Role of E-cadherin in Antimigratory and Antiinvasive Efficacy of Silibinin in Prostate Cancer Cells

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Abstract
The epithelial-to-mesenchymal transition (EMT) in prostate cancer (PCA) cells is considered prerequisite for acquiring migratory/invasive phenotype, and subsequent metastasis. We hypothesized that promoting the E-cadherin expression in PCA cells by using nontoxic phytochemicals, like silibinin, would prevent EMT and consequently invasiveness. Our results showed that silibinin treatment (5–90 μmol/L) significantly inhibits migratory and invasive potential of advance human PCA PC3, PC3MM2, and C4–2B cells in in vitro assays. Importantly, the antimigratory/antiinvasive efficacy of silibinin was not due to its cytotoxicity toward PCA cells. Molecular analyses showed that silibinin increases E-cadherin level that was localized mainly at cellular membrane as evidenced by subcellular fractional and confocal analyses in PC3 cells, which might be responsible for morphologically observed shift toward epithelial character. Silibinin also decreased the levels of Slug, Snail, phospho-Akt(ser⁴⁷³), nuclear β-catenin, phospho-Src(tyr⁴¹⁹) and Hakai; together they play an important role in regulating E-cadherin expression/function and EMT. Similar silibinin effects on E-cadherin, β-catenin, phospho-Src(tyr⁴¹⁹), and Hakai levels were also observed in PC3MM2 and C4–2B PCA cells. Selective Src inhibition by dasatinib also showed increased E-cadherin expression in PC3 cells suggesting a possible involvement of Src inhibition in silibinin-caused increase in E-cadherin level. Additional studies in PC3 cells with stable knock-down of E-cadherin expression revealed that antimigratory/antiinvasive efficacy of silibinin is in-part dependent on E-cadherin expression. Together, our results showing antimigratory/antiinvasive effects of silibinin and associated mechanisms suggest that silibinin should be tested further in clinically relevant animal models toward exploiting its potential benefits against metastatic PCA. Cancer Prev Res; 4(8): 1222–32. ©2011 AACR.

Introduction
Prostate cancer (PCA) is the most commonly diagnosed noncutaneous malignancy, and is second leading cause of cancer-related deaths in American men (1). Patients with localized PCA have a very high 5-year survival rate and a relatively low mortality to incidence ratio as compared with other cancers. Patients with clinically detectable metastatic disease, however, have median survival of 12–15 months, suggesting that cancer cell metastasis is the main cause of high mortality among PCA patients (2–4). Currently, chemotherapy, radiotherapy, hormone therapy, biological therapy, surgery, or a combination of these therapies are employed to treat advance stage PCA patients; but in most cases, these therapies provide only a marginal or no survival benefit. Further, exorbitant costs as well as unacceptable level of toxicity associated with current therapeutic regime call for newer measures to control PCA. One emerging approach in cancer management is the use of nontoxic and biologically effective dietary and nondietary agents. The list of these agents with efficacy against cancer metastasis is growing steadily and many of these agents have already entered clinical phase (5–7).

Silibinin, a flavonoid from milk thistle seed extract, is a widely consumed dietary supplement for its efficacy against liver disorders (8, 9). Silibinin has also shown strong efficacy both in vitro and in vivo against PCA cells, and is currently being evaluated in PCA patients (7, 10–14). Earlier, we reported the strong antimetastatic efficacy of silibinin in TRAMP (transgenic adenocarcinoma of the mouse prostate) model (12, 13); however, detailed mechanisms for its strong antimetastatic efficacy remains largely unknown.

Metastasis is one of the hallmarks of cancer cells and is considered responsible for more than 90% of cancer-associated deaths (15). This is an extremely complex biological event during which cancer cells acquire motility, invade locally and enter into systemic blood circulation, survive in circulation, arrest in microvasculature and subsequently
extravasate and grow at distant organs (2, 16, 17). In metastasis, acquisition of motility and invasiveness are the major earlier events during which cancer cells shed many of their epithelial characteristics, undergo drastic modifications in their cytoskeleton and acquire highly motile and invasive mesenchymal phenotype (18, 19). This phenomenon in cancer cells is known as epithelial-to-mesenchymal transition (EMT) and represents one major mechanism in cancer cell metastasis (18, 19).

The molecular basis of EMT is very complex and involves several interconnected pathways that downregulate the expression of epithelial molecule E-cadherin, which is well known for regulating cell-to-cell contact, cell shape, and polarity (18, 20). E-cadherin connects adjacent cells through homophilic interactions and is also linked to the cytoskeleton though multicatenin complex attached to their cytoplasmic tails (21, 22). In this complex, β-catenin and p120 are directly associated with E-cadherin, whereas α-catenin is the link between β-catenin and actin microfilament network of the cytoskeleton (21, 22). Importantly, the aberrant or decreased expression of E-cadherin is considered as one of the biomarkers for poor prognosis in PCA (23, 24). Therefore, promoting E-cadherin expression using nontoxic phytochemicals should be considered an ideal strategy toward preventing cancer cells from acquiring motility and invasiveness.

Lately, several transcriptional factors (Snail, Slug, Zeb1, Twist, etc.) have also been identified which negatively regulate E-cadherin expression and play important role in EMT induction and maintenance of migratory and invasive phenotype in cancer cells (18–20). A variety of kinases such as MAPKs, PI3K, and Src are also known to regulate EMT and metastasis of cancer cells (18, 19). Akt is also reported to upregulate Snail and β-catenin expression, thereby promoting EMT (18, 25). Src is a non-receptor tyrosine kinase whose overexpression and activation has been associated with numerous types of cancers including PCA (26, 27). Src is considered as an integrator of several cellular signaling cascades in PCA cells, thereby it affects a wide range of biological phenomena including proliferation, migration, adhesion, and metastasis (26, 27). Due to its pleiotropic roles in growth and progression, various Src inhibitors are being tested in clinic against PCA (26, 27). Src has also been reported to phosphorylate E-cadherin that facilitates its binding with Hakai, a RING finger-type E3 ubiquitin ligase, which leads to ubiquitination, endocytosis, and lysosomal-mediated degradation of E-cadherin (28, 29). Accordingly, in the present study, we assessed silibinin effect on migratory and invasive potentials of three human metastatic PCA cell lines namely PC3, PC3MM2, and C4–2B, and examined the role of E-cadherin and other EM regularulators in the biological efficacy of silibinin. Our findings showed a strong antimigratory and antiinvasive efficacy of silibinin against PCA cells, which was in-part through promoting E-cadherin expression and decreasing the level of Slug, phosphorylated-Akt, nuclear β-catenin, phosphorylated-Src, and Hakai.

Materials and Methods

Cell lines and reagents
PC3 cells were from American Type Culture Collection (ATCC). Highly metastatic PC3MM2 cell line was a kind gift (Dr. Isaiah J. Fidler, University of Texas M. D. Anderson Cancer Center) and was originally selected from PC3 cells. C4–2B cells were derived from the bone metastasis of LNCaP-variant cell line C4–2 and purchased from VirotMed Laboratories. All three cell lines were obtained during 2008, and tested and authenticated by DNA profiling for polymorphic short tandem repeat (STR) markers at University of Colorado Cancer Center DNA Sequencing & Analysis Core most recently in August 2010. Antibodies for β-catenin (sc-7199, 1:1000 dilution), Vimentin (sc-7557, 1:250 dilution), Zeb1 (sc-25388, 1:200 dilution), Histone H1 (sc-10806, 1:500 dilution), and Hakai (sc-101912, 1:200 dilution), and E-cadherin shRNA plasmid were from Santa Cruz Biotechnology. Antibodies for E-cadherin (#3195, 1:1000 dilution), phospho-Src( tyr419) (#2101, 1:1000 dilution), total Src (#2109, 1:1000 dilution), phospho-Akt(Ser473) (#9271, 1:500 dilution), and total Akt (#4685, 1:1000 dilution), and antirabbit peroxidase-conjugated secondary antibody (#7074, 1:1000 dilution) were obtained from Cell Signaling. Silibinin, mitomycin C, puromycin, and β-actin antibody (A2228, 1:2000 dilution) were from Sigma–Aldrich. Amershams Enhanced Chemiluminescence (ECL) detection system and antimouse horseradish peroxidase (HRP)-conjugated secondary antibody (NA931V, 1:1000 dilution) were from GE Healthcare. Antibodies for Snail (ab7732, 1:500 dilution) and Slug (ab27568, 1:500 dilution) were from Abcam. GeneJuice transfection reagent was from Novagen. Dasatinib was purchased from Selleck Chemicals. RPMI1640 media and other cell culture materials were from Invitrogen Corporation. Antibody for α-tubulin (MS-581, 1:1000 dilution) was from Lab Vision Corporation. All other reagents were obtained in their commercially available highest purity grade.

Cell culture and treatments
PC3, PC3MM2, and C4–2B cells were cultured in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum and 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate at 37°C in a humidified 5% CO2 incubator. Cells were treated with different concentrations of silibinin (5–90 μmol/L in medium), dissolved originally in dimethyl sulfoxide (DMSO), for described time periods. An equal amount of DMSO (vehicle) was present did not exceed 0.1% (v/v) in any treatment.

Wound healing assay
The antimigratory efficacy of silibinin was examined using the well-established wound healing assay (30, 31). Briefly, PC3 or PC3MM2 cells were grown to full confluence in six-well plates. In each case, cells were made quiescent by pretreating with 0.5 μmol/L mitomycin C for
2 hours to ensure that wounds are filled due to cell migration and not by cell proliferation (32, 33). Subsequently, cells were wounded by pipette tips and washed twice with media to remove detached cells, and photomicrographs of initial wounds were taken using Canon Power Shot A640 digital camera (at 100× magnification). Thereafter, cells were treated with DMSO or 5–90 μmol/L concentrations of silibinin in RPMI1640 media (10% FBS and 0.5 μmol/L mitomycin C). Experiment was terminated as soon as wound was completely filled in DMSO-treated controls and photomicrographs of final wounds were taken for each group. Initial and final wound sizes were measured using AxioVision Rel.4.7 software, and difference between the two was used to determine migration distance using the formula: Initial wound size minus final wound size divided by 2.

**Invasion assay**

Invasion assay was conducted using matrigel coated trans-well chambers from BD Biosciences as described earlier (34). Briefly, in this assay, bottom chambers were filled with RPMI media with 10% FBS and top chambers were seeded with 1 × 10^4 cells (PC3, PC3MM2, or C4–2B) per well in RPMI media (with 0.5% FBS) along with DMSO or 5–90 μmol/L concentrations of silibinin. After 22 hours of incubation under standard culture conditions, PCA cells on top surfaces of the membrane (noninvasive cells) were scraped with cotton swabs and cells on bottom sides of membrane (invasive cells) were fixed with cold methanol, stained with hematoxylin/eosin and mounted. Images were taken using Cannon Power Shot A640 camera on Zeiss inverted microscope and invasive cells were manually counted at 400× in 10 random fields on each membrane.

**Migration assay**

Migration assay for C4–2B cells was carried out similar to invasion assay described above, but in this assay the transwell chambers (BD Biosciences) lacked matrigel layer (30).

**Cell viability assay**

Approximately, 5 × 10^4 cells (PC3, PC3MM2, or C4–2B) were plated and treated with silibinin (5–90 μmol/L) in RPMI1640 media under normal serum condition. After 22 hours of treatment, cells were collected and total cell number was determined by counting each sample in duplicate using a hemocytometer under an inverted microscope.

**Western blotting**

Total or nuclear/cytoplasmic cell lysates were prepared as described earlier (35, 36) and subcellular fractionations were prepared as per vendor’s protocol (ThermoFisher Scientific). For Western blotting, 40–70 μg of protein per sample was denatured in 2× SDS–PAGE sample buffers and subjected to SDS-PAGE on 8 or 12% polyacrylamide tris-glycine gels followed by immunoblotting as described earlier (36, 37). In each case, protein loading was monitored by stripping and reprobing the blots with β-actin or α-tubulin antibody. The autoradiograms/bands were scanned and where mentioned, mean density of bands was determined using Adobe Photoshop 6.0 (Adobe Systems).

**Confocal imaging**

PC3 cells were grown on cover slips and treated with silibinin (30–90 μmol/L). After 72 hours, cells were fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked under 5% serum condition, and incubated overnight with E-cadherin antibody. Next, cells were incubated with FITC-tagged secondary antibody along with 4', 6 diamidino 2 phenylindole (DAPI) for 30 minutes. Images were captured at 1000× magnification on a Nikon inverted confocal microscope using 488/405 nm laser wavelengths to detect FITC (green) and DAPI (blue) emissions, respectively. Images were quantified for FITC-green color based upon following scoring criterion: 0+ (no color), 1+ (dim color), 2+ (moderate color), 3+ (bright color), 4+ (very bright color).

**Transfection**

PC3 cells were transfected at about 50% to 60% confluence using GeneJuice as per vendors’ protocol. Briefly, 0.5 μg of random siRNA plasmid or 0.5 μg of E-cadherin specific shRNA plasmid was incubated with GeneJuice/sodium free medium mixture for 15 minutes and then added drop-wise to cells. Transfected cells were selected through puromycin-based selection, and PC3 cells with stable knock-down of E-cadherin (ShEC-PC3 cells) and respective control cells (Sh-PC3 cells) were identified.

**Real-time reverse transcription-PCR**

Total RNA was isolated using PerfectPure RNA cultured cell kit from 5 Prime and E-cadherin mRNA level was quantified by real-time reverse transcription (RT)–PCR following the procedure detailed earlier (38). The primers used for E-cadherin were 5'-AGTGTCCCCCGGTATCTTCC-3' and 5'-CAGCCGCTTTCAGATTTTCAT-3'. Quantity of E-cadherin mRNA in each sample was normalized to the corresponding 18S rRNA level.

**Statistical analysis**

Statistical analysis was carried out using SigmaStat 2.03 software (Jandel Scientific). Data was analyzed using one-way ANOVA (Tukey test) and statistically significant difference was considered at P ≤ 0.05.

**Results**

**Silibinin exerts strong antimigratory and antiinvasive efficacy against prostate cancer cells**

Silibinin treatment (5–90 μmol/L) inhibited the migratory potential of PC3 and PC3MM2 cells by 30%–76% and 18%–80%, respectively, in a dose-dependent manner (Figs. 1A and 1B). In C4–2B cells, creation of wound irreversibly affected their migratory potential and wound was not filled within experimental duration; therefore, we
studied silibinin effect on migratory potential of C4–2B cells using migration chambers. In this assay, silibinin treatment inhibited (13%–87%) migratory potential of C4–2B cells in a dose-dependent manner (Fig. 1C).

Next, we examined effects of silibinin on the invasive potential of PC3, PC3MM2, and C4–2B cells in well-established invasion assay. This is an excellent in vitro model to study the potential of cancer cells to invade through extracellular matrix, which is the first barrier during cancer cells invasion. Results showed that silibinin inhibits invasive potential of PC3, PC3MM2, and C4–2B cells by 11%–65%, 20%–62% and 28%–55%, respectively, in a dose-dependent manner (Figs. 2A-2C). Next, we examined effect of silibinin treatment on the viability of PCA cells after 22 hours of treatment. As shown in Fig. 2D, silibinin treatment (5–90 μmol/L) decreased viability by 9%–34% in PC3, 8%–43% in PC3MM2 and 8%–29% in C4–2B cells (Fig. 2D). The cell viability data suggest that silibinin has antimigratory and antiinvasive efficacy at concentrations where it has minimal, if any, cytotoxicity toward PCA cells.

**Silibinin targets signaling molecules regulating epithelial-to-mesenchymal transition in PC3 cells**

To understand possible mechanism/s underlying silibinin antimigratory and antiinvasive efficacy, we first examined its effect on E-cadherin expression. As shown in Fig. 3A, silibinin treatment (30–90 μmol/L) increased the protein expression of E-cadherin after 24, 48, and 72 hours. Real-time RT-PCR assay showed that E-cadherin mRNA level was increased by approximately 1.8- fold after 72 hours of silibinin treatment, whereas no change in E-cadherin mRNA level was observed after 24 and 48 hours (data not shown).
Next, we examined silibinin effect on other major regulators and markers of EMT. As shown in Fig. 3A, silibinin treatment decreased the protein expression of Slug after 24, 48, and 72 hours whereas a decrease in Snail expression was observed only after 24 and 48 hours. Further, silibinin treatment decreased the Akt phosphorylation at ser-473 site after 48 and 72 hours but only marginally affected total Akt level. Silibinin treatment did not affect the levels of Zeb1 and mesenchymal marker protein Vimentin after 24, 48, and 72 hours in PC3 cells (data not shown).

Effect of silibinin on the expression and subcellular distribution of E-cadherin and \( \beta \)-catenin in PC3 cells

In epithelial cells, \( \beta \)-catenin is generally localized to basolateral plasma membrane, and in association with E-cadherin plays a critical role in cell–cell adhesion (22). Loss of E-cadherin during EMT releases \( \beta \)-catenin from the membranous pool, making it available for nuclear signaling, which then promotes cancer-cell proliferation and invasiveness (21, 39). Therefore, next we examined silibinin effect on \( \beta \)-catenin level in nuclear and cytoplasmic fractions of PC3 cells. As shown in Fig. 3B, silibinin treatment significantly decreased \( \beta \)-catenin level in nuclear fraction of PC3 cells after 24, 48, and 72 hours without affecting \( \beta \)-catenin level in cytoplasmic fraction. Next, we prepared cytoplasmic, membrane, and nuclear fractions to analyze silibinin effect on the localization as well as expression levels of E-cadherin and \( \beta \)-catenin. Silibinin treatment resulted in a significant increase in E-cadherin expression in the membrane fraction of PC3 cells (Fig. 3C), but caused a strong decrease in \( \beta \)-catenin level in nuclear fraction with only a slight decrease in cytoplasmic fraction and no significant change in membrane fraction (Fig. 3C). Blots were stripped and reprobed with Histone H1 to confirm purity and equal protein loading in nuclear fractions. Blots were also reprobed with Akt antibody as a loading control for cytoplasmic and membrane fractions. We selected this protein because silibinin treatment for 24 hours did not significantly alter...
the Akt level in total cell lysates (Fig. 3A). Confocal analysis also confirmed that silibinin (30–90 μmol/L) promotes E-cadherin expression especially at cellular membrane in a dose-dependent manner in PC3 cells (confocal pictures and FITC-quantification bar diagram are shown as part of Fig. 3C).

Next, we examined the effect of silibinin on E-cadherin and β-catenin levels in PC3MM2 and C4–2B cells. As
shown in Fig. 3D, silibinin treatment increased E-cadherin protein expression in both PC3MM2 and C4–2B cells, whereas slightly decreased β-catenin level in total cell lysates, supporting that silibinin effects on E-cadherin and β-catenin are not PCA cell line specific.

Silibinin decreases Hakai level and Src phosphorylation in prostate cancer cells

As shown in Fig. 4A, silibinin treatment (30–90 μmol/L) decreased the Hakai level in PC3 cells after 24, 48, and 72 hours. Similarly, silibinin treatment (30–90 μmol/L) for 24 hours decreased the Hakai level in both PC3MM2 and C4–2B cells; even though decrease was more prominent in the latter cell line (Fig. 4B). Next, we examined silibinin effect on Src phosphorylation at tyrosine-419 site that is considered surrogate marker for Src activity (27).

Silibinin treatment (30–90 μmol/L) strongly decreased phospho-Src(tyr419) after 24, 48, and 72 hours in PC3 cells without affecting total Src level (Fig. 4A). In PC3MM2 cells, decrease in phospho-Src(tyr419) was prominent only at 60 and 90 μmol/L concentrations of silibinin, whereas in C4–2B cells silibinin was also effective at 30 μmol/L concentration, without affecting total Src level in any treatment (Fig. 4B).

To further dissect Src role in the regulation of E-cadherin expression, we next used dasatinib, a well-known pharmacologic inhibitor of Src. As shown in Fig. 4C, dasatinib (25–100 nmol/L) strongly decreased the level of phospho-Src (tyr419) after 24 hours of treatment. Importantly, inhibition of Src phosphorylation by dasatinib was accompanied with increased E-cadherin level in PC3 cells (Fig. 4C). Cell morphologic analysis revealed that dasatinib and/or...
Silibinin treatment decreases the number of elongated and fibroblast-like cells (a mesenchymal characteristic) while increasing the number of small and round-type cells (an epithelial characteristic) compared with DMSO treated control PC3 cells (Fig. 4D). To dissect whether silibinin increases E-cadherin level by other pathways independent of Src, we inhibited Src phosphorylation by dasatinib together with silibinin treatment. Western blotting analysis of the lysates showed that when Src phosphorylation is selectively inhibited, silibinin treatment does not affect E-cadherin level, suggesting a key role of Src inhibition by silibinin in the observed increase in E-cadherin level.

Antimigratory and antiinvasive efficacy of silibinin is regulated in-part by upregulation of E-cadherin expression in PC3 cells

To further understand the role of silibinin-caused increase in E-cadherin in its antimigratory and antiinvasive potential, we generated PC3 cells with stable knockdown of E-cadherin expression (ShEC-PC3 cells) and respective control cells (Sh-PC3 cells). E-cadherin knockdown was confirmed in ShEC-PC3 cells with or without silibinin treatment through Western blotting (Fig. 5A). As shown in Fig. 5A, E-cadherin knock-down resulted in increased level of phospho-Src(tyr419), which was...
inhibited by silibinin treatment. Morphologic analysis showed that ShEC-PC3 cells were elongated, spindle-shaped, and more mesenchymal in appearance compared with Sh-PC3 cells (Fig. 5B).

Next, we compared the antimigratory efficacy of silibinin in Sh-PC3 and ShEC-PC3 cells using wound-healing assay. As shown in Fig. 5C, E-cadherin knock-down significantly increased the migratory potential of ShEC-PC3 cells compared with respective control Sh-PC3 cells ($P \leq 0.05$). In Sh-PC3 cells, silibinin treatment inhibited migratory potential by $28\%$ ($P \leq 0.001$), $39\%$ ($P \leq 0.001$), $69\%$ ($P \leq 0.001$), $75\%$ ($P \leq 0.001$), and $89\%$ ($P \leq 0.001$) at 5, 10, 30, 60, and 90 μmol/L concentrations, respectively (Fig. 5C). In ShEC-PC3 cells, however, silibinin treatment inhibited migratory potential by 19% (statistically nonsignificant), 26% (statistically nonsignificant), 48% ($P \leq 0.01$), 55% ($P \leq 0.001$), and 76% ($P \leq 0.001$) at 5, 10, 30, 60, and 90 μmol/L concentrations, respectively (Fig. 5C). These results showed that under E-cadherin knock-down conditions, silibinin antimigratory efficacy is partially compromised.

Next, we compared the antiinvasive potential of silibinin in Sh-PC3 and ShEC-PC3 cells using invasion assay. As shown in Fig. 5D, E-cadherin knock-down significantly increased the invasive potential of ShEC-PC3 cells compared with respective control Sh-PC3 cells ($P \leq 0.001$). In Sh-PC3 cells, silibinin treatment (90 μmol/L) inhibited invasive potential approximately by 66% ($P \leq 0.01$); whereas in ShEC-PC3 cells, silibinin (90 μmol/L) inhibited invasive potential approximately by 37% ($P \leq 0.01$) (Fig. 5D). These results showed that silibinin antiinvasive efficacy is also partially compromised under E-cadherin knock-down condition.

**Discussion**

In majority of PCA cases, death occurs due to cancer metastasis to bones (40, 41). Therefore, there is urgent need to develop new and better strategies toward preventing or treating metastatic stage of this disease. One approach in the management of advance PCA could be the use of natural agents also known as “nutraceuticals”, which are relatively nontoxic, cost-effective, physiologically bioavailable, and have multiple molecular targets in cancer cells (6, 42–44). Earlier, we reported in TRAMP mice that nutraceutical agent silibinin targets EMT-related regulators as well as many proteases and prevents metastatic spread of PCA cells to distant organs (12, 13). In cell culture studies, silibinin was reported to decrease migratory/invasive potential and to inhibit Vimentin and increase cytokeratin-18 expression in PCA cells (31, 45, 46). Despite these findings, the main molecular target/s responsible for silibinin’s strong antimigratory and antiinvasive efficacy remained inconclusive. In that perspective, results from present study are highly significant as we report for the first time that (a) silibinin inhibits invasive and migratory potential of three highly metastatic PCA cell lines at nontoxic concentrations; (b) silibinin increases E-cadherin expression at cellular membrane and inhibits nuclear β-catenin level; (c) silibinin decreases phospho-Src (tyr419), Hakai, Slug, Snail and phospho-Akt (ser473) levels; and (d) antiinvasive and antimigratory effects of silibinin are in-part through promoting E-cadherin expression in PCA cells. These findings have great translational relevance because the concentrations of silibinin used in these studies are within the range of free silibinin levels achieved in the plasma of PCA patients in our completed phase I clinical trial (14).

The present study has shown that silibinin significantly decreases nuclear β-catenin level without affecting its level on cellular membrane where it is known to bind with E-cadherin and promote cell-to-cell contact. Therefore, it seems plausible that silibinin treatment increases E-cadherin level at cellular membrane as well as promotes E-cadherin and β-catenin association; thereby restore cell-to-cell contact which is detrimental to migratory and invasive potential of PCA cells. Future studies are needed to understand silibinin’s effect on such molecular interactions between E-cadherin and β-catenin.

Receptor and nonreceptor kinases, such as Met and Src respectively, have been reported to promote the phosphorylation of intracellular tail of E-cadherin at tyrosine sites, which is then recognized by ubiquitin-ligase protein Hakai mediating its internalization and ubiquitin-dependent degradation (28, 29). Interestingly, overexpression of Hakai alone could enhance E-cadherin endocytosis, disrupts cell–cell adhesion, and could promote cell motility (28). Besides directly phosphorylating E-cadherin, activated Src could also phosphorylate β-catenin, reducing its association with E-cadherin and promoting its nuclear localization, thereby, severely disrupting the adhesion complex (21, 47). In Fig. 6, we have published online first May 5, 2011; DOI: 10.1158/1940-6207.CAPR-10-0370.
depicted these molecular interactions involving Src, Hakai, β-catenin and E-cadherin, which result in loss of cell–cell contact and enhanced migratory and invasive behavior in PCA cells. In the present study, we found that silibinin decreases Src activity in terms of its tyrosine phosphorylation and Hakai expression in PCA cells; thereby silibinin could limit Src capability to phosphorylate E-cadherin or β-catenin and inhibit the resultant E-cadherin degradation and nuclear translocation of β-catenin (Fig. 6). Overall, these molecular effects of silibinin’s might be responsible for observed enhanced cell–cell contact and decreased migratory and invasive potential of PCA cells with silibinin treatment (Fig. 6).

Results from present study showed that silibinin-caused upregulation of E-cadherin in-part contributes to antimigratory and antinvasive efficacy of silibinin, suggesting that other signaling molecules could also contribute toward silibinin’s biological efficacy against metastatic PCA cells. Our initial studies showed that E-cadherin knock-down results in increased Src phosphorylation that was significantly inhibited by silibinin treatment and might be responsible for the remnant biological effects of silibinin in the absence of E-cadherin. Further studies are needed in future to understand whether the effect of silibinin on Src activation is direct or through targeting the molecules that are upstream of Src such as integrins and epidermal growth factor receptor (EGFR) which are also known to get activated in response to E-cadherin loss (48).

In conclusion, present study for the first time showed that silibinin modulates E-cadherin, β-catenin, Snail, Slug, and Hakai level as well as Akt and Src phosphorylation, which possibly promotes cell–cell contact and inhibits migratory and invasive potential of PCA cells. Silibinin is nontoxic and widely consumed as a supplement showing its human acceptability, and therefore it is suggested that it should be studied further in clinically relevant animal models to exploit its potential benefits against metastatic PCA in future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by NCI R01 grant CA102514. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 10, 2010; revised April 14, 2011; accepted April 18, 2011; published online August 4, 2011.

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