United States. Preventing or inhibiting metastasis-related events through non-toxic agents could be a useful approach for lowering high mortality among PCA patients. We have earlier reported that natural flavonoid silibinin possesses strong anti-metastatic efficacy against PCA however, mechanism/s of its action still remains largely unknown. One of the major events during metastasis is the replacement of cell-cell interaction with integrins-based cell-matrix interaction that controls motility, invasiveness and survival of cancer cells. Accordingly, here we examined silibinin effect on advanced human PCA PC3 cells' interaction with extracellular matrix component fibronectin. Silibinin (50-200 μM) treatment significantly decreased the fibronectin (5 μg/ml)-induced motile morphology via targeting actin cytoskeleton organization in PC3 cells. Silibinin also decreased the fibronectin-induced cell proliferation and motility but significantly increased cell death in PC3 cells. Silibinin also inhibited the PC3 cells invasiveness in Transwell invasion assays with fibronectin or cancer associated fibroblasts (CAFs) serving as chemoattractant. Importantly, PC3-luc cells cultured on fibronectin showed rapid dissemination and localized in lungs following tail vein injection in athymic male nude mice; however, in silibinin-treated PC3-luc cells, dissemination and lung localization was largely compromised. Molecular analyses revealed that silibinin treatment modulated the fibronectin-induced expression of integrins (α5, αV, β1 and β3), actin-remodeling (FAK, Src, GTPases, ARP2 and cortactin), apoptosis (cPARP and cleaved caspase 3), EMT (E-cadherin and β-catenin), and cell survival (survivin and Akt) related signaling molecules in PC3 cells. Furthermore, PC3-xenograft tissue analyses confirmed the inhibitory effect of silibinin on fibronectin and integrins expression. Together, these results showed that silibinin targets PCA cells' interaction with fibronectin and

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inhibits their motility, invasiveness and survival; thus further supporting silibinin use in PCA intervention including its metastatic progression.

Keywords

Prostate cancer; Metastasis; Fibronectin; Integrin; Silibinin; EMT

1. Introduction

Prostate cancer (PCA) is the most common non-cutaneous cancer, and is the second leading cause of cancer-related deaths in American men after lung cancer. According to the American Cancer Society reports, 238,590 new cases and 29,720 deaths from PCA were estimated in the United States in 2013 [1]. Patients with localized PCA have a very high 5-year survival rate; however, in patients with clinically detectable metastatic disease, median survival is mostly reduced to 12-15 months. Therefore, preventing or inhibiting metastasis through nontoxic agents could be a rationalized approach for lowering high mortality among PCA patients. Now, there are ample evidences that epithelial-to-mesenchymal transition (EMT) is a major event during metastasis in epithelial cancer cells [2-4]. During EMT, E-cadherin based cell-cell interaction is replaced by integrins-based cell-matrix interaction which then controls motility, invasiveness and survival of cancer cells, and also confers chemoresistance [2, 5]. Therefore, preventing EMT and/or inhibiting integrins-based cell-matrix interaction could be important towards lowering metastasis incidences as well as associated morbidity and mortality in PCA patients.

Integrins are transmembrane heterodimeric receptors composed of α and β subunits [6]. Extracellular domain of integrins interacts with extracellular matrix (ECM) components, while the cytoplasmic portion associates with the cytoskeleton and numerous signaling molecules [2]. Integrins recognize and bind specific ligands (such as fibronectin, vitornectin, collagen and laminin) resulting in clustering of integrins and recruitment and activation of signaling/adaptor molecules such as focal adhesion kinase (FAK), Src, integrin-linked kinase (ILK), PI3K/Akt, Ras/MAPK and Rho family of GTPases (Rac, Rho and Cdc42 etc) [2, 7-11]. These signaling cascades regulate a variety of cellular processes including cell adhesion, shape, EMT, migration, proliferation, apoptosis, etc. [2, 9, 11, 12]. Importantly, integrins are aberrantly expressed in several epithelial tumors including PCA, and promote disease progression and metastatic spread [8, 13-18]. Specific integrins (α subunits 4, 5, and v, and β subunits 1, 3 and 6) are known to interact with fibronectin, which is a multifunctional extracellular glycoprotein that is also considered of critical relevance for the process of cell survival and metastasis [19]. Therefore, it is expected that targeting integrins expression and their interaction with fibronectin as well as downstream signaling molecules related to cytoskeletal remodeling through non-toxic phytochemicals should prevent cancer cells motility and metastasis. In the present study, we analyzed the efficacy of a natural flavonoid silibinin on fibronectin-induced integrins expression as well as activation of downstream signaling pathways in human PCA PC3 cells.

Silibinin is the major bioactive constituent present in silymarin which is mainly isolated from milk thistle (*Silybum marianum*), and widely used as a hepatoprotective agent and has

been marketed as a dietary supplement. Silibinin has shown strong efficacy against PCA cells both *in vitro* and *in vivo*, and is currently being tested in PCA patients [20-25]. Silibinin treatment has been reported to induce cell cycle arrest via activating cellular check points and cyclin dependent kinase inhibitors [20, 26, 27]; and to inhibit multiple signaling pathways such as androgen receptor, EGFR, IGF1R, IGFBP3, and NF-κB etc in human PCA cells [28-32]. In earlier studies, we have also reported that silibinin targets EMT-related molecules and inhibits PCA cells invasiveness and metastasis both *in vitro* and *in vivo* [3, 22, 23, 33]; however, the effect of silibinin treatment on PCA cells interaction with ECM component/s as well as integrin signaling remains unstudied. In the present study, for the first time, we examined the effect of silibinin treatment on advanced human PCA PC3 cells' interaction with ECM component fibronectin, and analyzed silibinin effect on fibronectin-induced motility, invasiveness and proliferation using PCA cell culture and animal models. Results clearly showed that silibinin targets fibronectin-integrins interaction as well as downstream signaling pathways, thereby inhibiting motility, invasiveness and survival of PC3 cells.

2. Materials & Methods

2.1 Cell lines and reagents

Human prostate carcinoma PC3 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate at 37°C in a humidified 5% CO_2 incubator. PC3-luc cells (expressing luciferase gene) were from Applied Biological Materials (ABM, British Columbia, Canada) and cultured in Prigrow IV media (from ABM, British Columbia, Canada) supplemented with 10%FBS and 100 U/ml penicillin G and 100 μg/ml streptomycin. FBS, penicillin and streptomycin were from Gibco, Life Technologies (Grand Island, NY). Prostate cancer associated fibroblasts (CAFs) were obtained and cultured as described earlier [34]. Antibodies for β-catenin, Rac, MMP9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for E-cadherin, Cdc42, ARP2, Integrins (α5, αv, β1, and β3), pSrc-tyr416, total Src, pFAK-Tyr925, total FAK, pAkt-Ser473, total Akt, cPARP, cleaved caspase 3, and anti-rabbit peroxidase-conjugated secondary antibody were obtained from Cell Signaling (Beverly, MA). Survivin antibody was from Novus Biologicals (Littleton, CO). Fibronectin, DAPI (4′,6-diamidino-2-phenylindole), carboxymethylcellulose (CMC), Harris hematoxylin, silibinin, and β-actin antibody were from Sigma-Aldrich (St Louis, MO). ECL detection system and anti-mouse HRP-conjugated secondary antibody were from GE Healthcare (Buckinghamshire, UK). Antibody for α-tubulin was from Lab Vision Corporation (Fremont, CA). Rhodamine-tagged phalloidin was obtained from Life Technologies. Protein assay kit was from Bio-Rad Laboratories (Hercules, CA). ECL detection system and anti-mouse HRP conjugated secondary antibody were from GE Healthcare (Buckinghamshire, UK). All other reagents were obtained in their commercially available highest purity grade.

2.2 Morphological analyses

Cell culture plates were coated with BSA (5 μg/ml) or fibronectin (5 μg/ml) overnight and washed with phosphate-buffered saline (PBS) just before use. Silibinin stock solution was prepared in DMSO and stored at -20°C. An equal amount of DMSO (vehicle) was present in each treatment, including control; DMSO concentration did not exceed 0.1% (v/v) in any treatment. For morphological analyses, PC3 cells were plated on fibronectin coated plates along with DMSO or different concentrations of silibinin (50-200 μM in medium) for desired duration and examined under a light microscope. The number of attached cells with defined morphological features (flattened morphology with lamellipodia) were counted and compared (between DMSO treated control and silibinin-treatment groups). PC3 cells plated on BSA (5 μg/ml) coated or uncoated plates served as relevant controls. Photomicrographs were captured using a Canon Power Shot digital camera.

2.3 Confocal imaging

PC3 cells were grown over cover slips coated with fibronectin in the presence of either DMSO or silibinin (50-200 μM doses). After 1 hr, cells were fixed in 3.7% formaldehyde overnight at 4°C, permeabilized with 0.1% Triton X-100 for 15 min and thereafter blocking was done with 5% serum. Cells were washed with PBS containing 0.2% Tween 20 and incubated with rhodamine-tagged phalloidin and DAPI for 30 min. Cell images were captured at 1500× magnification on a Nikon inverted confocal microscope using 688/405 nm laser wavelengths to detect rhodamine-phalloidin (red) and DAPI (blue) emissions, respectively.

2.4 Cell viability assay

PC3 cells were cultured in RPMI media (with 0.5% FBS) for 24 hrs; thereafter cells were collected and re-plated on fibronectin (5 μg/ml) coated plates with or without silibinin treatment. After 24 hrs, cells were collected by brief trypsinization and washed twice with PBS. Thereafter, cell number and cell death were assessed by trypan blue dye using a haemocytometer under an inverted microscope.

2.5 Migration assay

The effect of silibinin treatment on fibronectin-induced migration was assessed in Transwell migration assay using Falcon 24-well cell culture inserts (BD Biosciences, San Jose, CA) with 8-μm pores. In this assay, the bottom chambers of Transwell were filled with RPMI1640 media containing 0.5% FBS, and in the top chambers, PC3 cells (5×10^4) were seeded in 500 μL RPMI (0.5% FBS) supplemented with FN (5 μg/ml) with or without silibinin (50-200 μM) treatment. RPMI media containing 0.5% FBS supplemented with BSA (5 μg/ml) in the upper chambers served as negative control. After 10 hrs, cells that did not migrate through the pores and therefore remained on the upper side of the filter membrane were gently removed by scrubbing and the migrated cells on the lower side of the filter membrane were fixed with ice cold methanol for 5 min followed by staining with hematoxylin/eosin and mounted. Images were captured using Cannon Power Shot A640 camera on a Zeiss inverted microscope and total number of migrated cells was counted.

2.6 Invasion assay

The effect of silibinin treatment on CAFs-induced PC3 cells invasiveness was assessed in matrigel invasion assay using BD BioCoat Matrigel invasion chambers (BD Biosciences, San Jose, CA). In this assay, CAFs were cultured in the bottom chambers of Transwell in stromal basal medium (supplemented with 0.5% FBS) with or without silibinin (100 μM), and in the top chambers, PC3 cells (2×10^4) were seeded in 500 μL RPMI (supplemented with 0.5% FBS) with or without silibinin (100 μM). Only stroma basal media (supplemented with 0.5% FBS) in the lower chambers served as negative control. After 10 hrs, non-invasive cells which remained on the upper side of the filter membrane were gently removed by scrubbing and the invasive cells on the lower side of the filter membrane were fixed with ice cold methanol for 5 min followed by staining with hematoxylin/eosin and mounted. Images were captured using Cannon Power Shot A640 camera on Zeiss inverted microscope and total number of invasive cells was counted. In another invasion assay, the bottom chambers of Transwell were filled with RPMI media containing 0.5% FBS supplemented with or without fibronectin (40 μg/ml), and in the top chambers PC3 cells (2×10^4) were seeded in 500 μL RPMI (0.5% FBS) with or without silibinin (50 and 100 μM). After 10 hrs, invasive cells were stained and quantified as detailed above. Only RPMI media containing 0.5% FBS in the lower chambers served as negative control in this experiment.

2.7 Tail vein injection and animal imaging

Athymic (*nu/nu*) male nude mice were purchased from NCI (Frederick, Bethesda, MD) and housed at the University of Colorado Denver (UCD) animal care facility. Protocols were approved by UCD Institutional Animal Care and Use Committee. Mice were administered via oral gavage either vehicle (200 μL of 0.5% CMC [w/v] in sterile water) or silibinin 200 mg/kg body weight (200 μL in 0.5% CMC) for one week before PC-3-luc cells injection. Silibinin dose (200 mg/kg body weight) was based upon our earlier completed study [35] and unpublished animal studies where we have observed biological effect of silibinin at this dose. PC-3-luc cells were cultured in Prigrow IV (with 0.5% FBS) for 24 hrs; thereafter cells were collected and re-plated on fibronectin (5 μg/ml) coated plates with or without silibinin treatment. PC3 cells plated on BSA (5 μg/ml) coated plates served as a control in this experiment. After 24 hrs, cells were harvested, counted and injected (7×10^5 cells/mouse) into nude mice (3-5 mice in each group) through tail vein injection. Six hrs after injection, PC3-luc cells dissemination in mice was analyzed by bioluminescence imaging. Briefly, mice were intraperitoneally injected with 100 μL of 30 mg/mL D-luciferin and imaged using Xenogen IVIS200 imaging system.

2.8 Western blotting

Whole-cell extracts were prepared and Western blotting was performed as previously described [36, 37]. Approximately, 50–70 μg of protein lysate per sample was denatured in 2× sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 6 or 12% Tris–glycine gel (as required based upon the protein molecular weight). The separated proteins were transferred on to nitrocellulose membrane followed by blocking with 5% non-fat milk powder (w/v) in Tris-buffered saline (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature.

Membranes were probed for the protein levels of desired molecules using specific primary antibodies followed by the appropriate peroxidase-conjugated secondary antibody and visualized by ECL detection system. For certain proteins, membranes were also probed with appropriate secondary IR Dye-tagged antibodies and visualized using Odyssey infra-red imager (LI-COR Biosciences). Membranes were also stripped and re-probed again for protein of interest or with appropriate loading control. In each case, blots were subjected to multiple exposures on the film to make sure that the band density is in the linear range. The autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

2.9 Immunohistochemistry (IHC)

Paraffin-embedded PC3 tumor tissue sections from an earlier completed PC3 xenograft study [38] were used to determine the *in vivo* effect of silibinin administration on the levels of integrins (αV, α5, and β1), fibronectin and MMP9 by IHC as described before [35, 38, 39]. Briefly, sections were incubated with specific primary antibody, followed by a specific biotinylated secondary antibody, and then conjugated HRP streptavidin and DAB working solution, and counterstained with hematoxylin. Stained sections were analyzed by Zeiss Axioscope 2 microscope and images captured by AxioCam MrC5 camera at 400× magnifications. Immunoreactivity (represented by brown staining) was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), 4+ (very strong staining) as reported earlier [35, 39].

2.10 Statistical analysis

Statistical analysis was performed using SigmaStat 2.03 software (Jandel Scientific, San Rafael, CA). Data was analyzed using one way ANOVA (Tukey test) and a statistically significant difference was considered to be at $p<0.05$.

3. Results

3.1 Silibinin inhibits fibronectin-induced motile morphology in PC3 cells

First, we analyzed the effect of fibronectin on the attachment and morphology of the human PCA PC3 cells. PC3 cell were plated on fibronectin (5-15 μg/ml) coated plates. After 1 hr, cells were examined for attachment and morphology under a light microscope. PC3 cells plated on BSA (15 μg/ml) coated or uncoated (UC) plates served as relevant controls in this experiment. PC3 cells barely attached and most of the cells were round in shape without any morphological distinction on uncoated and BSA coated wells (Figure 1A). However, cells attached and showed a flattened morphology with lamellipodia-like structures suggesting a ‘motile morphology’ in fibronectin coated plates at all the concentrations of fibronectin used (Figure 1A). From this experiment, we selected a 5 μg/ml fibronectin dose for subsequent experiments, as this dose was sufficient for attachment and morphological distinction by PC3 cells.

Next, we examined effect of silibinin treatment on fibronectin-induced attachment and cell morphology. Silibinin (50-200 μM) treatment strongly inhibited the fibronectin (5 μg/ml)-induced attachment and motile morphology in PC3 cells in a dose-dependent manner

(Figure 1B). Since actin cytoskeleton is critical for the motile morphology, we next examined the effect of silibinin treatment on fibronectin-induced actin reorganization by analyzing F-actin distribution using rhodamine-tagged phalloidin. As shown in Figure 1C, PC3 cells plated over uncoated and BSA coated wells were round in shape after 1 hr of plating with an F-actin filament ring at one end of the cells. However, PC3 cells plated over fibronectin (5 μg/ml) coated plate showed robust actin cytoskeleton with F-actin filaments bundles forming lamellipodia and several filopodial like protrusions (Figure 1C). Silibinin treatment disrupted the fibronectin-induced strong actin filaments organization as well as filopodial protrusions (Figure 1C).

3.2 Silibinin inhibits fibronectin-induced proliferation and motility in PC3 cells

Fibronectin is known to enhance cell survival and motility in cancer cells, so next we examined silibinin effect on fibronectin-induced proliferation and motility in human PCA PC3 cells. The high serum levels found in supplemented growth media may obscure fibronectin-mediated cell proliferation; therefore, for these studies, PC3 cells were first cultured in RPMI media supplemented with 0.5% FBS for 24 hrs; thereafter, cells were collected and re-plated on fibronectin (5 μg/ml) coated plates with or without silibinin under low serum condition (0.5% FBS). PC3 cells plated on BSA (5 μg/ml) coated plates served as a control in this experiment. Under these culture conditions, 24 hrs after plating, PC3 cells were much elongated on fibronectin coated plates compared with BSA, and silibinin treatment resulted in rounding of the cells (Figure 2A). Importantly, with silibinin treatment especially at a dose of 200 μM, PC3 cells were not only rounded but also adhered to each other (Figure 2A). Silibinin (50-200 μM) treatment also significantly decreased the total cell number induced by fibronectin, while significantly increased the death in PC3 cells (Figure 2B and 2C). Silibinin effect on cell number and cell death was not dose-dependent at this time-point; still, in terms of its effect on cell morphology it is clear that higher doses of silibinin do have a better effect (Figure 2A).

Next, we examined the effect of silibinin on fibronectin-induced PC3 cells motility in a Transwell migration assay. Silibinin treatment (50-200 μM) for 10 hrs significantly decreased the fibronectin-induced motility of PC3 cells in a dose-dependent manner (Figure 2D-pictures and bar diagram).

3.3 Silibinin inhibits fibronectin- and CAFs-induced invasiveness of PC3 cells

Next we examined the effect of silibinin treatment on the invasiveness of PC3 cells in Transwell invasion assay with fibronectin (40 μg/ml) used as a chemoattractant in the lower chambers. As shown in Figure 3A, fibronectin significantly enhanced the invasiveness of PC3 cells through the matrigel layer compared with BSA control, while silibinin (50 and 100 μM) treatment strongly reduced the invasiveness of PC3 cells.

In the tumor microenvironment, CAFs release ECM components including fibronectin and are involved in ECM remodeling, facilitating movement of cancer and endothelial cells. Therefore, next we analyzed the effect of CAFs presence in the lower chambers as chemoattractant on the invasiveness of PC3 cells cultured in the upper chambers of Transwells. As shown in Figure 3B, CAFs strongly induced the invasiveness of PC3 cells

while silibinin presence either in the upper chamber (with PC3 cells) or in the lower chambers (with CAFs) significantly reduced the invasiveness of PC3 cells.

3.4 Silibinin inhibits fibronectin-induced dissemination of PC3-luc cells in vivo

Activated integrin signaling (following fibronectin exposure) protects the cells during metastatic spread in the blood through conferring anoikis resistance and play a critical role in intravasation at distant metastatic sites. We next employed tail vein injection metastatic model, mimicking the hematogenous dissemination of cancer cells, to understand the *in vivo* capability of PCA cells to survive and colonize at the metastatic site when cultured on fibronectin coated plates with or without silibinin treatment. For this study, we used PC3-luc cells (expressing luciferase gene) to non-invasively image and monitor the dissemination of cells when injected in mice. PC3-luc cells were cultured in RPMI media supplemented with 0.5% FBS for 24 hrs; thereafter cells were collected and re-plated on fibronectin (5 μg/ml) coated plates with or without silibinin (200 μM) treatment. PC3 cells plated on BSA (5 μg/ml) coated plates served as a control in this experiment. After 24 hrs, cells were harvested, and 7×10^5 cells were injected in each male nude mouse through tail vein. Thereafter, mice were imaged 6 hrs later by Xenogen IVIS200 imaging system. As shown in the Figure 4A, 2/3 mice showed faint bioluminescence signal in mice injected with PC3-luc cells cultured on BSA-coated plates; however, 4/4 animals showed strong bioluminescence signal and lung localization in mice injected with PC3-luc cells cultured on fibronectin-coated plates (Figure 4B). Importantly, in mice injected with PC3-luc cells cultured on fibronectin-coated plates along with silibinin treatment, 2/5 mice showed no bioluminescence signal and other 3/5 mice showed extremely dim bioluminescence signal (Figure 4C).

3.4 Silibinin targets multiple signaling pathways down-stream of fibronectin-integrin in PC3 cells

Fibronectin-integrin interaction is known to activate and/or modulate several signaling pathways regulating adhesion, motility, invasiveness, survival, anoikis as well as chemoresistance of cancer cells [9, 13, 19, 40]. Our results revealed that silibinin (200 μM) treatment for 24 hrs decreased the fibronectin-induced expression of integrins (α5, αV, β1 and β3) in PC3 cells (Figure 5A). One of the key molecules recruited downstream of integrins is non receptor tyrosine kinase FAK, which is autophosphorylated, and then binds and activates Src, that in turn phosphorylate FAK at tyrosine residue [2, 9]. As shown in Figure 5A, silibinin treatment decreased the phosphorylation of FAK at Tyr-925 site, while total FAK level was only slightly affected by silibinin treatment. Silibinin treatment also reduced the fibronectin-induced phosphorylation of Src kinase without affecting the total Src level (Figure 5A). Integrins clustering on plasma membrane also activates Rho family of small GTPases that play a critical role in actin cytoskeleton remodeling and formation of various motile structures such as lamellipodia and filopodia [9-11, 41]. Our results also showed that fibronectin activated while silibinin reduced the expression of GTPases Cdc42 and Rac (Figure 5A). Rho family GTPases are known to activate ARP2/3 and cortactin, and both of these molecules (ARP2/3 and cortactin) play an important role in actin polymerization and the branching that is required for the formation of motile structures such as lamellipodia, filopodia, etc [42-45]. Importantly, silibinin treatment also decreased the

levels of ARP2 and cortactin (Figure 5A). Silibinin treatment also reduced the level of matrix metalloproteinase 9 (MMP9) that belongs to family of zinc and calcium dependent endopeptidase involved in ECM degradation and considered important during invasion and cancer metastasis [46].

Next, we examined the effect of silibinin treatment on apoptosis and survival related signaling molecules as fibronectin-integrins interaction is known to promote survival and proliferation as well as prevents cell death [2, 6, 12]. As shown in Figure 5B, silibinin treatment slightly increased the level of cPARP and cleaved caspase 3, molecular markers of apoptosis, compared with PC3 cells cultured over fibronectin coated plates alone. Importantly, silibinin treatment strongly decreased the fibronectin-induced survivin level, which is an established negative regulator of apoptosis and is also reported to promote metastasis [47].

Integrin based signaling is also known to provide cells a mesenchymal phenotype associated with increased invasiveness [2]. Our results showed that silibinin treatment resulted in higher E-cadherin expression (Figure 5B), an important adherens junction protein, which also correlates with the round morphology and greater adhesion between cells by silibinin treatment as shown in Figure 2A. Silibinin treatment also reduced the fibronectin-induced β-catenin expression in PC3 cells (Figure 5B). Next, we examined silibinin effect on fibronectin-induced Akt phosphorylation which is known to play an important role in promoting cell survival, proliferation, EMT, and inhibiting apoptosis [2, 48]. Importantly, Akt phosphorylation at Ser-473 site was significantly increased in the presence of fibronectin while silibinin treatment inhibited that without affecting the total Akt level in PC3 cells (Figure 5B).

3.5 Silibinin feeding inhibits integrins, fibronectin and MMP9 expression in PC3 tumors

Next, we confirmed our cell culture results *in vivo* by analyzing PC3 xenograft tissues from a completed study [38]. Earlier, we have reported that silibinin feeding (0.5%, w/w in diet) strongly inhibited the growth of PC3 tumors in nude mice [38]. IHC analyses of PC3 tumor tissues showed that silibinin feeding moderately decreased the integrins α5 and αV expression while only slightly but significantly decreased the integrin β1 expression (Figure 6). Furthermore, we observed a slight but significant decrease in fibronectin level, and a moderate decrease in MMP9 level in PC3 tumor tissues (Figure 6). These *in vivo* results further support our cell culture findings that silibinin targets fibronectin-integrin signaling in PCA cells.

4. Discussion

Integrin-ECM interaction as well as associated signaling cascades help cancer cells in acquiring invasiveness and motility, survival in the circulation as well as extravasation at metastatic sites [2, 13, 19]. In the past, several studies in pre-clinical models have shown that blocking integrin/ECM interaction could be effective in inhibiting cancer growth and metastasis [6, 8, 17, 19]. However, serious doubts have been raised over the selectivity of agents targeting integrins or integrins-ECM interaction, as these proteins and their interactions are also involved in several cellular activities essential for normal cellular

functions [8]. Therefore, there is a need to develop agent/s targeting integrins, ECM components and/or their interactions specifically in the tumors. One attractive option could be to test the efficacy of non-toxic natural agents to target integrins, ECM and their interaction in cancer cells to prevent their growth, invasiveness and metastasis. In this direction, here we tested the efficacy of a natural non-toxic dietary supplement silibinin against PCA cells' interaction with fibronectin. Results are encouraging as silibinin inhibited the fibronectin-induced motile morphology, migration, invasion, and dissemination *in vivo* as well as inhibited multiple fibronectin-induced signaling pathways in human PCA PC3 cells.

Fibronectin expression has been reported to be increased in several types of malignancies including PCA, and its expression positively correlates with an invasive and metastatic phenotype [49-51]. Similarly, integrins are aberrantly expressed in several cancers including PCA [16, 52, 53]. Therefore, fibronectin-integrin axis has been suggested as a target for preventive and therapeutic approaches against cancer progression [17-19, 54, 55]. Results from present study showed that silibinin inhibited the fibronectin-induced expression of integrins as well as downstream signaling molecules including FAK/Src/GTPases/Akt/MMP9. There have been several reports highlighting the important role of these signaling molecules in survival, anoikis resistance, invasiveness and metastasis of cancer cells [2, 7-9]. For example, Lee *et al* reported that integrin β1 is upregulated in human PCA cells which is associated with higher FAK auto-phosphorylation and correlated with anoikis resistance and metastasis [8]. Earlier, Zeng *et al* have also reported the important role of FAK and PI3K in integrin-fibronectin mediated MMP1-dependent invasion of metastatic PCA cells [7]. Therefore, silibinin mediated inhibition of fibronectin-induced motility, invasiveness and survival as well as *in vivo* dissemination could be through inhibition of integrins and downstream signaling pathways.

In earlier studies, we and others have reported that silibinin targets multiple signaling pathways associated with PCA invasiveness and metastasis [3, 33]. Silibinin inhibited the migration and invasiveness of human PCA cells through enhancing E-cadherin expression [56]. In other cell culture studies, silibinin was also reported to decrease migratory/invasive potential and to inhibit vimentin and increase cytokeratin-18 expression in PCA cells [57-59]. We have also reported in transgenic adenocarcinoma of the mouse prostate (TRAMP) model that silibinin feeding strongly reduces the expression of fibronectin corresponding to a significant decrease in the metastatic progression of the disease [22]. Importantly, in the same study we also observed that silibinin feeding decreased the MMPs (2, 3 and 9) and increased the E-cadherin expression in the prostate tissues [22]. In the present study, we showed for the first time that silibinin treatment inhibited the fibronectin-induced integrins expression while enhanced the E-cadherin expression, and significantly reduced the fibronectin-induced motile morphology in PC3 cells. Supporting these results, Handorean *et al* have earlier reported that silibinin (at 100 μM dose) inhibited the human PCA PC3M cells adhesion to fibronectin [60]. Silibinin treatment also inhibited the high-glucose mediated increase in matrix-associated peri-cellular fibronectin as well as soluble fibronectin in human mesangial cells, suggesting that silibinin could inhibit fibronectin production or enhance its degradation [61]. Based upon previous studies and results from the

present study, it seems that silibinin targets multiple signaling including the integrins, fibronectin and their interaction, thus promoting epithelial characteristics and inhibiting invasiveness of PCA cells.

Fibronectin is primarily secreted by fibroblasts and plays an important role in wound healing. Similarly, in the tumor microenvironment, CAFs promote cancer cells survival and metastasis as well as angiogenesis through secreting ECM components including fibronectin as well as growth factors and cytokines [62]. Importantly, increased fibronectin level is an important event during preparation of pre-metastatic niches, supporting its important role in facilitating metastasis [63]. In the present study, we observed that CAFs or fibronectin in the Transwell assays acted as a strong chemoattractant and significantly enhanced the invasiveness of PC3 cells. Importantly, silibinin treatment inhibited the CAF-induced PC3 invasiveness, probably through targeting the CAFs ability to secret fibronectin or other ECM components. Our unpublished data also support this notion as silibinin treatment strongly reduced the fibronectin secretion in the media by CAFs. Also, we have recently reported that silibinin treatment inhibits the capacity of PCA cells to induce a CAF-like phenotype in prostate stromal cells by acting on PCA cells' capacity to secrete TGFβ2 [34]. Also, PC3 tumor tissues from silibinin-fed mice exhibited significantly reduced TGFβ2 expression as well as decreased CAF biomarkers such as α-SMA, vimentin and FAP expression [34]. Analyses of tumor tissues from the same study also suggested that fibronectin expression was also decreased with silibinin treatment, which could be due to inhibition of CAFs activation by silibinin in these tumors. Overall, in prostate tumor microenvironment, silibinin could target PCA cells ability to activate CAFs and/or inhibit CAFs ability to secrete fibronectin; thereby, silibinin could inhibit the survival and invasiveness of PCA cells.

In conclusion, our results showed that silibinin inhibits fibronectin-induced integrin signaling as well as motility and invasiveness of PCA cells (Figure 7). Further studies are warranted to understand the mechanism through which silibinin targets fibronectin-integrin interaction and decreases fibronectin expression *in vivo*. Since silibinin was also effective in lowering the fibronectin-induced survival in PCA cells (Figure 7), silibinin should be further tested for its potential to overcome chemotherapeutic resistance. Overall, these completed studies suggest that non-toxic [3, 24, 64] silibinin could be useful in preventing and inhibiting PCA metastatic progression.

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Highlights

- Silibinin inhibits fibronectin-induce motile morphology in PC3 cells
- Silibinin inhibits fibronectin-induced migration and invasion in PC3 cells
- Silibinin targets fibronectin-induced integrins and downstream signaling molecule

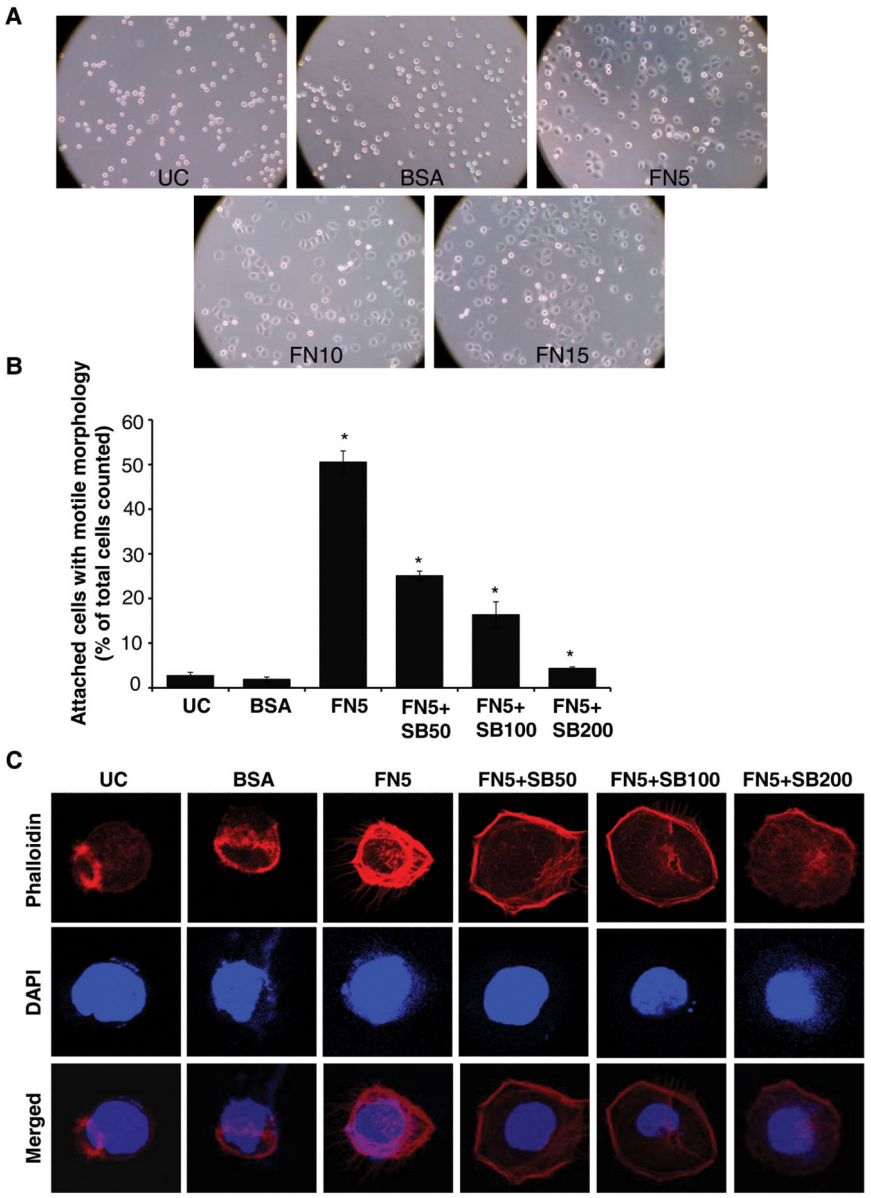

Figure 1. Silibinin inhibits fibronectin-induced motile morphology in PC3 cells

(A) PC3 cells were plated on cell culture plates coated with different concentrations of fibronectin (5-15 μg/ml). After 1 hr, cells were examined under a light microscope (at 100× magnification). PC3 cells plated on BSA (15 μg/ml) coated or uncoated plates served as relevant controls. **(B)** PC3 cells (1.5×10^4 per well) were plated on fibronectin coated culture plates (5 μg/ml fibronectin) along with DMSO or different concentrations of silibinin (50-200 μM). After 1 hr, attached cells with defined motile morphology were counted under a light microscope. PC3 cells plated on BSA (5 μg/ml) coated or uncoated plates served as relevant control. Data shown in bar diagram is mean±SEM of three samples. **(D)** PC3 cells (1.5×10^4 per well) were plated on fibronectin (5 μg/ml) coated cover slips along with DMSO or different concentrations of silibinin (50-200 μM). After 1 hr, cells were fixed and analyzed for F-actin distribution through confocal microscopy detailed in 'Materials and

methods'. In confocal pictures shown (at 1500×), rhodamine-phalloidin (in red) represents cellular F-actin distribution, while DAPI (in blue) stains nuclei. Abbreviations: UC: Uncoated; BSA: Bovine serum albumin; FN: Fibronectin; SB: Silibinin. *, $p \leq 0.001$

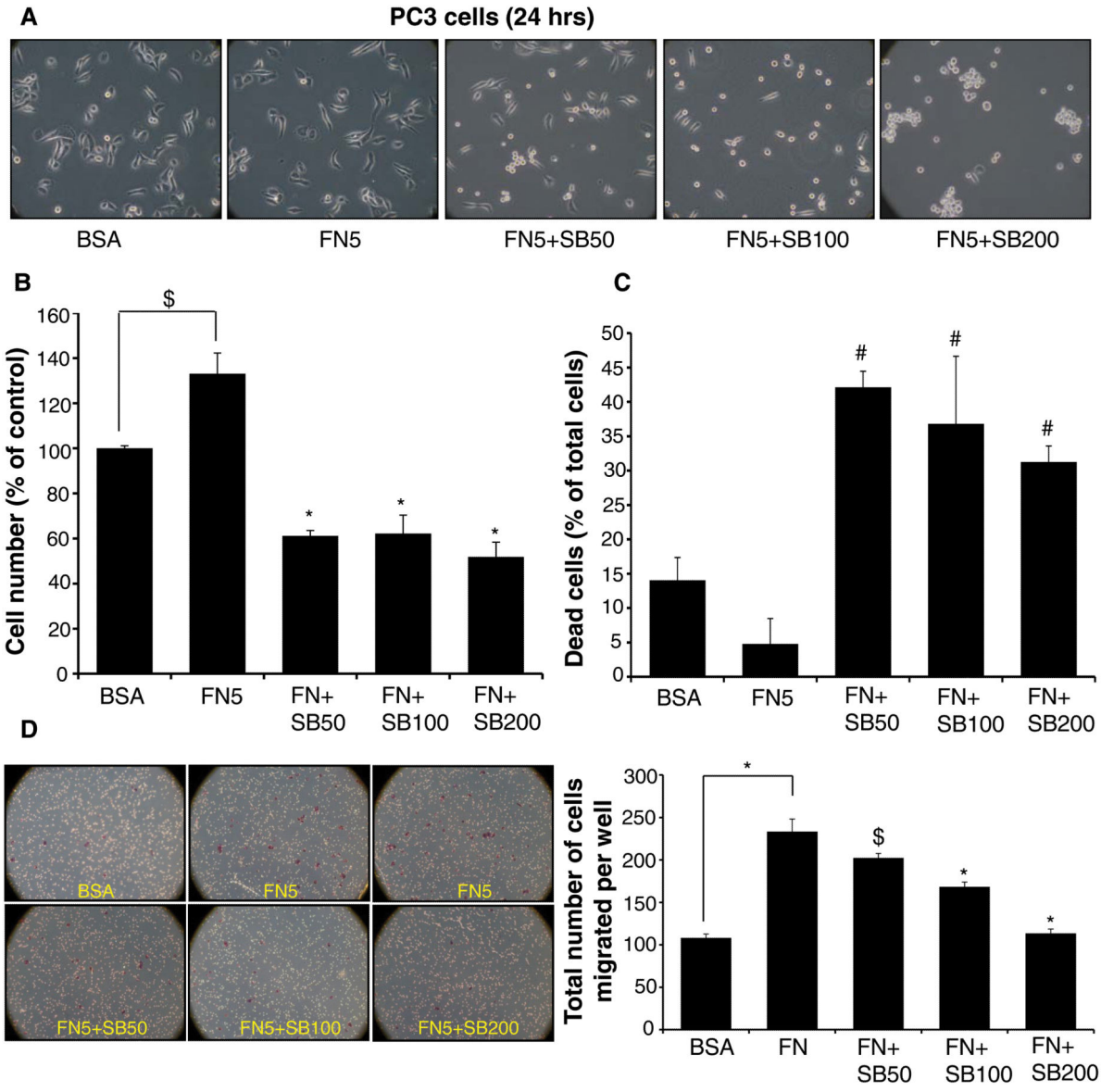

Figure 2. Silibinin inhibits fibronectin-induced proliferation and motility in PC3 cells

PC3 cells were cultured in RPMI media (with 0.5% FBS) for 24 hrs; thereafter cells were collected and re-plated on fibronectin (5 μg/ml) coated plates with or without silibinin (50-200 μM). PC3 cells plated on BSA (5 μg/ml) coated plates served as relevant control. After 24 hrs, cells were analyzed for **(A)** morphology under a microscope; and **(B-C)** total cell number and dead cells (% of total cells). **(D)** Effect of silibinin treatment on fibronectin-induced motility was analyzed in a Transwell migration assay. Data shown in bar diagram is mean±SEM of three samples.

Abbreviations: BSA: Bovine serum albumin; FN: Fibronectin; SB: Silibinin. *, $p \leq 0.001$; #, $p \leq 0.01$; $, $p \leq 0.05$

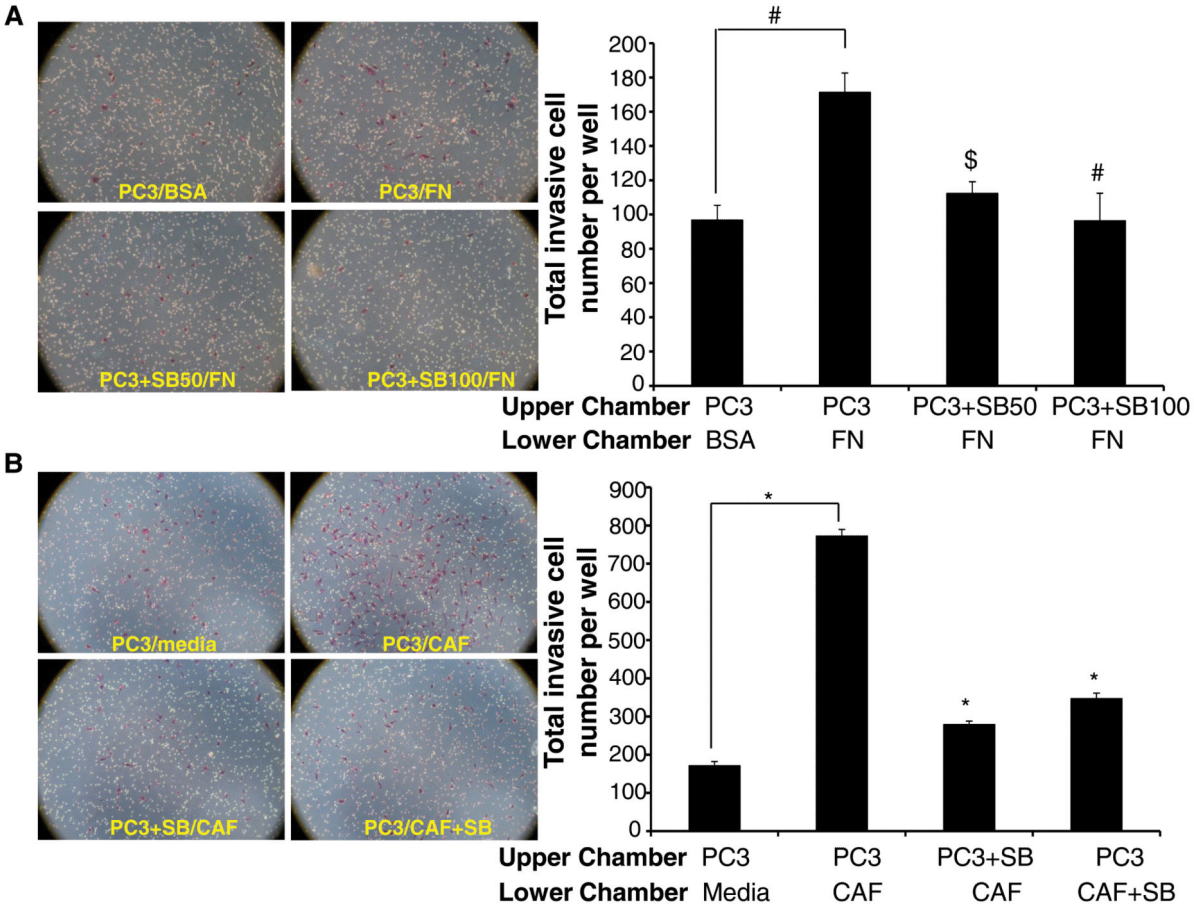

Figure 3. Silibinin inhibits fibronectin- and CAFs-induced invasiveness in PC3 cells

(A) The bottom chambers of Transwell were filled with RPMI media containing 0.5% FBS supplemented with BSA or fibronectin (40 μg/ml), and in the top chambers PC3 cells (2×10^4) were seeded in RPMI media containing 0.5% FBS with or without silibinin (50 and 100 μM). After 10 h, PC3 cells invasiveness was measured. **(B)** CAF cells were grown in the bottom chambers of Transwell with or without silibinin (100 μM), and PC-3 cells (2×10^4) were seeded in the top chambers with or without silibinin (100 μM). After 10 hrs, PC3 cells invasiveness was measured. Cell invasion data shown are means±SEM of three samples. Representative photomicrographs are shown at 100×. Abbreviations: BSA: Bovine serum albumin; FN: Fibronectin; SB: Silibinin; CAF: Cancer-associated fibroblasts. *, $p \leq 0.001$; #, $p \leq 0.01$; $, $p \leq 0.05$

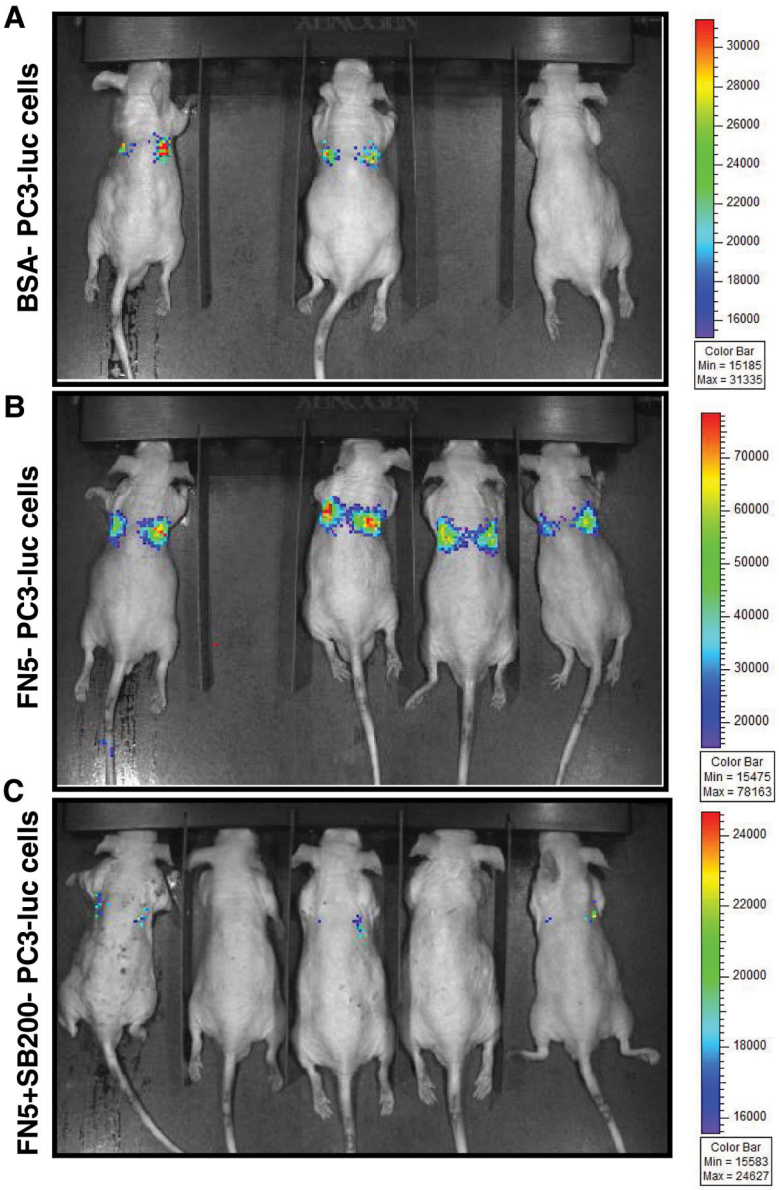

Figure 4. Silibinin inhibits fibronectin-induced dissemination of PC3-luc cells in athymic nude mice

(A-C) PC3 cells were cultured in RPMI media (with 0.5% FBS) for 24 hrs; thereafter cells were collected and re-plated on fibronectin (5 μg/ml) coated plates with or without silibinin (200 μM). PC3 cells plated on BSA (5 μg/ml) coated plates served as relevant control. After 24 hrs, cells were harvested, and 7×10^5 cells were injected in each male athymic nude mouse. Mice were imaged 6 hrs later as described in the 'Materials and methods'. Representative mice images as well as bioluminescence intensity scale bar for each image are presented. Abbreviations: BSA: Bovine serum albumin; FN: Fibronectin; SB: Silibinin

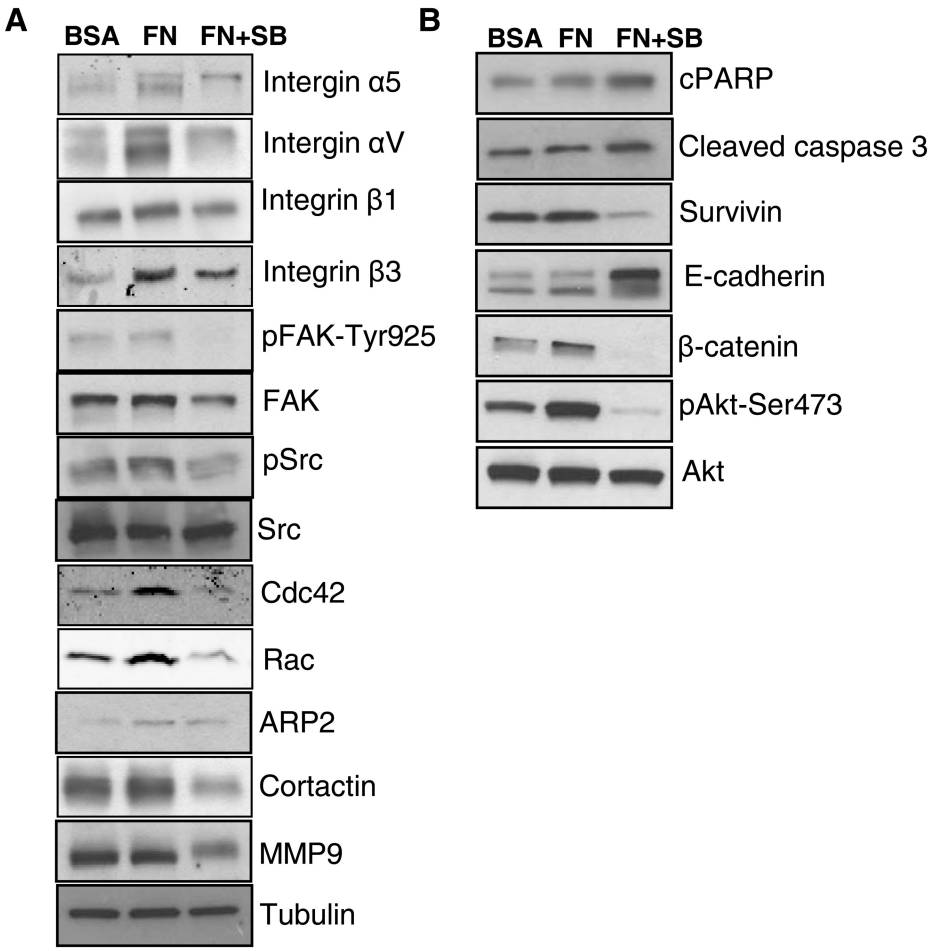

Figure 5. Silibinin targets multiple signaling pathways down-stream of fibronectin-integrin in PC3 cells

(A-B) PC3 cells were cultured in RPMI media (with 0.5% FBS) for 24 hrs; thereafter cells were collected and re-plated on fibronectin (5 μg/ml) coated plates with or without silibinin (200 μM). PC3 cells plated on BSA (5 μg/ml) coated plates served as relevant control. After 24 hrs, cells were harvested and whole cell lysates were prepared and analyzed for Integrins (α5, αV, β1 and β3), phosphorylated and total FAK and Src, Cdc42, Rac, ARP2, cortactin, MMP9, cPARP, cleaved caspase 3, survivin, E-cadherin, β-catenin, and phosphorylated and total Akt. Tubulin was used as a loading control

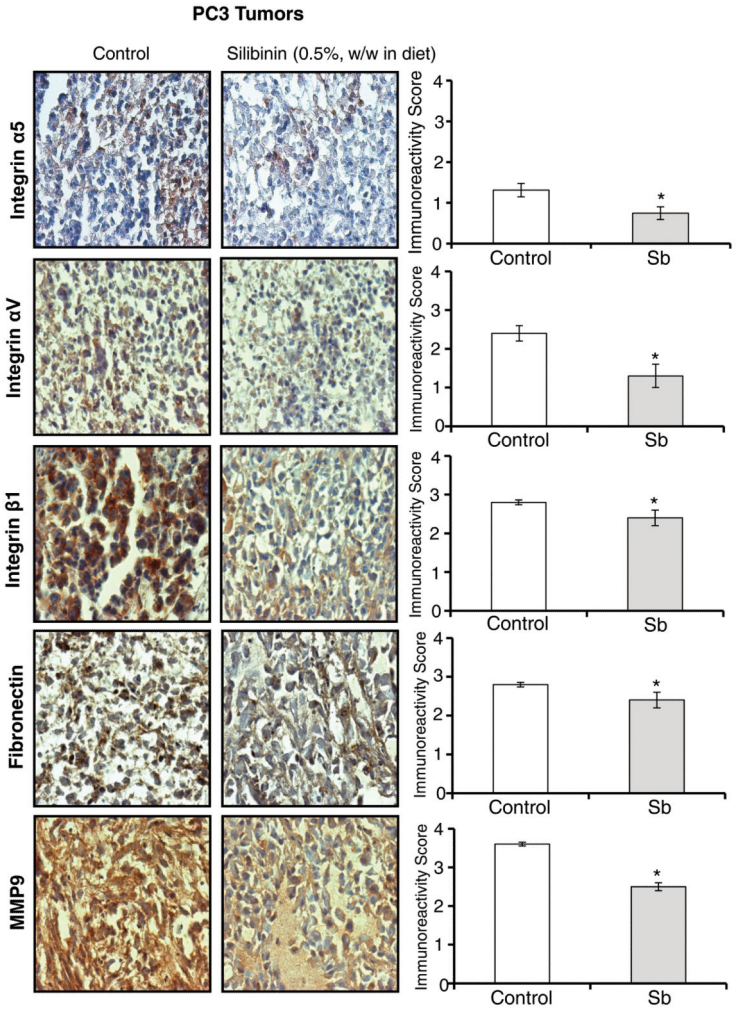

Figure 6. Effect of silibinin feeding on the expression of integrins, fibronectin and MMP9 in PC3 tumors

PC3 tumor tissues were analyzed by IHC to determine silibinin's effects on the expression of integrins (α5, αV, and β1), fibronectin and MMP9. Immunoreactivity (represented by brown staining) of these biomarkers was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), and 4+ (very strong staining). Data shown in bar diagram represent mean ± SEM of 6-9 samples for each group. SB: Silibinin. *, $p \leq 0.001$

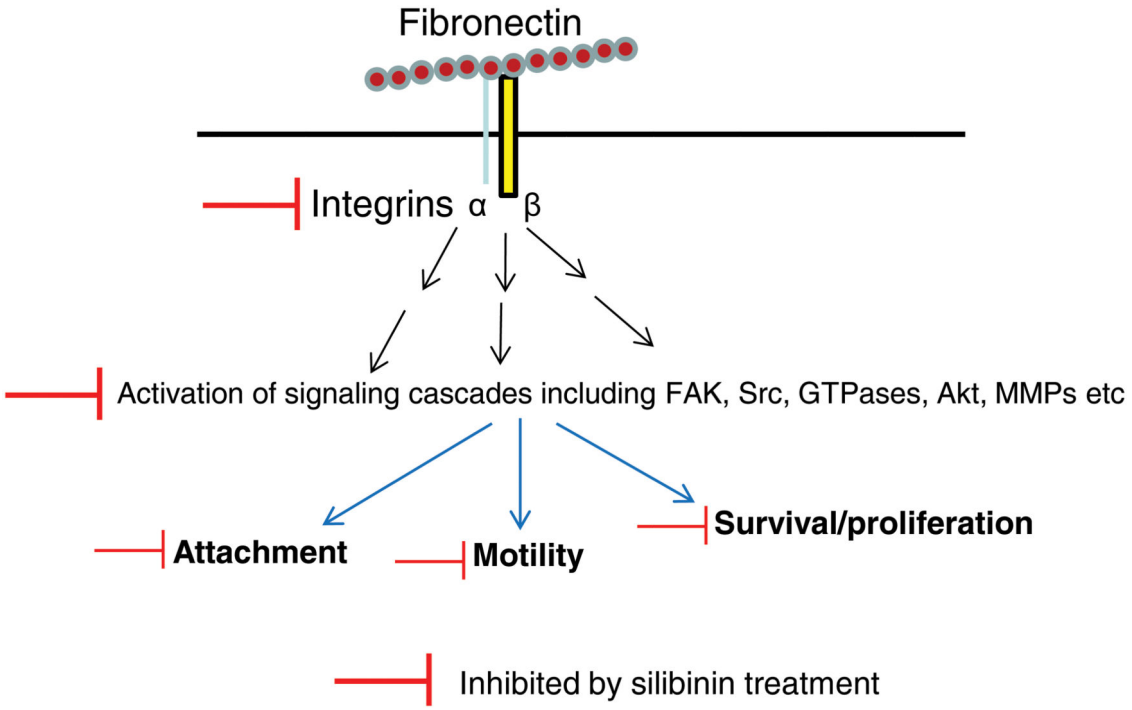

Figure 7. A summary of silibinin effect on integrin signaling in prostate cancer cells

Silibinin targets the fibronectin induced integrin signaling together with several additional signaling cascades, thereby inhibiting the attachment, motility and proliferation of prostate cancer cells.