A Study of High-Dose Oral Silybin-Phytosome Followed by Prostatectomy in Patients With Localized Prostate Cancer

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BACKGROUND. Silibinin is a polyphenolic flavonolignan derived from milk thistle (*Silybum marianium*) with anti-oxidant properties. The purpose of the current trial was to determine the tissue and blood effects of high-dose silybin-phytosome in prostate cancer patients.

METHODS. Subjects with localized prostate cancer planning for a prostatectomy were eligible to enroll. Six patients received 13 g of silybin-phytosome daily with six additional participants serving as control subjects.

RESULTS. Patients in the treatment arm received silybin-phytosome for 14–31 days (mean was 20 days) prior to surgery. Silibinin blood levels were measured 1 hr after the first silybin-phytosome dose with a mean value of 19.7 μM. Trough silibinin levels were assessed at the end of the trial with an average concentration of 1.2 μM. In contrast to the high peak levels of silibinin observed in blood, the highest silibinin level observed in the harvested prostate tissue was 496.6 pmol/g. There were no significant differences noted in baseline and post-treatment blood levels of IGF-I and IGFBP-3. One of the treated patients developed a grade 4 post-operative thromboembolic event. The other observed toxicities in the treatment group were mild: four subjects had diarrhea and one had asymptomatic grade 2 hyperbilirubinemia which was transient.

CONCLUSIONS. High-dose oral silybin-phytosome achieves high blood concentrations transiently, but low levels of silibinin are seen in prostate tissue. Silibinin's lack of tissue penetration may be explained by its short half-life, the brief duration of therapy in this study or an active process removing silibinin from the prostate.

KEY WORDS: silibinin; prostate cancer

INTRODUCTION

The crude extract of milk thistle (Silybum marianium) is known as silymarin and has a long history of use in humans, especially as a liver protectant [1], with ongoing human trials to define its utility in treating chronic hepatitis [2] and primary sclerosing cholangitis [3]. Silibinin is a specific polyphenolic flavonolignan isolated from silymarin. (Fig. 1) Silibinin has both anti-oxidant and anti-cancer properties. In vivo data support silibinin's anti-cancer activity in several cancer types including prostate [4], colon [5], lung [6], skin [7], and bladder cancer [8]. This activity has led to the evaluation of silibinin as a potential cancer preventative and therapeutic agent.

There is an extensive body of medical literature examining silibinin's biological activity. One of silibinin's primary actions is the induction of G1 cell cycle arrest [9] with inhibition of retinoblastoma protein (Rb) phosphorylation, increased Rb-E2F complex formation [10], and an associated increase in p21 and p27 [11]. Additionally, silibinin has multiple cancer-associated targets including inhibition of the epidermal growth factor receptor (EGFR) [9], inhibition of NF-κB [12], down regulation of survivin [13], and suppression of angiogenesis [14]. Importantly, in response to DNA damage (e.g., ultraviolet B light), silibinin preferentially triggers apoptosis via the DNA protein-kinase-dependent pathway, suggesting a role for silibinin as a sensitizing agent with DNA-damaging therapy [15].

In prostate cancer, silibinin demonstrates disease specific targeting and in vivo activity in several models. In vitro, silibinin treatment of LNCaP cells decreases prostate-specific antigen (PSA) levels and promotes histologic neuroendocrine differentiation [16]. Constitutive activation of the signal transducer and activator of transcription (STAT)-3 is common in prostate cancer; silibinin has been shown to directly inhibit this factor in prostate cancer cells [17]. Isosilybin B, a related flavonolignan also derived from milk thistle, potentiates androgen receptor degradation in prostate cancer cells, while the crude extract of milk thistle (silymarin) reduces androgen receptor activity by suppressing its nuclear localization [18]. Using the transgenic adenocarcinoma of the mouse

prostate (TRAMP) model, oral silibinin inhibits prostate cancer growth with increased apoptosis and decreased cellular proliferation [19]. This effect was confirmed in follow-up studies showing a decrease in both the size and number of high-grade prostate cancer tumors that developed in a TRAMP model treated with silibinin as compared to control mice [20]. Using xenograft models of DU 145 prostate cancer in mice, silibinin supplementation in the diet significantly inhibited tumor growth without any observed toxicity [21]. Kohno et al. utilized a 3,2′-dimethyl-4-aminobiphenyl model of prostate cancer to study the effects of silymarin. After 60 weeks, silymarin reduced the appearance of prostate cancer with a 50% incidence of cancer in the control animals, compared to only 18% of the silibinin-treated animals [22].

The insulin-like growth factors (IGF) and their associated binding proteins have been correlated with prostate cancer in well-designed epidemiologic studies. In one recent analysis of this association, high levels of IGF-I were linked with a moderately increased risk of developing prostate cancer [23]. High levels of IGF-I and low levels of IGF binding protein-3 (IGFBP-3) correlated with a more advanced stage of prostate cancer in other investigations [24]. In animal studies, silibinin treatment increased IGFBP-3 levels in the plasma [21,25], tissue [19,26], and the mRNA levels of IGFBP-3 in the blood [25]. Silibinin's effects on the IGF-axis have led to significant interest in silibinin as a preventative agent in prostate cancer.

Recently, a phase I dose finding study of silybin-phytosome (Siliphos™) was completed in men with prostate cancer [27]. In this trial, asymptomatic grade 1–2 hyperbilirubinemia was frequently noted at doses of 15 g or more daily. The recommended phase II testing dose was therefore 13 g daily, as three divided doses. It is hypothesized that silibinin's known inhibition of Uridinediphosphoglucuronate-glucuronosyl-transferase-1A1 (UGT-1A1) is responsible for this finding, with UGT-1A1 activity critical for the glucuronidation and processing of bilirubin [28].

The goal of the current trial was to determine the tissue and blood effects of high-dose silybin-phytosome in prostate cancer patients. The primary objective was to establish the concentration of silibinin in prostate tissue after administration of high-dose silybin-phytosome. The secondary objectives were to characterize the histologic and blood marker changes with respect to the IGF pathway, apoptosis and proliferation.

MATERIALS AND METHODS

Patients and Treatment

Twelve patients were recruited from the urologic oncology clinic at the University of Colorado Hospital
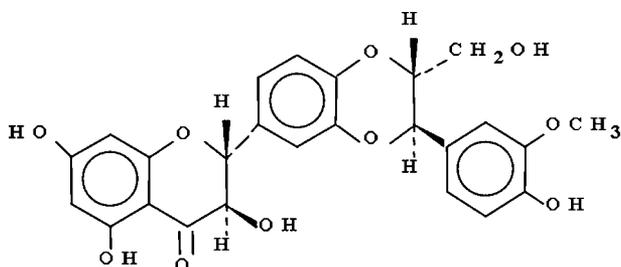

Fig. 1. The structure of silibinin.

between October of 2006 and October of 2007. All patients were newly diagnosed with prostate cancer and planning to pursue surgical radical prostatectomy. Study subjects could choose to be in the silybin-phytosome treatment group or in the non-treatment control group, with tissue collection at the end of the trial and selected blood tests. Six patients selected the control arm and six selected the silybin-phytosome treatment arm.

This protocol was approved by the local institutional review board and all patients provided written informed consent prior to participation. The Clinical-Trials.Gov identifier for this trial is NCT00487721. To qualify, participants were planning for radical prostatectomy, were 18 years or older, had histologic documentation of prostate cancer on biopsy, an ECOG performance status of ≤ 2, and adequate organ function (including a total bilirubin ≤ 1.5 mg/dl, aspartate transaminase and alanine transaminase $< 1.5\times$ the upper limits of normal, and creatinine ≤ 1.5). Patients were excluded from participation if they had received previous treatment for prostate cancer or if their surgery was scheduled within 14 days. Toxicity was graded with the National Cancer Institute's Common Terminology Criteria for Adverse Events version 3.0 (http://ctep.cancer.gov/forms/CTCAEv3.pdf).

Subjects in the treatment group received silybin-phytosome for 2–10 weeks, depending on the time from enrollment until the time of the prostatectomy. Silybin-phytosome (Siliphos^{TM}) was obtained from the Indena Corporation (Indena USA, Inc., Seattle, WA) in powdered form. Silybin-phytosome contains silibinin with phosphatidylcholine and is approximately 30% silibinin by weight. The dose of silybin-phytosome was 13 g daily, in three divided doses, based on the recommended phase II testing dose from the phase I trial [27]. Patients mixed 4.3 g of silybin-phytosome powder into six tablespoons of applesauce for each dose. The silybin-phytosome was taken on an empty stomach (30 min before eating and at least 2 hr after the previous meal) and was continued until the patients were told to stop taking their oral medication as part of their pre-operative instructions. In most cases, the last dose of silybin-phytosome was at bedtime, the night before surgery. The timing of the prostatectomy was determined by routine scheduling considerations and was not influenced by participation in this study. Subjects in the control arm did not receive any treatment or placebo.

Specimen Collection and Immunohistochemistry. Directly after removal of the prostate by the surgeon, about 100 mg of tissue was collected from the ex vivo specimen by needle biopsy (10–15 biopsies, 15 gauge needle, Boston Scientific, Natick, MA), tissue was snap frozen in liquid nitrogen and stored at -80°C. The prostatectomy specimen was formalin-fixed, paraffin-embedded, and sectioned for routine histological evaluation.

Five-micron thick sections of formalin-fixed paraffin-embedded prostatectomy specimens with representative tumor and normal areas were stained with the following antibodies and dilutions: COX-2 at 1:25 (clone SP21, Cell Marque, Rocklin, CA), pre-diluted Ki-67 (clone K-2, Ventana Medical Systems, Inc., Tucson, AZ), and cleaved Caspase-3 at 1:200 (Rabbit Polyclonal, Biocare Medical, Concord, CA). Heat-induced epitope retrieval for COX-2 was performed in 10 mM citrate buffer for 10 min (125°C; 22 psi in a pressure cooker). Ki-67 and Caspase-3 antigens required 5 min retrieval in pH 9.5 BORG Decloaker solution (Biocare) also at pressure (125°C; 22 psi). All immunoreactions were performed by the Ventana NexES immunostainer using the I-VIEW enhanced DAB kit. The kit contained universal biotinylated goat anti-mouse and goat anti-rabbit antibodies and streptavidin horseradish peroxidase for secondary antibody detection. An Avidin-Biotin kit (Ventana) was used to block endogenous biotin after incubation of COX-2 and Caspase-3 antibodies. Antigens were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB). The slides were counterstained in Mayer's hematoxylin for 60 sec, blued with 1% ammonia hydroxide, dehydrated, cleared, and cover slipped for light microscopy.

Serum samples for IGF axis assessments were collected in standard red top blood collection tubes. After sitting and forming a clot at room temperature, the samples were spun down and the serum was collected. From this, 0.5–0.75 ml of serum was placed in a 1 ml screw top freezer vial and stored at -80°C until processing. IGF-I and IGFBP-3 concentrations were determined by use of standard ELISA and a single production lot of reagents (Diagnostic Systems Laboratories, Beckman Coulter, Webster, TX) in the laboratory of Dr. Michael Pollak.

Analysis of Silibinin in Human Plasma, Urine, and Prostate Tissue. Analysis of silibinin in human fluid and tissue samples was carried out by LC/MS/MS following liquid extraction. Briefly, 200 µl of sample (plasma, urine, or 100 mg/ml prostate tissue homogenate in water) was extracted in 1 ml acidified (0.1% formic acid) ethyl acetate by vortex mixing for 10 min. Following centrifugation, the organic layer was evaporated to dryness in a rotary evaporator (Savant SpeedVac$^\circledR$). Dried samples were dissolved in acetonitrile/10 mM ammonium acetate with 0.1% acetic acid (1:1, v/v) for analysis. Sample analysis was done using an ABI 3200 QTrap$^\circledR$ triple quadrupole mass spectrometer with an Agilent 1100 LC system and

HTC-PAL Leap Autosampler. Chromatography conditions used included a YMC ODS-AQ 5 μm (120 Å, 50 × 2.0 mm²) column, and a gradient mobile phase of acetonitrile (Solvent B) and 10 mM ammonium acetate with 0.1% acetic acid (Solvent A) going from 20% B to 90% B over a 3 min period. The solvent flow rate was 500 μl/min, 10 μl of sample was injected for each run, and the total run time was 5.5 min. The mass spectrometry conditions were multiple reaction monitoring (MRM) operating in negative ion mode for the ion transitions $481 \rightarrow 125$ m/z for silibinin and $271 \rightarrow 119$ m/z for the internal standard naringenin. Voltages, temperatures and gas conditions were optimized for the analyte (silibinin) using MRM optimization algorithms included in the Analyst Software (Applied Biosystems, Foster City, CA). Quantitation of silibinin in samples was done by internal standard reference and batch analysis verified by the inclusion of spiked quality control samples in the appropriate matrix.

RESULTS

Twelve patients were enrolled in this study from October of 2006 until October of 2007. Participants selected their involvement in either the treatment or control group in a non-randomized fashion, with six subjects in each group. Table I describes the patient characteristics. The mean PSA was 7.58 ng/ml in the control group versus 5.42 ng/ml in the treatment arm. The mean duration of silybin-phytosome consumption was 20 days with a range of 14–31 days. Therapy with silibinin was generally well tolerated. Three of the six treated participants reported grade 1 diarrhea with one reporting grade 2 diarrhea. There was one case of grade 2 hyperbilirubinemia (maximum level of 1.6 mg/dl) that was not clinically significant. One patient did

experience a grade 4 post-operative thromboembolic event after he stopped silybin-phytosome, from which he fully recovered.

Silibinin levels were assessed in the blood, urine and tissue. The blood was assessed at three timepoints: 1 hr after the first dose, at an end of study trough, and an end of study level 1 hr post-silybin-phytosome treatment. The end of study assessment was done in the clinic a day or 2 before surgery. Evaluable blood samples for silibinin concentration determination are available for 5, 4, and 4 of the patients treated with silybin-phytosome at these timepoints, respectively. Some end of study blood samples were not obtained due to the timing of surgery, although tissue was successfully obtained in all participants. A large degree of inter-patient variability in the silibinin blood levels was noted at all timepoints. At the first assessment point 1 hr after the first dose of silybin-phytosome, the mean blood level was 19.7 μM, but the range was wide (4.6–34.5 μM) (Table II). Notably, the trough levels at the end of the trial revealed relatively low silibinin blood levels (1.2 μM), without evidence of silibinin accumulation in the blood after 2 or more weeks of therapy. Urine samples were also obtained at the beginning and the end of the trial. The urine silibinin levels were lower than the plasma levels and also showed significant inter-patient variability. Unlike the high peak concentrations of silibinin observed in the blood, tissue levels of silibinin were low. Of the six treated patients, silibinin was only detected in three with levels ranging from 14.9 to 496.6 pmol/g tissue.

IGF-I and IGFBP-3 levels were assessed prior to the start of treatment and at the end of the trial. Pre-clinical data demonstrate that IGFBP-3 levels increase with silibinin treatment in xenograft models of prostate cancer. As demonstrated in Figure 2, there were no significant changes in either the IGF-I or IGFBP-3 levels in either the control or treatment groups. The PSA was also assessed at baseline and at the end of the trial. There was no significant difference in baseline PSA (mean 5.4 ng/ml) and the end of study PSA (mean 5.1 ng/ml) in the treatment group.

Based on tissue changes observed in pre-clinical animal modeling, immunohistochemical assessment of the harvested tissue was performed. No clear trends were noted with respect to the markers of apoptosis, proliferation, and inflammation. The percent of Ki-67 positive cells was low in both the groups with a range of 3–12% and 2–27% in the silibinin and control arms, respectively. Apoptosis, as assessed by caspase-3 staining, was low with an average of <5% positive cells in either group. No clear trends were seen in the Cox-2 staining, with notable inter-patient variability in both patient groups. Representative images are shown in Figure 3.

TABLE I. Patient Characteristics

	Control group	Treatment group
Number of patients	6	6
Age (years)		
Mean	57.5	56.5
Range	49–65	46–66
Initial PSA (ng/ml)		
Mean	7.58	5.42
Range	4.38–14.4	1.04–14.8
Surgical Gleason Score		
Median	7	6.5
Range	6–9	6–9
Duration of study involvement/treatment (days)		
Mean	17	20
Range	15–21	14–31

TABLE II. Silibinin Level

	Day 1—1 hr level (μM)	Pre-operative trough level (μM)	Pre-operative 1 hr level (μM)
Blood (mean)	19.7 ± 12.3	1.2 ± 1.2	22.7 ± 4.2
Blood (range)	4.6–34.5	0.1–2.9	18.0–28.3
Urine (mean)	7.4 ± 7.5	1.2 ± 0.6	7.7 ± 5.1
Urine (range)	0.1–19.6	0.6–1.7	4.7–17.1

Mean $\pm$ SD for patients.

DISCUSSION

High-dose silybin-phytosome achieves notable peak blood concentrations of silibinin, although comparable levels of silibinin are not observed in the prostate tissue after 2 or more weeks of therapy. In broad agreement with the phase I trial, the peak serum levels of silibinin in this trial were as high as 35 μM. However, silibinin's half-life at these doses was just a few hours [27], such that even when dosing silibinin three times a day, the trough levels at the end of the study remained low. The primary objective of this trial was to determine the
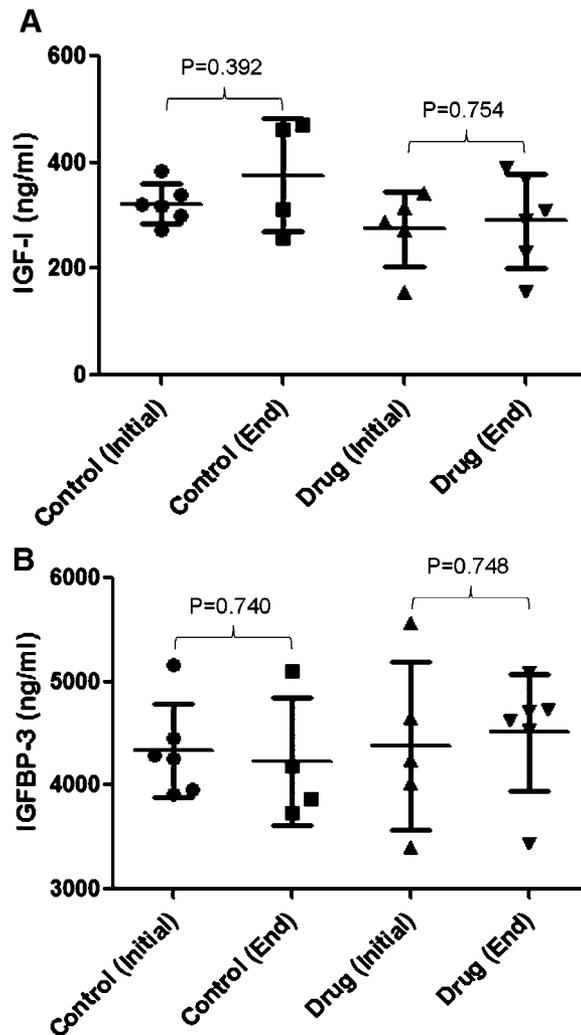
Fig. 2. ELISA blood assessment performed at the start and end of the study for IGF-I (**A**) and IGFBP-3 (**B**) in both the control and treatment arm. The vertical bars represent the standard deviation.
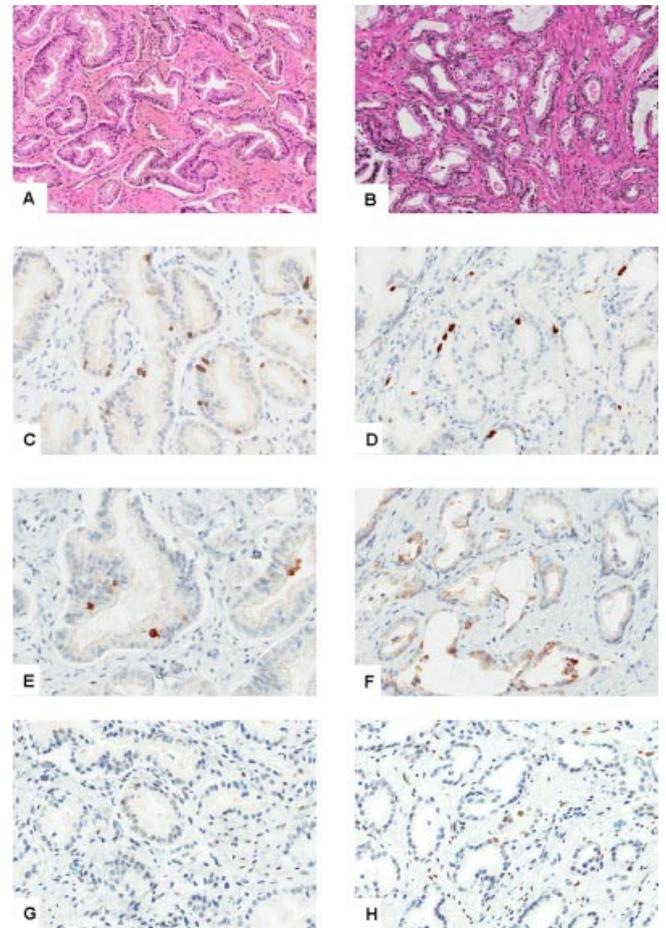
Fig. 3. Immunohistochemistry. Control slides (**A**, **C**, **E**, **G**) from a non-treated subject and slides from a silibinin treated subject (**B**, **D**, **F**, **H**) were obtained from a region of Gleason 3 prostate cancer. Representative images are shown for each of the staining techniques utilized: Hematoxylin and eosin (A, B: 100×), Ki-67 (C, D: 200×), COX-2 (E, F: 200×), and Caspase 3 (G, H: 200×). The overall staining for all three markers was similar between the control and treatment groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

prostate tissue concentration of silibinin in patients with prostate cancer; disappointingly, low levels of silibinin in the prostate tissue were observed.

There are several possible explanations for the discordance observed between the serum and tissue levels of silibinin. In pre-clinical animal studies, athymic mice with DU 145 xenografts were given 0.05 and 0.1% silibinin (w/w) in their diet. After 60 days of dietary silibinin treatment (0.05–0.1%), silibinin plasma concentrations from 14 to 27 μM and prostate tissue levels of 6–10 μmol/g tissue were observed [21]. In the current study, the peak blood levels of silibinin were approximately 20 μM at 1 hr, but the tissue levels were <500 pmol/g of tissue. Thus the relationship between blood and prostate tissue levels of silibinin was much more favorable in the mouse model than observed in this human study. Plausibly, while the peak serum levels observed in the present study appear adequate considering the pre-clinical modeling, the transient nature of silibinin serum levels in humans may not be sufficient for tissue penetration. Additionally, silybin-phytosome was only administered for 2–4 weeks in this study, with the duration limited and dictated by the normal considerations of surgical scheduling. Perhaps a longer duration of treatment may be needed, similar to the 60 day treatment used in the murine model.

An earlier study examined the effect of a different silibinin-phosphatidylcholine formulation (Silipide or IdB 1016) in colorectal patients at lower doses than the current study [29]. With 7 days of therapy at the dose of 1.44 g daily, Hoh et al. report higher tissue levels of silibinin in normal colorectal tissue (141 nmol/g) compared to normal hepatic tissue (2.5 nmol/g). Additionally, malignant colorectal tissue showed lower levels (68 nmol/g) than the normal colorectal tissue. These findings suggest a difference in silibinin tissue accumulation that may be due to organ-specific processes (e.g., active efflux of silibinin), leading to different tissue levels in different organs. The current study analyzed unselected prostate tissue containing normal and possibly cancerous prostate tissue, but tissue from other organs was not assessed.

The pre-clinical animal modeling demonstrated increased IGFBP-3 blood levels after silibinin treatment. Previous human trials with silibinin failed to show any change in IGF-I or IGF-BP3, however, the current study utilized maximally dosed silybin-phytosome. Even though silibinin did not accumulate in any notable fashion in the prostate tissue, its effect on the IGF-axis would not necessarily be dependent on this event. Nonetheless, no significant change was observed in IGF-I or IGFBP-3 levels in treated subjects in this study. Since this trial was primarily designed to determine if silibinin would be concentrated in the prostate tissue, subtle differences in the IGF-axis could certainly be obscured with the small sample size of this study.

CONCLUSION

In summary, silibinin has a strong pre-clinical rationale for its use in the prevention and treatment of prostate cancer. This study demonstrated high peak silibinin blood levels in humans, without any notable prostate tissue accumulation after 2 weeks of therapy. Despite the low tissue levels of silibinin noted in this study, there are several treatment approaches with silibinin that may be considered in future human studies. A longer duration of treatment with silibinin, beyond the 2 weeks required in this study, may be needed. In the pre-clinical studies demonstrating an increase in IGFBP-3, for example, the mice were treated continuously with silibinin in their diet. Silibinin does show additive and synergic anti-cancer activity in prostate cancer models when combined with cisplatin, carboplatin [30], doxorubicin [31], and mitoxantrone [32]. It is possible that silibinin's transient blood concentration will be adequate in combination with chemotherapy. Additionally, sustained-release formulations of silymarin are being developed [33], which have the potential of prolonging the half-life of orally administered silibinin and improving its prostate tissue kinetics.

ACKNOWLEDGMENTS

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