Advances in Brief

Silibinin Up-Regulates Insulin-like Growth Factor-Binding Protein 3 Expression and Inhibits Proliferation of Androgen-independent Prostate Cancer Cells[1]

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Abstract

Silibinin, a naturally occurring flavonoid antioxidant found in the milk thistle, has recently been shown to have potent antiproliferative effects against various malignant cell lines, but the underlying mechanism of action remains to be elucidated. We investigated the effect of silibinin on androgen-independent prostate cancer PC-3 cells. At pharmacologically achievable silibinin concentrations (0.02–20 μM), we observed increased insulin-like growth factor-binding protein 3 (IGFBP-3) accumulation in PC-3 cell conditioned medium and a dose-dependent increase of IGFBP-3 mRNA abundance with a 9-fold increase over baseline at 20 μM silibinin. An IGFBP-3 antisense oligodeoxynucleotide that attenuated silibinin-induced IGFBP-3 gene expression and protein accumulation reduced the antiproliferative action of silibinin. We also observed that silibinin reduced insulin receptor substrate 1 tyrosine phosphorylation, indicating an inhibitory effect on the insulin-like growth factor I receptor-mediated signaling pathway. These results suggest a novel mechanism by which silibinin acts as an antiproliferative agent and justify further work to investigate potential use of this compound or its derivatives in prostate cancer treatment and prevention.

Introduction

Silymarin is a standardized extract of flavanolignans from milk thistle (*Silybum marianum*), and silibinin is the main component of this plant extract (1). For the past three decades, silymarin and silibinin have been used clinically for the treatment of certain liver diseases (2, 3). This clinical experience provides convincing evidence of safety of both acute and chronic administration of the compound to humans (2, 3).

Recently, silibinin and silymarin have received attention regarding their antiproliferative and anticarcinogenic effects with respect to prostate, breast, and skin neoplasia in both short-term cell culture models and long-term *in vivo* protocols (4–12). Although we and others (8, 9) have demonstrated that silibinin and silymarin are potent antiproliferative agents for both androgen-dependent (LNCaP) and androgen-independent (DU145) prostate cancer cells, the underlying mechanisms have not been fully characterized.

Peptide growth factors including IGFs,[3] epidermal growth factor, transforming growth factors, fibroblast growth factors, and keratinocyte growth factor are known to have important roles in normal prostate development and in prostate cancer progression (13, 14).

Up-regulation of expression of growth factors by neoplastic prostate epithelial cells may represent an adaptive response to androgen deprivation and may contribute to the evolution of androgen-independent prostate cancer. There is evidence that in the normal prostate, IGFs are produced by stromal cells, whereas normal epithelial cells express the IGF-IR, suggesting a paracrine mode of regulation (15). IGFs are mitogens for androgen-independent cancer cell lines such as DU145 and PC-3, but the androgen-dependent prostate cancer cell line LNCaP is only responsive to IGF-I in the presence of dihydrotestosterone (16, 17).

The activities of IGFs are modulated by a family of high affinity specific IGFBPs (14, 18). IGFBP-3 is the major serum carrier protein for the IGFs. More than 90% of IGFs in the intravascular compartment are found in a ternary complex composed of the IGF, IGFBP-3, and an acid-liable subunit (18). Outside the circulation, IGFBP-3 has been found to be a negative regulator of cell proliferation in prostatic and other tissues (19, 20). This growth inhibition has been attributed not only to the reduction of IGF-I and/or IGF-II bioactivity (18) but also to an IGF-independent growth-inhibiting action that appears to involve cell surface receptors for IGFBP-3 (20). Recent demonstrations that the growth-inhibitory activity of transforming growth factor β1 (21), vitamin D-related compounds (22), retinoic acid (23), antiestrogens (24), antiandrogens (25), p53 (26), and tumor necrosis factor α (27) all involve up-regulation of IGFBP-3 gene expression and production suggest that IGFBP-3 may be involved in many growth-inhibiting signaling pathways. We present the first evidence that the inhibitory effect of silibinin on prostate cancer PC-3 cells involves increased IGFBP-3 gene expression and secretion as well as inhibition of IGF-I-induced signaling.

Materials and Methods

Cell Culture and Silibinin Treatment. Silibinin pure compound was purchased from Sigma. It was dissolved in DMSO at 20 mM. The DMSO in culture media never exceeded 0.1% (v/v), a concentration known not to affect cell proliferation, IGFBP-3 gene expression or production, or IRS-1 tyrosine phosphorylation. PC-3 cells were plated at 4×10^3 cells per well in 24-well plates in RPMI 1640 containing 10% FBS. The growth-inhibitory effect of silibinin was studied using thymidine incorporation under both serum-free and 10% FBS conditions, as described previously (24).

For the Western ligand and Western blotting and Northern blotting, PC-3 cells were cultured in RPMI 1640 with 10% FBS and 1% gentamicin. At 60% confluence, cells were washed three times with serum and phenol-free RPMI 1640 and then incubated in this media for 48 h with vehicle alone or with varying concentrations of silibinin (0.02, 0.2, 2, and 20 μM). Conditioned media were collected, clarified, and frozen at −70°C until assayed for IGFBPs by Western ligand and Western blotting. Total cellular RNA was isolated from cells by the RNazol B method (Tel-Test, Friendswood, TX) as detailed previously (24).

For studies assessing the effects of a 2-h exposure to silibinin or to exogenous rhIGFBP-3 (kindly provided by Celtrix Pharmaceuticals, Santa Clara, CA) on IRS-1 activation by IGF-I, 70–80% confluent cultures were washed twice with PBS and then cultured in serum- and phenol-free medium for 36 h. During the last 2 h of culture, the cultures were treated with either

vehicle or 2 μM silibinin or 500 ng/ml rhIGFBP-3. For the last 15 min of culture, cells were exposed to PBS alone or to IGF-I (100 ng/ml; Celtrix Pharmaceuticals) at 37°C. Monolayers were quickly washed twice with cold PBS and lysed with 0.5 ml of lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP40, and 0.2 unit/ml aprotinin] per plate. Cell lysates were collected and assayed for protein concentration as detailed previously (9), and Western blotting was carried out as described below.

For studies to determine whether silibinin interferes with the IGF-IR signaling pathway and whether IGFBP-3 plays a functional role, 70–80% confluent cultures were washed three times with serum- and phenol-free RPMI 1640 and then incubated in this media for 48 h with vehicle alone or 2 μM silibinin or 2 μM silibinin with 5 μg/ml IGFBP-3 antisense IGFBP-3 oligodeoxynucleotides (synthesized by Sheldon Biotechnology Center, Montreal, Quebec, Canada). At the end of these treatments, cultures were exposed to PBS or 100 ng/ml IGF-I and incubated for 15 min at 37°C. Cell lysates were collected and used for Western blotting or immunoprecipitation.

Effect of Silibinin and IGFBP-3 Antisense Oligodeoxynucleotides on Cell Proliferation. The effect of sense and antisense IGFBP-3 oligodeoxynucleotides (synthesized by Sheldon Biotechnology Center) on cellular proliferation was studied under serum-free conditions. The sense oligodeoxynucleotide was a 20-mer corresponding to the 20 NH_2-terminal nucleotides of the human IGFBP-3 mRNA (28), and the antisense oligodeoxynucleotide was complementary to this sequence. Each was used at a concentration of 5.0 μg/ml. Proliferation of cells was quantitated by [^{3}H]thymidine incorporation as described previously (24). The [^{3}H]thymidine incorporation data are highly correlated with the cell number end point in our PC-3 cell culture system. Experiments were performed in triplicate.

Western Ligand Blotting. IGF-I was radiolabeled using Na^{125}I (ICN Biochemical Inc., Irvine, CA) and the chloramine-T method to a specific activity of 350–500 μCi/μg (22). The 20× concentrated conditioned media were subjected to 10% SDS-PAGE under nonreducing conditions, and proteins were transferred onto nitrocellulose membranes. The membranes were blocked and probed with ^{125}I-IGF-I and exposed to X-ray film as described previously (22).

Western Blotting. The 20× concentrated conditioned media were resolved by 10% SDS-PAGE, and proteins were transferred onto nitrocellulose membranes. Immunoblot analysis with polyclonal antibodies against human IGFBP-3, IRS-1 (Upstate Biotechnology Inc., Lake Placid, NY), and horseradish peroxidase-conjugated antirabbit antiserum (Amersham, Oakville, Ontario, Canada) was performed using standard protocols. Western blots were visualized by enhanced chemiluminescence (22).

Immunoprecipitation. Cell lysate (400 μg) was immunoprecipitated with 2 μg of anti-IRS-1 at 4°C for 4 h. Protein A-agarose beads (25 μl) were added and incubated overnight at 4°C. Immunoprecipitated proteins were washed four times with lysis buffer, electrophoresed by SDS-PAGE, and analyzed by Western blotting as described previously (9).

Northern Blotting. RNA (50 μg) from each sample was separated on 1% agarose-formaldehyde gels and then transferred on to Zeta-Probe membrane (BioRad) in 50 mM NaOH. Blotted RNAs were hybridized with human IGFBP-3 cDNA probe (kindly provided by Dr. S. Shimasaki, University of California, San Diego, CA) as well as a human GAPDH cDNA probe (Clontech, Palo Alto, CA) to control for equal loading of wells. The cDNA probes were labeled with [^{32}P]dCTP by the T7 Quick-Prime kit (Pharmacia) following the kit instructions (24). Prehybridization, hybridization, and washing were performed as described previously (24). Bands were visualized by autoradiography after exposure to X-ray film with intensifying screens at −70°C.

Results and Discussion

PC-3 cells (29) express IGF-IR and produce both IGFs and IGFBPs (17, 19). IGFBPs play a central role in regulating IGF availability and bioactivity (18). Consistent with published results (16, 22), we detected by ^{125}I-IGF-I Western ligand blot a very weak doublet band at M_r 46,000 in the conditioned medium under control conditions (Fig. 1A), indicating the presence of a low concentration of IGFBP-3. Silibinin treatment for 48 h at 2 or 20 μM concentration significantly increased IGFBP-3 protein accumulation in PC-3 cell-conditioned
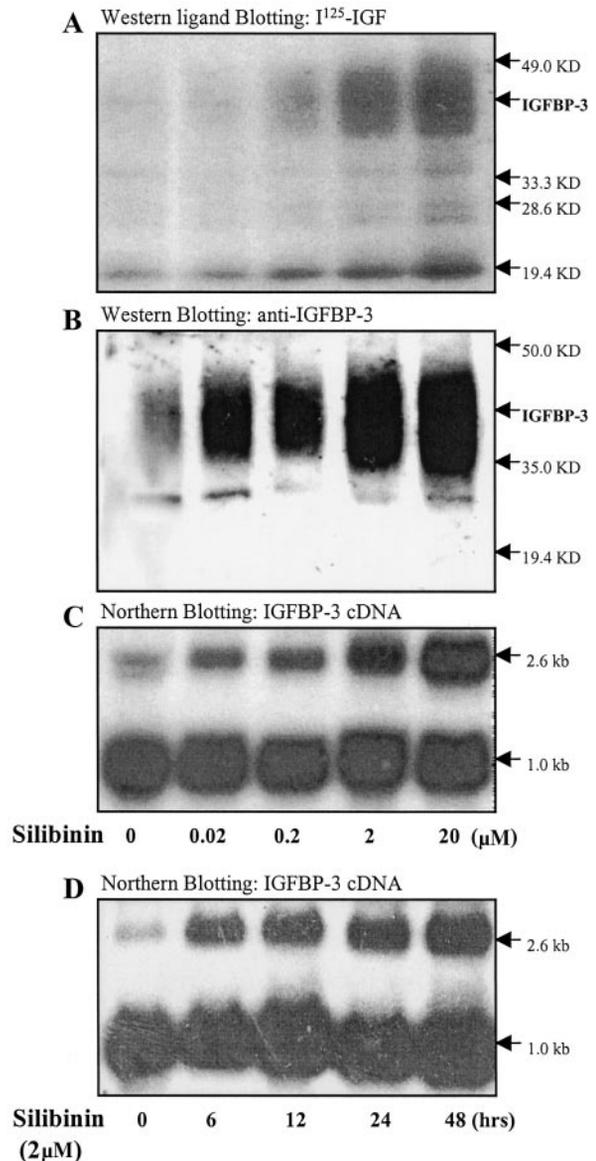

Fig. 1. Silibinin up-regulates IGFBP-3 gene expression and increases its accumulation in media conditioned by human prostate cancer PC-3 cells. PC-3 cells were cultured in RPMI 1640; after reaching 70–80% confluence, the cultures were treated with the indicated concentrations of silibinin for 48 h (A–C) or with 2 μM silibinin for the indicated time (D) in serum- and phenol red-free RPMI 1640. A, conditioned medium was collected and concentrated 20 times, and the Western ligand blots were performed as described previously (24). B, conditioned medium was collected and concentrated 20 times, and the Western blots were performed as described previously (22). C, and D, total RNA was extracted by RNazol B, Northern blotting of 50 μg was carried out, and blots were hybridized with a 475-bp human IGFBP-3 and a human GADPH cDNA probe to control for equal loading of wells as described previously (24). The 2.6-kb IGFBP-3 and 1.0-kb GADPH mRNA are shown.

media (Fig. 1A). Densitometric scanning estimated the increase to be 4- and 6.5-fold for the 2 and 20 μM concentrations, respectively.

To confirm this observation, Western blots were performed. The major finding using an antiserum to IGFBP-3 was that silibinin induced accumulation of the expected M_r 39,000–46,000 IGFBP-3 (Fig. 1B). To determine whether increased IGFBP-3 protein accumulation associated with exposure to silibinin is related to up-regulation of IGFBP-3 gene expression, we performed Northern blot analysis of total cellular RNA. Fig. 1C shows that a cDNA probe for human IGFBP-3 hybridized to a 2.6-kb mRNA species (24) and that treatment of cells with silibinin for 48 h resulted in a dose-dependent increase in IGFBP-3 gene expression. Densitometry revealed a 9-fold

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increase in expression over control in the presence of 20 μM silibinin. A time-course experiment shows that silibinin induces an increase in IGFBP-3 mRNA expression as early as 6 h after treatment (Fig. 1*D*).

In the PC-3 model, there is evidence that autocrine secretion of IGFs contributes to maintenance of these cells in the proliferative state in the absence of exogenous mitogens (16). Compared to untreated or vehicle controls, treatment of PC-3 cells with 2 μM silibinin resulted in 54% growth inhibition ($P < 0.001$, Student's *t* test; Fig. 2*A*) under the serum-free condition. Dose response data demonstrate higher cell growth inhibition up to a concentration of 20 μM silibinin, at which 74% inhibition was seen (Fig. 2*A*). In medium supplemented with 10% FBS, compared with vehicle or untreated controls, treatment of PC-3 cells with 2 and 20 μM silibinin resulted 17.3% and 54% growth inhibition, respectively ($P < 0.05$; Fig. 2*A*). According to the reported pharmacokinetics of silibinin in human and animals (3, 30), this concentration range is pharmacologically achievable and nontoxic.

We have reported previously that exogenously added IGFBP-3 reduced proliferation of prostate cancer PC-3 cells (22). We used an antisense strategy to investigate to what extent, if any, induction of IGFBP-3 expression by silibinin mediates the inhibitory action of this compound on PC-3 cell proliferation. IGFBP-3 antisense oligodeoxynucleotides attenuated silibinin-induced IGFBP-3 gene expression and protein accumulation, but no effect was found with the sense oligodeoxynucleotide (data not shown). As shown in Fig. 2*B*, in the presence of 2 μM silibinin, 5 μg/ml antisense IGFBP-3 oligodeoxynucleotide attenuated the antiproliferative action of silibinin by approximately 50% ($P < 0.05$). No significant change was seen with sense treatment.

IGF-I interacts with the IGF-IR to stimulate cell growth (31, 32). IRS-1 is a key signaling molecule activated by IGF-I. Increased IRS-1 tyrosine phosphorylation by IGF-I is directly correlated with increased activation of the downstream effector molecules phosphatidylinositol 3' kinase and mitogen-activated protein kinase, and the growth response to IGF-I is mediated by these pathways (31, 32). To evaluate the effects of silibinin or exogenously added IGFBP-3 on IGF-I-induced IRS-1 tyrosine phosphorylation, PC-3 cells were starved for 36 h and, for the last 2 h, exposed to vehicle or 2 μM silibinin or 500 ng/ml rhIGFBP-3. After this, cells were incubated for 15 min with or without 100 ng/ml IGF-I, as shown in Fig. 3*A*. We observed that 36 h of serum starvation of PC-3 cells resulted in an absence of tyrosine phosphorylation of IRS-1, as evidenced by the absence of reactivity of immunoprecipitated IRS-1 with antiphosphotyrosine antibody in Western blotting (Fig. 3*A*, *Lane 1*). On the other hand, treatment with IGF-I for 15 min resulted in a highly significant activation of IRS-1, as evidenced by a very strong reactivity with the antiphophotyrosine antibody (Fig. 3*A*, *Lane 2*). A 2-h pretreatment with silibinin resulted in only a slight decrease in IGF-I-induced IRS-1 tyrosine phosphorylation (Fig. 3*A*, *Lane 3 versus Lane 2*), whereas an 80% inhibition of IGF-I-induced IRS-I phosphorylation was seen with a 2-h pretreatment with 500 ng/ml rhIGFBP-3 (Fig. 3*A*, *Lane 4 versus Lane 2*). These results suggested that a 2-h exposure to exogenous IGFBP-3 had an important inhibitory effect on IRS-1 tyrosine phosphorylation, but that a 2-h exposure to silibinin did not.

Because our results demonstrated that silibinin induces IGFBP-3 expression and that IGFBP-3 reduces IRS-1 tyrosine phosphorylation, we next carried an experiment to determine whether prolonged exposure to silibinin would reduce IRS-1 tyrosine phosphorylation and whether this was dependent on IGFBP-3 induction. As shown in Fig. 3*B*, when we exposed cells to 100 ng/ml IGF-I for 15 min, a highly significant activation of IRS-1 was observed in vehicle control (Fig. 3*B*, *Lane 1 versus Lane 2*). In cells pretreated with 2 μM silibinin for 48 h, the 15-min IGF-1 incubation resulted in 70% less activation of IRS-1 than that seen in cells unexposed to silibinin (Fig. 3*B*, *Lane 3 versus Lane 2*). When cells were pretreated for 48 h with both 2 μM silibinin and IGFBP-3 antisense oligodeoxynucleotide, the reduction in IRS-1 tyrosine phosphorylation by silibinin was attenuated (Fig. 3*B*, *Lane 4 versus Lane 3*). None of the treatments affected IRS-1 protein levels as shown in Fig. 3, *A* and *B*, *bottom panels*.

A review of the data in Fig. 3, *A* and *B*, allows comparison of the effect of a 2-h *versus* 48-h pretreatment with silibinin on IRS-1 activation after IGF-I stimulation and reveals that the dominant effect requires more than 2 h; therefore, the silibinin effect is likely to be indirect. This is compatible with a hypothesis that silibinin-induced IGFBP-3 expression reduces the amount of bioavailable ligand available for interaction with the IGF-IR.

These data provide evidence that the antiproliferative action of silibinin for androgen-independent PC-3 prostate cancer cells involves increased IGFBP-3 accumulation and inhibition of IGF-I-induced IRS-1 phosphorylation. To the best of our knowledge, this study constitutes the first demonstration that a naturally occurring flavonoid influences cellular IGFBP-3 physiology. The possibility that at least a portion of the *in vivo* antiproliferative and chemoprotective actions of flavonoids is attributable
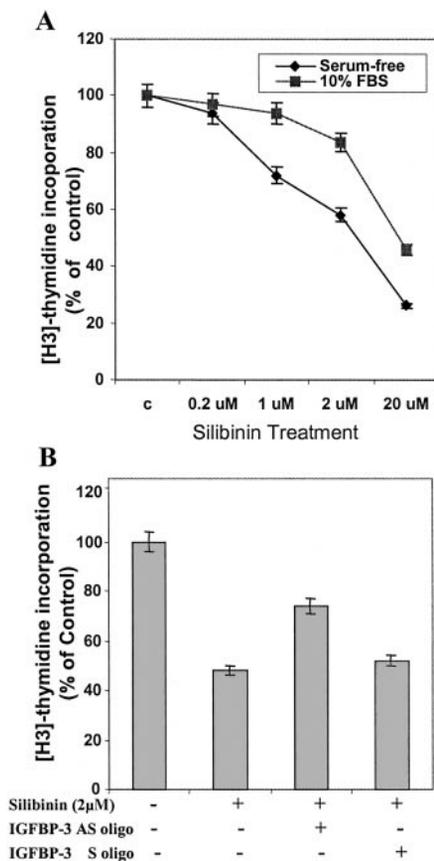

Fig. 2. Inhibitory effect of silibinin on PC-3 cell proliferation and attenuation of this inhibition by IGFBP-3 antisense oligodeoxynucleotide. *A*, 3×10^3 cells were plated in 24-well plates. After reaching 70–80% confluence, in the presence of 10% FBS, cells were treated with vehicle or with various concentrations of silibinin (0.2, 1, 2, and 20 μM) for 48 h; for serum-free experiments, the cells were washed three times with serum- and phenol red-free RPMI 1640 and then incubated in this medium for 48 h in the presence or absence of various concentrations of silibinin as indicated in the figure. Proliferation of cells was quantitated by [^{3}H]thymidine incorporation as described previously (24). Experiments were performed in triplicate, and SEs were less than 10%. *B*, the effect of sense (*S*) and antisense (*AS*) IGFBP-3 oligodeoxynucleotides on cellular proliferation was studied under serum-free conditions. The sense oligodeoxynucleotide was a 20-mer corresponding to the 20 NH$_2$-terminal nucleotides of human IGFBP-3 (24), and the antisense oligodeoxynucleotide was complementary to this sequence. Each was used at a concentration of 5.0 μg/ml for 2 days; the silibinin concentration was 2 μM. Proliferation of cells was quantitated by [^{3}H]thymidine incorporation as described previously (24). Experiments were performed in triplicate. The difference in thymidine incorporation levels between antisense and sense oligodeoxynucleotides or no oligodeoxynucleotide controls was significant ($P < 0.05$, Student's *t* test).
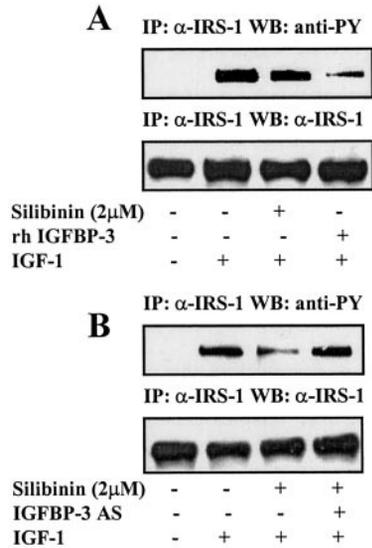
Fig. 3. Exposure of PC-3 cells to silibinin for 48 h reduces IGF-I-induced IRS-1 tyrosine phosphorylation, and this reduction is attenuated by IGFBP-3 antisense oligodeoxynucleotide. *A*, after reaching 70–80% confluence, PC-3 cells were washed and cultured under serum-free conditions for 36 h. During the last 2 h of serum starvation, they were treated either with vehicle alone or with 2 μM silibinin or with 500 ng/ml rhIGFBP-3. After this treatment, cells were incubated with PBS or 100 ng/ml IGF-I for 15 min at 37°C. Cell lysates were prepared, IRS-1 was immunoprecipitated using anti-IRS-1 antibody, and then SDS-PAGE and Western blotting were carried out. Membranes were probed with antiphosphotyrosine or anti-IRS-1 and then with peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was done using the enhanced chemiluminescence detection system. *B*, cells were incubated for 48 h in serum- and phenol-free medium without or with 5 μg/ml IGFBP-3 antisense (*AS*) oligodeoxynucleotide in the presence or absence of 2 μM silibinin and then stimulated for 15 min with 100 ng/ml IGF-I. Cell lysates were prepared, and immunoprecipitation and Western blotting were performed as detailed in *A*.

to this action as compared with the better-known antioxidant properties of this class of compounds deserves investigation.

It is relevant that the IGFBP-3-related actions of silibinin were observed at concentrations previously shown to be nontoxic in humans, indicating the possibility that this compound or its derivatives may be useful in prostate cancer treatment. Furthermore, silibinin and/or silibinin derivatives also deserve study in the context of prostate cancer prevention because we (33, 34) and others (35) have recently shown in population studies that higher circulating IGF-I levels and/or lower IGFBP-3 levels are associated with increased risk of prostate cancer. Experiments to determine the effects of silibinin on PC-3 tumor growth *in vivo*, circulating IGFBP-3 levels, normal prostatic tissue IGFBP-3 expression, and *in vivo* prostate carcinogenesis are now justified. However, it will also be necessary to undertake pharmacodynamic studies to determine the relationships between oral silibinin intake and steady-state tissue levels.

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