Silibinin inhibits prostate cancer invasion, motility and migration by suppressing vimentin and MMP-2 expression

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Aim: Silibinin is known to exert growth inhibition and cell death together with cell cycle arrest and apoptosis in human prostate cancer cells. Whether silibinin could inhibit the invasion, motility and migration of prostate cancer cells remains largely unknown. This study was designed to evaluate this efficacy and possible mechanisms using a novel highly bone metastatic ARCaP M cell model.

Methods: Four prostate cancer cell lines, LNCaP, PC-3, DU145, and ARCaP M, were used in this study. These cells were treated with increasing concentrations of silibinin (50, 100, and 200 μmol/L) for different periods of time. After treatment, cell viabilities of four prostate cancer cells were compared by MTT assay. Alterations of ARCaP M cell invasion, motility and migration were assessed by cell invasion, motility and wound healing assays. The changes of vimentin expression were observed by Western blotting and immunofluorescence staining, and the expression of MMP-2, MMP-9, and uPA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR).

Results: ARCaP M cells showed less sensitivity to the growth inhibition of pharmacological doses of silibinin than LNCaP, PC-3, and DU145 cells. However, silibinin exerted significant dose- and time-dependent inhibitory effects on the invasion, motility and migration of ARCaP M cells. Furthermore, the expression of vimentin and MMP-2, but not MMP-9 or uPA, was down-regulated in a dose- and time-dependent manner after treatment of silibinin.

Conclusion: This study shows that silibinin could inhibit the invasion, motility and migration of ARCaP M cells via down-regulation of vimentin and MMP-2 and therefore may be a promising agent against prostate cancer bone metastasis.

Keywords: silibinin; prostate cancer; invasion; motility; migration; vimentin; MMP-2

Introduction

Prostate cancer is one of the most commonly diagnosed cancers and the second leading cause of death in men in Western countries[1]. Surgical and hormonal therapies have shown beneficial effects for early-stage, hormone-responsive disease. However, few treatment options are available for more aggressive hormone-independent or hormone-refractory prostate cancer that is resistant to chemotherapy and radiotherapy[2]. Therefore, more effort must be devoted to developing novel agents that target these unique characteristics of prostate cancer. In this regard, several nontoxic phytochemicals, including silibinin, have shown promising therapeutic and preventive efficacy against prostate cancer[3].

Silibinin, the major active constituent of silymarin isolated from milk thistle (Silybum marianum), has shown strong anticancer efficacy against both androgen-dependent and androgen-independent prostate cancer. This phytochemical is currently being used in a phase I/II clinical trial for treatment of prostate cancer[4]. Silibinin inhibited the proliferation of human prostate cancer LNCaP, PC-3, and DU145 cells in vitro, and oral silibinin suppressed the growth of PC-3 and DU145 xenografts in nude mice and transgenic adenocarcinoma of mouse prostate (TRAMP) in vivo[5–10]. Extensive studies have shown the multi-targeted molecular mechanisms underlying therapeutic actions of silibinin in prostate cancer, and silibinin could target the IGF1-IGFBP3 axis and the cyclin-dependent kinase (CDK)-cyclin-CDK inhibitor axis to inhibit cell proliferation, as well as activate the caspase pathway to induce apoptosis[5–10]. In addition, several recent studies have also shown the anti-metastatic activities of silibinin in prostate cancer. It
of carcinoma. ARCaP cells were treated with silibinin (50, 100, and 200 μmol/L) for 24, 48, and 72 h, and then cells were harvested and their in vitro invasiveness was determined using a Transwell chamber (Corning, NY, USA). Matrigel (Sigma, St Louis, MO, USA) was diluted by serum-free medium to a final concentration of 2 mg/mL, and 8 μm pore polycarbonate membrane filters were coated with 50 μL of Matrigel. Treated cells were then seeded into the upper chamber with 5×10^4 cells/well in 100 μL of serum-free medium, and 1 ml medium containing 20% FBS was added to the lower chamber as a chemoattractant. After incubation for 48 h at 37 °C in 5% CO₂, the Matrigel coating on the upper surface of the filter was wiped with a cotton swab. Cells that invaded to the lower surface of the filter were fixed by 4% paraformaldehyde and stained with Giemsa. Cell numbers were counted in three random fields (×100) per filter. The cell motility assay was conducted with 2.5×10^3 cells/well in a similar fashion in a Transwell chamber without coating Matrigel.

Wound healing assay
To determine the effect of silibinin on migration of ARCaPM cells in vitro, cells were seeded at a density of 5×10^3 cells in 60 mm-dishes and grew to about 90% confluence after 48 h. Medium was removed and cell monolayers were wounded by manually scraping the cells with a 1 ml plastic pipette tip. Debris was removed from the culture by washing with PBS twice, and cells were then cultured with fresh medium containing silibinin at different concentrations for 24, 48, and 72 h. Images were captured immediately after wounding and 24 h post wounding, and wound closure was monitored with a UOP DSZ500X inverted microscope (UOP Microscope, Chongqing, China). Wound sizes were verified with the scale of the images to ensure that all wounds were the same width at the beginning. The migration distance and migration inhibitory rate were calculated by the following formula: migration distance=(wound width at the beginning–wound width after treatment)/2 (μm); migration inhibitory rate=(average migration distance in the control group–average migration distance in the treatment group)/average migration distance in the control group×100%.

Western blotting analysis
After the indicated silibinin treatment, the medium was removed and ARCaPM cells were washed with cold PBS twice; then, total cellular protein lysates were prepared with RIPA buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.1% SDS,
1% NP40 and 0.5% sodium deoxycholate) containing proteinase inhibitors (1% Cocktail and 1 mmol/L PMSF, both from Sigma, St Louis, MO, USA). A total of 30 μg of protein was separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 1 h with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (pH 7.6, TBST). Monoclonal vimentin antibody (V9, Santa Cruz, CA, USA) was applied at 1:2000 dilutions by 5% skim milk in TBST at room temperature for 1.5 h. After being washed with TBST, membranes were incubated with secondary antibodies coupled to horseradish peroxidase at room temperature for 1 h and visualized with an ECL chemiluminescent detection system (Pierce, Rockford, IL, USA). Loading differences were normalized using a monoclonal GAPDH antibody. The mean density for each band was analyzed using Glyko BandScan software (Glyko, Novato, USA).

Immunofluorescence staining

After the indicated silibinin treatment, ARCaPM cells on coverslips were washed with PBS and fixed in 4% paraformaldehyde for 20 min. Cells were permeabilized with PBS containing 0.25% Triton X-100 for 30 min and blocked with horse serum for 20 min. Cells were then incubated with a 1:1000 dilution of monoclonal vimentin antibody in PBS buffer containing 10% bovine serum overnight at 4 °C and washed and incubated with a 1:200 dilution of TRITC conjugated anti-mouse IgG. The specimens were observed under Olympus IX-50 fluorescence inverted microscope (Olympus, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

After the indicated silibinin treatment, total RNA was isolated from ARCaPM cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quantitated by absorbance at 260 nm. The RNA (2 μg) was reverse transcribed using ReverTaid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St Leon-Rot, Germany) according to the manufacturer’s instructions. All PCR analyses were subsequently performed with 2 μL of the cDNA reaction utilizing conditions as follows: 94 °C, 5 min, 32 cycles of 94 °C, 30 s; 58 °C, 30 s; 72 °C, 45 s. Reactions were finished with 72 °C, 7 min extension. Primers were used for MMP-2, MMP-9, uPA, and β-actin: MMP-2 (475 bp): 5’-GGCCCTGTCACTCCTGAGAT-3’ (forward) and 5’-GGCATCCAGGTTATCGGGGA-3’ (reverse); MMP-9 (482 bp): 5’-CAACATCACCTATTGGATCC-3’ (forward) and 5’-CGGGTGTAGAGTCTCTCGCT-3’ (reverse); uPA (348 bp): 5’-TTGCCGCCCACCTACTACAGGAG-3’ (forward) and 5’-ATCCCTTCGGAATGATGTTCA-3’ (reverse); β-actin (318 bp): 5’-ATCATGTTTGAGACCTTCAACA-3’ (forward) and 5’-CATCTCTGTCGGAAGTCCA-3’ (reverse). PCR products were analyzed by 2% agarose gel electrophoresis and visualized using ethidium bromide staining. The mean density for each band was also analyzed using Glyko BandScan software.

Statistical analysis

All assays were repeated in triplicate in three independent experiments, and all data were expressed as means±SEM. Analysis of variance (ANOVA) for multiple comparisons was used as noted. In all cases, P<0.05 was considered significant. All statistical tests were performed with statistical analysis software (SPSS, Chicago, IL, USA).

Results

Decreased sensitivity of ARCaPM cells to growth inhibition by silibinin

We exposed four human prostate cancer cell lines (LNCaP, PC-3, DU145, and ARCaPM) to increasing concentrations of silibinin for different time periods and compared their relative sensitivities to the growth inhibition of silibinin by MTT assay. These cells displayed marked heterogeneity in responsiveness. Consistent with the previous data[5–7], silibinin significantly inhibited the cell proliferation of LNCaP, PC-3, and DU145 cells in a dose- and time-dependent manner, whereas it had a weaker inhibitory effect on the cell viability of ARCaPM cells (Figure 1). In dose-dependent experiments, the lower dose of 50 μmol/L silibinin could reduce the cell viabilities of LNCaP, PC-3, and DU145 cells by 18.5%, 26.7%, and 15.0%, respectively, whereas only 0.7% growth inhibition was observed in ARCaPM cells after 48 h of treatment. Even at a concentration as high as 200 μmol/L, only 18.5% growth inhibition was
observed in ARCaP_M cells, but 48.7%, 60.0%, and 73.8% in LNCaP, PC-3, and DU145 cells, respectively (Figure 1A). Similarly, in time-course experiments, 100 μmol/L silibinin treatment for 24 h led to 24.8%, 21.8%, and 27.3% growth inhibition of LNCaP, PC-3, and DU145 cells, respectively, but only 5.3% of ARCaP_M cells. The growth inhibition reached its maximum at 96 h, and 100 μmol/L silibinin treatments resulted in 47.2%, 71.3%, and 41.1% growth inhibition of LNCaP, PC-3, and DU145 cells, respectively, but only 22.2% of ARCaP_M cells (Figure 1B). These data indicated that highly metastatic ARCaP_M cells were less sensitive to growth inhibition by silibinin than LNCaP, PC-3, and DU145 cells.

Inhibition on invasion, motility and migration of ARCaP_M cells by silibinin

Using a Transwell chamber coated with or without Matrigel, we found that silibinin could significantly reduce the invasion and motility of ARCaP_M cells (Figure 2). As low as 37% of the invasive capability and 50% of motility capability were retained after 100 μmol/L silibinin treatment for 48 h (Figure 2A). Moreover, this tremendous inhibitory effect occurred in a dose- and time-dependent manner. Different concentrations of silibinin (50, 100, and 200 μmol/L) after 48 h treatment reduced the invasion of ARCaP_M by 15%, 63%, and 92% and reduced the motility by 20%, 50%, and 25% (Figure 2B). As such, 100 μmol/L silibinin for 24, 48, and 72 h reduced the invasion of ARCaP_M cells by 41%, 53%, and 87.5%, respectively, and reduced the motility by 35%, 50%, and 75%, respectively (Figure 2C).

Additionally, we also obtained similar results in a wound healing assay. Silibinin inhibited migration in vitro for ARCaP_M cells (Figure 3). After treatment with 200 μmol/L silibinin for 72 h, the cells remained creviced, whereas the wounds with mock treatment healed completely (Figure 3A). This inhibitory effect was dose- and time-dependent (Figure 3B). In a quantitative analysis, different concentrations of silibinin (50, 100, and 200 μmol/L) after 24 h treatment reduced the migration of ARCaP_M cells by 45%, 70.1%, and 85%, respectively. Similarly, after 72 h of treatment with 50, 100, and 200 μmol/L silibinin, 30.6%, 47.2%, and 69.4% inhibition of migration was observed in ARCaP_M cells. Our results strongly suggest that pharmacological doses of silibinin could lead to significant inhibition of invasion, motility and migration of ARCaP_M cells.

Silibinin inhibits vimentin and MMP-2 expression in ARCaP_M cells

To elucidate the possible underlying mechanisms of anti-metastatic activities of silibinin on ARCaP_M cells, we detected changes in vimentin, MMP-2, MMP-9, and uPA expression by Western blotting, immunofluorescence staining and RT-PCR. As shown in Figure 4, Western blotting revealed silibinin treatment significantly decreased the expression of vimentin in a dose- and time-dependent manner when GAPDH served as a loading control (Figure 4A and 4B). Treatment with 50, 100, and 200 μmol/L of silibinin after 24 h showed 32.4%, 54.9%, and 79% decreases in vimentin protein compared with controls; 100 μmol/L silibinin treatment for 24, 48, and 72 h showed 58.6%, 74.3%, and 86.9% decreases in vimentin protein. Furthermore, we observed the similar decreasing tendency of vimentin in the cytoplasm after 100 μmol/L silibinin treatment by immunofluorescence staining (Figure 4C). In addition, 100 μmol/L silibinin treatment for 24, 48, and 72 h decreased MMP-2 mRNA by 42.3%, 34.5%, and 33.3%,
but had no significant effects on MMP-9 and uPA mRNA expression when β-actin served as a loading control (Figure 4D). Together, these findings suggest that down-regulation of vimentin and MMP-2 might be involved in the inhibition of invasion, motility and migration of ARCaP_M cells after silibinin treatment.

Discussion
In this study, utilizing a well-characterized prostate cancer cell line, we have demonstrated a novel anticancer effect of silibinin and provided possible mechanisms that are responsible for its anti-metastatic effect in vitro.

The anti-proliferative effect of silibinin has been well documented in the androgen-dependent and androgen-independent prostate cancer LNCaP, 22Rv1, PC-3, and DU145 cell lines, and studies have shown that silibinin could inhibit the growth of prostate cancer by inducing cell cycle arrest or caspase-dependent apoptosis[5-7]. In agreement with previous studies, we also observed strong inhibitory effects of silibinin on LNCaP, PC-3, and DU145 cell proliferation, whereas ARCaP_M cells showed a much weaker response to the anti-proliferative effect of silibinin. As a derivative subclone from androgen-repressed prostate cancer, Xu et al have demonstrated that ARCaP_M cells exhibited an aggressive mesenchymal phenotype, which was fast-growing and more resistant to chemotherapeutic drugs, such as doxorubicin, etoposide and paclitaxel[14]. Here, we revealed that ARCaP_M cells showed stronger resistance to growth inhibition following silibinin treatment.

Several studies have silibinin might represent a potential anti-metastatic agent inhibiting cancer cell invasion and motility, and this efficacy has been demonstrated in A549 lung cancer cells, SCC-4 tongue cancer cells, osteosarcoma MG-63 cells and MCF-7 breast cancer cells[16-20]. Recently, it has been reported that silibinin exerts inhibitory effects by silibinin on viability, migration and adhesion of androgen-independent PC-3 cells, such that α2β1-integrin may be the target[11]. Singh et al also found that silibinin treatment inhibited prostate tumor growth, progression, local invasion and distant metastasis using the TRAMP mouse model[22]. Here, we are the first to show that pharmacological doses of silibinin may exert inhibitory effects on the invasion, motility and migration of ARCaP_M cells in a dose- and time-dependent manner.

To investigate the molecular effects of silibinin treatment on ARCaP_M cells, we further analyzed the alterations of several molecules related to cancer invasion and metastasis, such as vimentin, MMP-2, MMP-9, and uPA. Vimentin is a mammalian structural cytoskeletal protein constituting type III mesenchymal filaments, and its elevated and aberrant expression correlates well with up-regulated cell invasion or migration both in the embryo and in malignancy[21]. Several studies have shown that vimentin could affect the invasion and motility of prostate cancer cells and is a promising marker for predicting aggressive and metastatic prostate cancer[22-23]. Consistent with their highly metastatic features, ARCaP_M cells display higher expression of vimentin and other mesenchymal markers[14]. Here, it was demonstrated that silibinin markedly decreased vimentin protein expression in a dose- and time-dependent manner. Similar results were reported in Singh's findings, which revealed anti-metastatic activities of silibinin in TRAMP mice together with a concomitant strong decrease in the level of vimentin[12].

In addition, we found silibinin significantly suppressed MMP-2 expression, but not MMP-9 or uPA in ARCaP_M cells. MMPs are known proteolytic enzymes that degrade the extracellular matrix and basement membrane of cells, and MMP-2 and MMP-9 are the most vital enzymes for degradation[24, 25]. Therefore, they are considered major factors in tumor invasion and metastasis. uPA, which is an upstream enzyme of MMPs, might activate a series of protein degradation reactions to regulate or activate MMPs[26]. A series of studies demonstrated that silibinin could inhibit invasion and motility of A549 cells and SCC-4 cells by down-regulating MMP-2 and uPA and up-regulating tissue inhibitor of metalloproteinase-2 (TIMP-2) and PAI-1 expressions[16, 17]. Moreover, in A549 lung cancer cells, silibinin inhibited MMP-2 and uPA expression through reducing ERK1/2 and Akt phosphorylation[18]. In human osteosarcoma MG-63 cells, silibinin inhibited uPA and MMP-2 expressions, IL-6-induced ERK1/2 and c-Jun phosphorylation, and cell invasiveness[19]. In addition, silibinin also reduced...
PMA-induced invasion of MCF-7 breast cancer cells through the specific inhibition of AP-1-dependent MMP-9 gene expression[20]. In our studies, we observed a suppression effect of silibinin on MMP-2 expression, but not MMP-9 or uPA in ARCaP_M cells. Thus, it is possible that silibinin could suppress prostate cancer invasion and metastasis through the inhibition of vimentin and MMP-2 expression.

In conclusion, here we are the first to demonstrate a novel mechanism by which silibinin acts as an anti-metastatic agent via inhibiting invasion, motility and migration in novel, highly bone metastatic ARCaP_M cells by down-regulating the vimentin and MMP-2 expression. Furthermore, we will use nude mice to verify the in vivo anti-metastatic effects of silibinin in human prostate cancer and explore additional molecular mechanisms of silibinin on prostate cancer bone metastasis.

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Author contribution
Kai-jie WU, Jin ZENG, and Da-lin HE designed this research; Kai-jie WU, Guo-dong ZHU, Lin-lin ZHANG, and Dong ZHANG performed this research, Lei LI, Jin-hai FAN, and Xinyang WANG contributed new analytical tools and reagents; Kai-jie WU and Jin ZENG analyzed data, wrote and revised the paper.

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