

Milk Thistle and Prostate Cancer: Differential Effects of Pure Flavonolignans from *Silybum marianum* on Antiproliferative End Points in Human Prostate Carcinoma Cells

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Abstract

Extracts from the seeds of milk thistle, *Silybum marianum*, are known commonly as silibinin and silymarin and possess anticancer actions on human prostate carcinoma *in vitro* and *in vivo*. Seven distinct flavonolignan compounds and a flavonoid have been isolated from commercial silymarin extracts. Most notably, two pairs of diastereomers, silybin A and silybin B and isosilybin A and isosilybin B, are among these compounds. In contrast, silibinin is composed only of a 1:1 mixture of silybin A and silybin B. With these isomers now isolated in quantities sufficient for biological studies, each pure compound was assessed for antiproliferative activities against LNCaP, DU145, and PC3 human prostate carcinoma cell lines. Isosilybin B was the most consistently potent suppressor of cell growth relative to either the other pure constituents or the commercial extracts. Isosilybin A and isosilybin B were also the most effective suppressors of prostate-specific antigen secretion by androgen-dependent LNCaP cells. Silymarin and silibinin were shown for the first time to suppress the activity of the DNA topoisomerase II α gene promoter in DU145 cells and, among the pure compounds, isosilybin B was again the most effective. These findings are significant in that isosilybin B composes no more than 5% of silymarin and is absent from silibinin. Whereas several other more abundant flavonolignans do ultimately influence the same end points at higher exposure concentrations, these findings are suggestive that extracts enriched for isosilybin B, or isosilybin B alone, might possess improved potency in prostate cancer prevention and treatment. (Cancer Res 2005; 65(10): 4448-57)

Introduction

Prostate cancer is the most commonly diagnosed invasive malignancy and second leading cause of cancer death in American men, accounting for an estimated 220,900 new cases and 28,900 deaths in the United States in 2003 (1). Dietary factors have been

associated with increased prostate cancer incidence, thereby leading to studies of dietary modification and supplementation as preventative approaches (2; reviewed in ref. 3). Naturally occurring polyphenolic antioxidants are recognized as one of the most effective classes of cancer preventive agents (4-8), as they exhibit little or no systemic toxicity (9) and because oxidative stress is a known contributor to carcinogenesis (10).

Over the last several years, extracts from the seeds of milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)] have shown efficacy in arresting human prostate carcinoma proliferation in a number of *in vitro* and *in vivo* preclinical models. This traditional botanical agent has been used since antiquity as a hepatoprotectant against *Amanita* mushroom poisoning and oxidative damage by xenobiotics, particularly in Europe (11). Interest in its utility in cancer followed a report in 1994 showing its effectiveness against tumor progression in a rodent skin cancer model (12). Collectively, the milk thistle flavonolignan mixtures silymarin and silibinin have been shown to possess significant antiproliferative effects in cultured cancer cells (13), antiproliferative and antiangiogenic activities *in vivo* in a number of rodent and human cancer models (14, 15), and synergy with cytotoxic chemotherapeutics *in vitro* and in xenografts of human prostate and ovarian cancer (16, 17). Therefore, these and other chemopreventive agents have become the focus of emerging interest in cancer treatment (18).

The proximal molecular target of milk thistle compounds has proven elusive, but a number of cancer cell signaling pathways are influenced by these agents that consequently suppress mitogenic and cell survival programming (7). Silibinin and silymarin inhibit extracellular signal-regulated kinase (ERK1/2) activation in prostate carcinoma cells, which is associated with decreased [¹²⁵I]-EGF ligand binding (19). Silibinin also inhibits the constitutive activation of nuclear factor κ B in DU145 cells by reducing p65 and p50 levels, increasing I κ B α levels, and decreasing I κ B α kinase activity (20). Silibinin stimulates production by PC3 cells of the insulin-like growth factor binding protein, IGFBP-3, and antisense IGFBP-3 oligonucleotides attenuate the growth inhibitory effects of silibinin (21). Silibinin influences the cell cycle to cause G₁ arrest in many but not all cases. Silibinin treatment of LNCaP or DU145 cells causes hypophosphorylation of Rb/p107 and Rb2/p130 and enhanced Rb-E2F association with a concomitant decrease in abundance of several E2F family members (22, 23). In LNCaP cells, silibinin reduces levels of cyclin D1, CDK4, and CDK6, while inducing p21^{Cip1} and p27^{Kip1}, which causes G₁ growth arrest and cellular differentiation but little indication of apoptosis (24). Longer treatment of some cancer cell lines with silymarin or silibinin will ultimately trigger apoptosis, and silibinin was shown recently to reduce mRNA levels of survivin, an apoptotic inhibitor,

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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in bladder transitional carcinoma cells (25). The most direct effect of milk thistle extracts observed to date has been the *in vitro* displacement by silymarin of radiolabeled 17β -estradiol from ER β but not ER α at concentrations similar to those causing cancer cell growth arrest (26). However, the functional significance of this *in vitro* observation on prostate carcinoma survival and growth remains unknown.

Understanding how compounds from milk thistle affect cell cycle and cell signaling to arrest cancer cell growth has been confounded by the lack of standardized nomenclature for the crude plant extracts and their individual pure compounds (27). Silymarin and silibinin are the most common forms of milk thistle extract sold commercially and the terms are often used interchangeably, albeit incorrectly (27). Recently, the Research Triangle Institute Natural Products Laboratory isolated and purified from silymarin (crude milk thistle extract) a series of seven distinct flavonolignans and one flavonoid (28). Silibinin has now been shown to be a 1:1 mixture of

two stereoisomers, denoted silybin A and silybin B. Of greatest significance is that two other regioisomers have been isolated as well that possess the same molecular mass, isosilybin A and isosilybin B (Fig. 1). These four compounds share the same chemical formula but differ in structure only with respect to the stereochemical positions of the terminal aryl(3-methoxy-4-hydroxyphenyl) and hydroxymethyl groups at the α and β carbons (Fig. 1). Isolation of these two pairs of diastereomers and their stereochemical assignments has also been accomplished and confirmed independently (29). Interestingly, evidence had existed as early as 1979 (30) to indicate that silybin and isosilybin were each mixtures of diastereomers, but pure reference standards and experimental quantities had not been available previously for biological studies. The resolution and purification of each of these compounds in milligram quantities now permits a dissection of the molecular actions of milk thistle extracts in prostate cancer suppression. The current report represents a first step in addressing the hypothesis

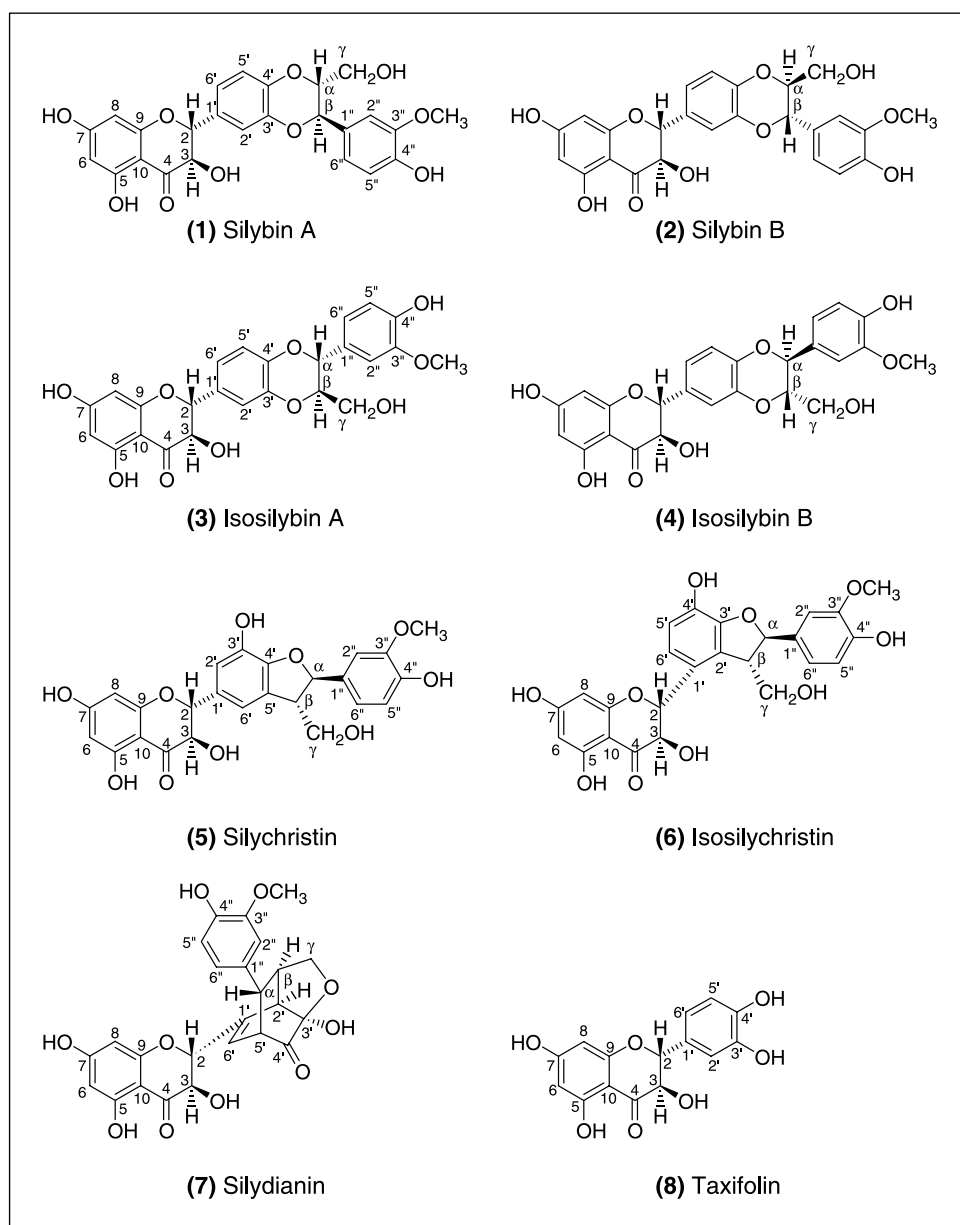


Figure 1. Chemical components of milk thistle extracts (*S. marianum*). These eight compounds have been identified in three milk thistle extracts available commercially as silymarin (28). Composition of each extract is listed in Table 1. The seven flavonolignan compounds share a formula weight of 482.1. Taxifolin, also called dihydroquercetin, is the only flavonoid detected in commercial silymarin products. Silibinin, thought previously to be a single compound, is known now to be a 1:1 mixture of the diastereomers, silybin A and silybin B.

of whether each individual milk thistle compound possesses distinct or overlapping biochemical actions that contribute to the overall, broad anticancer activity of the natural product extracts.

A number of end points were investigated that result from the antiproliferative effects of each milk thistle compound, including prostate cancer cell growth suppression, cell cycle distribution, as well as the suppression of prostate-specific antigen (PSA) secretion, and DNA topoisomerase II α (topo II α) promoter activity. Topo II α , a nuclear enzyme involved in DNA replication, chromosomal segregation, and recombination, is absolutely required for cellular proliferation, as its DNA decatenation activity is essential to successful chromosomal segregation (31). A recent meta-analysis of gene expression data sets from normal versus cancerous human tissue specimens revealed that *topo II α* is one of the most commonly activated genes across a large majority of human cancers (32). The current report details the first demonstration of suppression of *topo II α* gene expression by either crude milk thistle extracts or pure compounds. Silibinin had also been shown to decrease in androgen-dependent LNCaP prostate carcinoma cells the secreted levels of PSA, a clinical diagnostic serum marker for monitoring the presence and progression of prostate cancer (33, 34). This diagnostic tool provided us with a well-characterized end point for further dissection of the action of individual pure milk thistle compounds.

Materials and Methods

Isolation of reference standards from silymarin. Preparative high-performance liquid chromatography (HPLC) was used to isolate flavonolignans from silymarin (Madaus AG, Cologne, Germany) as described previously (28). Silymarin was obtained also from Aldrich (Milwaukee, WI) and Indena SpA (Rome, Italy), and silibinin was obtained from Sigma (St. Louis, MO).

For studies involving silibinin, the diastereomeric mixture of silybin A and silybin B, molar calculations were employed as if the mixture was a single compound with a formula weight of 482.1. For experiments involving silymarin, the crude mixture of eight polyphenolic compounds, molar concentrations are expressed based on a formula weight of 482.1 (the formula weight common to each of the seven flavonolignans; the 1.6-2.2% contribution of the smaller taxifolin compound was considered negligible). These molar calculations are recognized as imprecise (27) but are presented here as such to enable comparison with previous literature on the biological effects of these mixtures. It is suggested in the future that molar calculations be reserved for the pure milk thistle compounds and that w/v concentrations be employed when referring to silibinin or silymarin.

Analysis of silymarin and silibinin. HPLC analyses were done with a Star Chromatography Workstation (version 5.5.1; Varian, Inc., Walnut Creek, CA) at a flow rate of 1 mL/min using Varian ProStar 210 pumps with a YMC ODS-A column (S 5 μ m, 120 \AA , 4.6 \times 150 mm; Waters Co., Milford, MA). Signals were monitored at UV 280 nm using a Varian ProStar 330 PDA detector, and all injections of reference standards, silymarin, and silibinin were of 50 μ L by a Varian ProStar 410 autosampler. A quality control standard was used to monitor the consistency of the chromatographic analyses by preparing a solution of 0.100 mg/mL biochanin A (Aldrich) in DMSO (DMSO-B). All standards and study materials were prepared in DMSO-B, and the measured concentration of biochanin A did not vary more than $\pm 2\%$ in all determinations. To prepare the standard curves (Supplementary Fig. S1), stock solutions of the reference standards were prepared in DMSO-B and serially diluted to concentrations of ~ 0.50 , 0.20, 0.050, and 0.0125 mg/mL; the absolute concentration of each standard in DMSO-B was calculated based on the purity of each reference standard (range, $>87\%$ to $>99\%$ pure). The R^2 value for each standard curve was >0.99 . To verify the presence and confirm the retention time of each of the reference standards in the silymarin and silibinin samples, one of the

silymarin samples was added to each standard in turn, and the observed change in peak area for a distinct peak was observed by HPLC. Supplementary Fig. S2 illustrates the retention time of each of the reference standards and biochanin A in one of the silymarin samples (Indena). Stock solutions of the silymarin samples and silibinin were prepared at 12.0 mg/mL in DMSO-B, and each was diluted with DMSO-B to a concentration of 1.2 mg/mL for analysis. The concentration of the compounds in the silymarin and silibinin samples was calculated from the measured area under the peak correlating to each reference standard and extrapolated using the equation for the standard curve of that particular standard.

Cell culture. Frozen tumor cell stocks were obtained from the American Type Culture Collection (Manassas, VA): LNCaP (CRL-1740, an androgen-dependent line derived from a lymph node metastasis of prostate adenocarcinoma), DU145 (HTB-81, an androgen-independent line derived from a central nervous system metastasis of prostate adenocarcinoma), and PC-3 (CRL-1435, an androgen-independent line derived from a bone metastasis of prostate adenocarcinoma). All cell lines were cultured in RPMI 1640 (Life Technologies/Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT), 100 units/mL penicillin G, and 100 μ g/mL streptomycin sulfate and incubated in a humidified 37°C/5% CO₂ incubator. Cells were maintained in a logarithmic growth state by subculturing every other day at an initial density of 1×10^6 cells per 75 cm² flask in 30 mL medium.

Cell survival and flow cytometry assays. Logarithmically growing prostate carcinoma cells were seeded at 5×10^4 per well of 24-well plates in 0.5 mL of supplemented RPMI 1640 and allowed to attach to substrate overnight. The following day, stock solutions of silibinin, silymarin, or pure milk thistle compounds were prepared in DMSO at 50 mmol/L and diluted into RPMI 1640 at twice the intended final concentration of 30, 60, or 90 μ mol/L. A 0.5-mL aliquot of each compound in medium, or DMSO at 0.2%(v/v), was added to each well (in triplicate for each final concentration). Cells were permitted to grow for 72 hours at which time 10 μ L of the mitochondrial dehydrogenase substrate, WST-8 [(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, in combination with phenazine methosulfate; Cell Counting Kit-8, Dojindo, Gaithersburg, MD] was added to each well and incubated 1 to 2 hours further. Cell-free wells of medium were incubated similarly to correct for nonenzymatic hydrolysis of the substrate to its formazan product. Mitochondrial respiratory activity was quantified by spectrophotometry at 450 nm using a Tecan Ultra microtiter plate reader (Tecan US, Research Triangle Park, NC) and cellular survival expressed as a percentage relative to corrected control values.

Cell cycle distribution of DU145 cells (1×10^5 cells per 60-mm tissue culture plate) treated with silibinin or pure compounds was determined after propidium iodide staining by fluorescence-activated cell sorting (FACS) analysis at the Duke University Comprehensive Cancer Center Cell Sorting Facility (Durham, NC).

Topoisomerase II α promoter-reporter transfections and reporter activity assays. Logarithmically proliferating DU145 cells were plated at 4×10^4 cells per well in 12-well tissue culture plates and allowed to adhere for 5 to 6 hours in a humidified 37°C/5% CO₂ incubator. Cells were transiently transfected with the human *topo II α* gene promoter-reporter construct -562TOP2LUC (35) using 0.5 μ g DNA per well and 3:1 GeneJuice (EMD Biosciences/Novagen, Madison, WI) transfection reagent/DNA ratio according to manufacturer's instructions. Cells were incubated with the transfection mixture overnight (~ 16 hours). Subsequently, purified flavonolignans, taxifolin, silibinin, silymarin (all dissolved in DMSO) or vehicle alone were diluted in complete medium at a 3 \times stock concentration and added to transiently transfected cells in triplicate to final concentrations of 30 and 60 μ mol/L. In studies to compare relative potencies of silybin A, silybin B, isosilybin A, and isosilybin B, compounds were tested in triplicate using final concentrations of 30, 60, 90, and 120 μ mol/L. Cells were incubated further for 24 hours before harvest.

After harvesting, cells were assayed for luciferase enzymatic activity using the Enhanced Luciferase Assay Kit (BD Biosciences PharMingen, San Diego, CA) according to manufacturer's instructions with a dual-injector Monolight 3010 luminometer (PharMingen). Forty microliters of lysate were

assayed and triplicate points averaged and reported with their respective SDs. In initial experiments, a pCMV plasmid encoding β -galactosidase activity (pCMV β , BD Biosciences Clontech, Palo Alto, CA) was used as internal control for transfection activity but proved an inadequate one, as it was activated by some flavonolignans and suppressed by others. Therefore, subsequent transfection results were expressed as total luciferase light units and repeated at least thrice to assure uniformity and confirm the rank order of compound potency.

Western immunoblotting for secreted prostate-specific antigen.

LNCaP cells were plated at a density of 3×10^5 cells per 60-mm dish containing each purified flavonolignan or silibinin at final concentrations of 0, 15, 30, 60, and 90 μ mol/L. After 24 hours of exposure, the medium was replaced with serum-free medium containing the corresponding flavonolignan or silibinin at the same concentrations. Cells were incubated 48 hours further and the conditioned medium was collected from each sample. Sixty microliters of conditioned media for each sample were resolved by 12% SDS-PAGE and proteins transferred onto polyvinylidene difluoride membranes (Amersham Biosciences Co., Piscataway, NJ) by semidry electroblotting. Blots were blocked with 5% nonfat dry milk in PBS for 15 minutes and the membranes were incubated with primary antibody (goat antihuman PSA, C-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:500 dilution in 2.5% milk in PBS containing 0.05% Tween 20 (PBST) overnight at 4°C with rocking. After washing 3×10 minutes in PBST, blots were incubated with rabbit anti-goat IgG-horseradish peroxidase conjugate (1:500) secondary antibody (Bio-Rad Laboratories, Hercules, CA) in PBST with 2.5% milk for 2 hours at room temperature. After washing membranes in PBST as described above, the blots were incubated with Enhanced Chemiluminescence Plus Western Blotting Detection Reagent (Amersham Biosciences) and PSA signals were detected using a Storm 860 imaging system (Amersham Biosciences).

Statistical analysis. Group means were assessed relative to vehicle controls by one-way ANOVA followed by a multiple comparison post-test (Bonferroni's correction) using Prism v3.02 software (GraphPad Software, Inc., San Diego, CA).

Results

Composition of commercial milk thistle extracts. HPLC analysis using reference standards showed clearly that silibinin from Sigma was exclusively an equal mixture of silybin A and silybin B and was devoid of any of the five other flavonolignans or taxifolin (ref. 28; Table 1). In contrast, each commercial silymarin extract contained all eight compounds in varying but relatively constant amounts (Table 1). Of note, silymarin, but not silibinin, contained the two other flavonolignans most relevant in this report, isosilybin A and isosilybin B. Among silymarin

products, the only large difference observed was the greater amount of silydianin in the Indena and Madaus extracts relative to that from Aldrich. As shown below in the cellular growth inhibition assays, silydianin alone was a relatively inactive component.

Isosilybin B exhibits preferential growth inhibition of hormone-independent DU145 human prostate carcinoma cells. As a first test of the individual activities of each of the four milk thistle flavonolignans, DU145 cells were exposed to each compound at 30, 60, and 90 μ mol/L and cell growth assessed after 3 days using the mitochondrial succinate dehydrogenase substrate, WST-8. The activity of each compound was also compared with that of the other three flavonolignans (silychristin, isosilychristin, and silydianin) as well as the flavonoid, taxifolin.

The most significant finding (Table 2) was that isosilybin A and isosilybin B were the only compounds capable of significant growth inhibition in DU145 cells at 30 μ mol/L after a 72-hour exposure, with isosilybin B ranking as the most potent. Silybin A, silybin B, and silibinin had no growth inhibitory activity at this concentration. Moreover, the three silymarin extracts were also devoid of activity with the exception of 20% growth suppression by the Madaus extract. This observation is notable in that silibinin, well known for its *in vivo* activity against DU145 xenografts (14), was without effect at a concentration where isosilybin A and isosilybin B produced 37.5% and 68.9% growth inhibition, respectively. Of course, higher concentrations produced similar growth inhibition among the four major flavonolignans, with silybin B being the least efficacious. A direct comparison with silymarin is somewhat complicated by the fact that it contains all eight of the compounds, as a crude extract, along with other fatty acids and polyphenols. However, on a mass basis, it is clear that isosilybin B was more efficacious than any of the three silymarin preparations or silibinin (Fig. 2). This trend was also observed with isosilybin A and silybin A in DU145 cells. Because most studies on milk thistle extract effectiveness in prostate cancer *in vitro* and *in vivo* have employed either mixture (silibinin or silymarin), it is notable that three of the four major flavonolignans given alone exhibited substantially greater efficacy in the DU145 growth inhibition assay. Moreover, a clear rank order of potency emerged from this study: isosilybin B > isosilybin A > silybin A > silybin B. It should be stressed that each of these compounds share the same chemical formula and structure with

Table 1. Composition (% of total material) of commercial milk thistle products

	Silibinin (Sigma)	Silymarin (Aldrich)	Silymarin (Indena)	Silymarin (Madaus)
Silybin A	46.9	14.8	13.1	16.0
Silybin B	49.6	23.3	21.6	23.8
Isosilybin A	—	4.9	6.6	6.4
Isosilybin B	—	2.1	3.9	4.4
Silydianin	—	0.8	9.4	16.7
Silychristin	—	16.5	15.5	11.6
Isosilychristin	—	0.6	1.5	2.2
Taxifolin	—	1.7	2.2	1.6

NOTE: Note that silymarin product composition totals are <100%; each of these products are labeled as 70% to 80% silymarin with the remainder consisting of uncharacterized polyphenols and aliphatic fatty acids such as oleic and palmitic acids.

Table 2. WST-8 cell survival assay with milk thistle compounds and extracts in prostate carcinoma cells after 72 h of continuous exposure

	LNCaP ($\mu\text{mol/L}$)			DU145 ($\mu\text{mol/L}$)			PC3 ($\mu\text{mol/L}$)		
	30	60	90	30	60	90	30	60	90
Silybin A	91.0 \pm 2.6	75.9 \pm 2.4*	55.9 \pm 3.1*	93.1 \pm 6.0	22.7 \pm 1.6*	15.2 \pm 0.9*	80.6 \pm 2.6*	40.3 \pm 9.5*	21.9 \pm 7.9*
Silybin B	109.9 \pm 2.1	88.2 \pm 7.0 [†]	29.2 \pm 6.3*	96.2 \pm 7.3	67.1 \pm 6.6*	33.9 \pm 3.6*	67.1 \pm 3.2*	23.1 \pm 0.5*	12.7 \pm 1.6*
Isosilybin A	107.2 \pm 4.4	85.3 \pm 5.4 [‡]	54.4 \pm 10.5*	62.5 \pm 1.5*	17.2 \pm 1.8*	19.9 \pm 0.5*	86.8 \pm 3.6 [†]	43.9 \pm 4.7*	22.9 \pm 4.0*
Isosilybin B	70.4 \pm 3.1*	46.4 \pm 3.3*	26.2 \pm 4.3*	31.1 \pm 0.6*	15.2 \pm 0.9*	16.0 \pm 0.3*	60.5 \pm 1.7*	37.5 \pm 0.9*	18.1 \pm 3.1*
Silibinin (Sigma)	90.1 \pm 3.7	72.0 \pm 3.6*	36.8 \pm 1.1*	105.1 \pm 2.4	61.3 \pm 8.2*	19.5 \pm 1.8*	69.7 \pm 3.6*	22.9 \pm 2.8*	13.3 \pm 0.6*
Silymarin (Aldrich)	90.6 \pm 2.9	91.4 \pm 3.1	47.1 \pm 17.6*	100.7 \pm 2.4	75.6 \pm 4.9*	11.4 \pm 0.4*	81.0 \pm 4.4*	35.9 \pm 11.1*	20.5 \pm 1.6*
Silymarin (Madaus)	120.0 \pm 8.6*	96.0 \pm 1.2	71.5 \pm 5.0	80.7 \pm 4.0	68.6 \pm 5.4*	30.8 \pm 0.9*	84.0 \pm 0.5 [‡]	38.2 \pm 2.1*	21.9 \pm 1.0*
Silymarin (Indena)	106.1 \pm 2.7	95.8 \pm 1.4	58.5 \pm 27.5 [‡]	93.2 \pm 6.3	83.2 \pm 0.5 [†]	22.7 \pm 3.0*	84.2 \pm 8.6 [‡]	34.4 \pm 1.8*	22.8 \pm 2.3*
Silychristin	110.4 \pm 5.7	99.7 \pm 2.4	100.4 \pm 3.0	106.8 \pm 6.0	59.0 \pm 5.9*	16.6 \pm 2.5*	101.1 \pm 3.1	45.4 \pm 5.9*	21.4 \pm 0.9*
Isosilychristin	111.7 \pm 3.9	102.9 \pm 4.2	94.8 \pm 1.5	98.1 \pm 1.8	98.1 \pm 1.9	99.3 \pm 1.5	98.5 \pm 1.3	92.7 \pm 6.0	76.9 \pm 3.2*
Silydianin	101.4 \pm 4.2	100.4 \pm 0.7	99.1 \pm 2.4	102.8 \pm 4.9	97.8 \pm 6.6	76.7 \pm 3.0*	102.4 \pm 2.8	82.0 \pm 4.2 [‡]	51.3 \pm 4.3*
Taxifolin	86.8 \pm 3.8	57.6 \pm 0.7*	30.3 \pm 1.4*	131.8 \pm 23.7 [‡]	75.5 \pm 6.0*	34.2 \pm 1.9*	52.4 \pm 1.8*	36.2 \pm 1.2*	25.9 \pm 0.4*

NOTE: Results are presented as mean percent survival \pm SD ($n = 3$) relative to vehicle controls. The original number of cells plated exhibited a substrate conversion rate of 11% to 15% that of 3-day growth controls, such that survival values in this range indicate 100% growth inhibition.

*Statistically significant differences from DMSO controls ($P < 0.001$).

[†]Statistically significant differences from DMSO controls ($P < 0.05$).

[‡]Statistically significant differences from DMSO controls ($P < 0.01$).

the exception of the stereochemical positions of the terminal aryl(3-methoxy-4-hydroxyphenyl) and hydroxymethyl groups at the α and β carbons (Fig. 1).

In other experiments (data not shown), the potency of the compounds increased as DU145 cell density decreased (and, therefore, as a function of population growth fraction increasing). Under such conditions, 50% growth inhibition was observed with as little as 5 $\mu\text{mol/L}$ isosilybin B, although the rank order of potency remained unchanged.

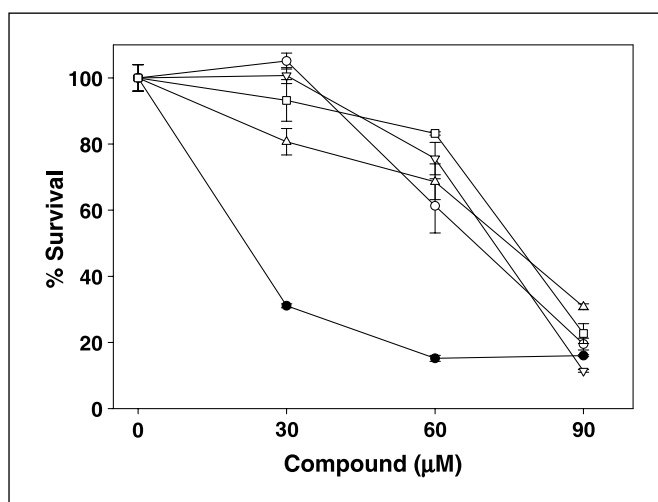


Figure 2. Isosilybin B is more potent than commercial milk thistle extracts in inhibiting the growth of DU145 prostate carcinoma cells. Data selected from the growth inhibitory experiments in Table 2 (WST-8 reduction after 72 hours of exposure to compounds/extracts) is depicted here graphically to illustrate the potency of pure isosilybin B relative to silymarin or silibinin.

Isosilybin B also inhibits cell growth in androgen-independent PC3 and androgen-dependent LNCaP human prostate carcinoma cells. In parallel experiments using PC3 cells (Table 2), isosilybin B and silybin B exhibited the greatest growth inhibition at 30 $\mu\text{mol/L}$, but there was less distinction among the compounds than in DU145 cells. One notable exception to DU145 cells was that the silibinin and silymarin mixtures were comparable in activity to the pure flavonolignans against PC3 cells.

In androgen-dependent LNCaP cells grown in complete media, isosilybin B was also the most effective growth suppressor at all concentrations and, as with DU145 cells, was superior to the silibinin or silymarin mixtures. However, LNCaP cells were generally less sensitive to growth inhibition by pure compounds or mixtures than either DU145 or PC3 cells. Taken together, isosilybin B was the most potent growth inhibitory constituent of silymarin-based milk thistle extracts and was often more effective than silibinin or silymarin mixtures.

Antiproliferative effect of other milk thistle components. Among the other flavonolignans, silychristin, but not isosilychristin, inhibited DU145 and PC3 cell growth (Table 2). This observation is suggestive that the positioning of the 1'-linkage in silychristin is critical for antiproliferative activity. It is notable that this linkage in silychristin is shared by the four primary flavonolignans but not by the inactive isosilychristin (Fig. 1).

Interestingly, silydianin was slightly growth inhibitory only in androgen-independent DU145 and PC3 cells but not in androgen-dependent LNCaP cells. Finally, the flavonoid taxifolin (also known as dihydroquercetin), which constitutes the flavonoid moiety in many of these flavonolignans, also inhibited growth in all three cell types, with PC3 being more sensitive than either DU145 or LNCaP. Given this observation, we also tested the growth inhibitory activity of the corresponding lignan moiety of

the bifunctional flavonolignans by exposing cells to coniferyl alcohol; however, this compound was without growth inhibitory activity at concentrations up to 200 $\mu\text{mol/L}$ in DU145 cells (data not shown).

Cell cycle effects of milk thistle components. Silibinin is known to cause G_1 arrest in most prostate cancer cell lines (7, 24). To determine how the four major flavonolignans influenced cell cycle progression, FACS analysis was done on DU145 cells treated in duplicate for 72 hours with silybin A, silybin B, isosilybin A, isosilybin B, and silibinin at 60 and 90 $\mu\text{mol/L}$ (Table 3). At 60 $\mu\text{mol/L}$, only silybin B exerted a modest but significant G_1 arrest on DU145 cells (6-7% increase relative to vehicle control), whereas the other compounds had no appreciable effect. At 90 $\mu\text{mol/L}$, silybin B led to a greater (11-14%) increase in the G_1 population (relative to control) followed by isosilybin B and isosilybin A. The slight effect of silybin A on the G_1 population at this concentration failed to achieve statistical significance. With silibinin treatment, the percent of cells accumulating in G_1 increased roughly by the average of that for silybin A and silybin B, consistent with the demonstration that silibinin comprises nearly equivalent amounts of each compound. Most notable was that silybin B had the most pronounced effects on the cell cycle whereas being the least effective in growth inhibition of DU145 cells. However, the other compounds and silibinin all exhibited a small but reproducible degree of subdiploid DNA fragmentation at 90 $\mu\text{mol/L}$. This observation is consistent with a weak apoptotic effect that does not seem shared by silybin B.

DNA topoisomerase II α promoter inhibition by milk thistle components in DU145 cells. The growth inhibitory action of milk thistle extracts and pure compounds would be consistent with suppressed expression of topo II α , a nuclear enzyme required for chromosomal segregation whose DNA-decatenating activity is indispensable for cellular proliferation (31). However, this variable had not been investigated previously even for milk thistle extract mixtures, thereby providing an opportunity for these agents to be

assessed for their action on a transcriptional target. A human *topo II α* promoter-reporter plasmid, -562TOP2LUC, in which a section of the 5' -flanking region of the *topo II α* gene was subcloned upstream of the firefly luciferase cDNA (35), was used to analyze the effect of the individual flavonolignans on promoter activity.

The effects of each of the seven flavonolignans, taxifolin, silibinin, and three preparations of silymarin were compared at 30 and 60 $\mu\text{mol/L}$ on DU145 cells transiently transfected with this construct. As illustrated in Fig. 3A, all four of the major flavonolignans had suppressive effects on *topo II α* gene promoter activity. However, only the suppression with isosilybin A and isosilybin B achieved statistical significance, with isosilybin B the most potent and effective. Silibinin and the three silymarin formulations had comparable *topo II α* promoter suppression activities that were intermediate compared with the active pure flavonolignans. In contrast, silydianin, silychristin, isosilychristin, and taxifolin lacked any suppressive effect on promoter activity (Fig. 3B) despite the fact that silychristin and taxifolin exhibited growth inhibitory effects on these cells (Table 2).

The rank order of potency of the four most active flavonolignans on *topo II α* promoter activity was determined in subsequent experiments with each at 30, 60, 90, or 120 $\mu\text{mol/L}$. In this case, each of the four compounds significantly suppressed *topo II α* promoter activity at concentrations as low as 30 $\mu\text{mol/L}$ (Fig. 4). These results deviated somewhat from those observed in Fig. 3A, most likely owing to the greater variability of this transient transfection assay relative to other less intrusive methods employed in this study. However, the rank order for *topo II α* promoter suppression was comparable: isosilybin B > silybin B > isosilybin A \approx silybin A. Most notable, again, was that isosilybin B was the most active of these four compounds.

Flavonolignan inhibition of prostate-specific antigen secretion by LNCaP cells. PSA is used to monitor androgen action in androgen-dependent prostate carcinoma and serves as a clinical tool in screening, diagnosis, and treatment decisions (3). As both

Table 3. FACS analysis of DU145 cells treated with each of the four major flavonolignans or silibinin

	Sub- G_0 - G_1	G_0 - G_1	S	G_2 -M
DMSO control	2.2 \pm 2.1	49.2 \pm 1.5	19.4 \pm 2.1	29.4 \pm 1.6
60 $\mu\text{mol/L}$				
Silybin A	1.1 \pm 0.3	50.2 \pm 1.6	19.2 \pm 0.5	29.7 \pm 1.9
Silybin B	1.1 \pm 0.7	56.3 \pm 1.8*	18.9 \pm 0.1	23.9 \pm 1.0 [†]
Isosilybin A	1.6 \pm 1.2	52.5 \pm 0.4	19.9 \pm 0.7	26.1 \pm 1.0
Isosilybin B	1.0 \pm 0.0	51.5 \pm 0.7	18.7 \pm 0.4	28.9 \pm 0.4
Silibinin	1.4 \pm 0.2	52.2 \pm 1.0	18.1 \pm 0.8	28.5 \pm 0.5
90 $\mu\text{mol/L}$				
Silybin A	3.3 \pm 1.1	51.7 \pm 1.3	18.6 \pm 1.3	26.6 \pm 0.4
Silybin B	0.9 \pm 1.2	62.9 \pm 0.5*	15.4 \pm 0.1 [‡]	20.9 \pm 0.7*
Isosilybin A	3.8 \pm 0.5	56.9 \pm 0.4*	16.4 \pm 1.9	23.0 \pm 0.4*
Isosilybin B	2.5 \pm 0.3	57.0 \pm 0.7*	16.4 \pm 0.8	24.3 \pm 1.4*
Silibinin	4.5 \pm 0.1	56.5 \pm 1.8*	16.1 \pm 0.5	23.1 \pm 0.2*

NOTE: Exponentially growing DU145 cells were treated with the indicated compounds/mixtures at 60 or 90 $\mu\text{mol/L}$ for 72 hours. Final DMSO concentrations were 0.09% (v/v) in all samples. The results are presented as the mean percent and SD of triplicate samples in each cell cycle phase.

* Statistically significant differences from the DMSO control group ($P < 0.001$).

[†] Statistically significant differences from the DMSO control group ($P < 0.01$).

[‡] Statistically significant differences from the DMSO control group ($P < 0.05$).

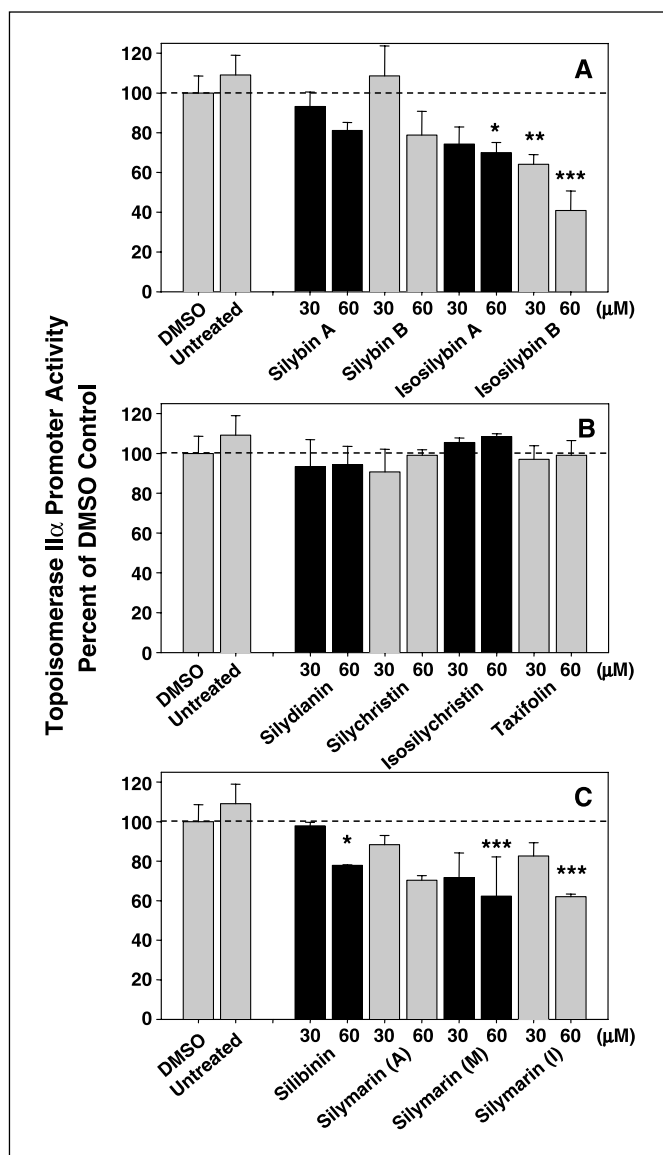


Figure 3. Milk thistle extracts and pure compounds suppress the activity of the human *topo II α* gene promoter in DU145 prostate carcinoma cells. The *topo II α* promoter-reporter construct -562TOP2LUC was transiently transfected into DU145 cells and samples exposed to the indicated concentrations of extracts and pure compounds for 24 hours as described in Materials and Methods. Expressed relative to the mean reporter expression of DMSO vehicle control-treated cells ($5.28 \pm 0.45 \times 10^6$ relative light units). **A**, four major flavonolignans: silybin A, silybin B, isosilybin A, and isosilybin B. **B**, silydianin, silychristin, isosilychristin, and taxifolin. **C**, silibinin and the three silymarin preparations (A, Aldrich; M, Madaus; I, Indena). Untreated and DMSO controls as means and SD of six replicates; experimental samples as means and SD of three replicates. All samples, except untreated controls, contained 0.06% (v/v) DMSO. Statistically significant differences from the DMSO controls are noted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

silibinin and silymarin have been shown to inhibit androgen-induced PSA secretion (34), we examined the relative effects of the major four flavonolignans in inhibiting PSA secretion into the culture medium by LNCaP cells (Fig. 5). No appreciable suppression of PSA secretion was observed at a concentration of 15 $\mu\text{mol/L}$ for any of the compounds. At 30 $\mu\text{mol/L}$, both isosilybin A and isosilybin B caused a slight reduction in PSA secretion relative to the silybin A and silybin B, and these differential effects

became more striking at $\geq 60 \mu\text{mol/L}$. Isosilybin A and isosilybin B reduced PSA secretion to nearly undetectable levels at 90 $\mu\text{mol/L}$, whereas some PSA remained evident with silybin A, silybin B, or the silibinin mixture.

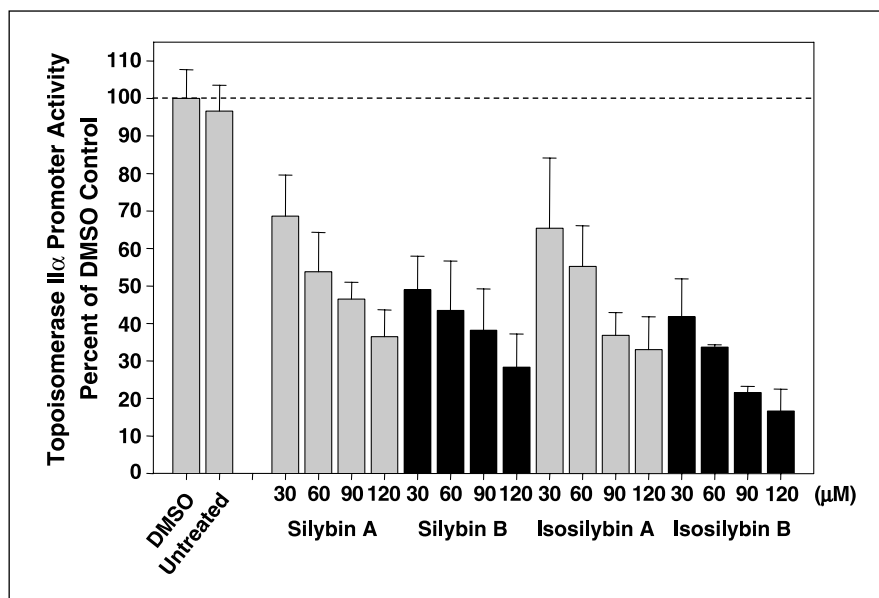
Discussion

A major challenge in ascribing biological significance to botanical extracts with potential anticancer activity has been their chemical complexity and variability in composition. The work of Agarwal et al. (14, 24, 34) has clearly established the potential utility of milk thistle extracts in suppressing prostate cancer cell growth *in vitro* and tumor formation *in vivo*, with no toxicity to normal prostate epithelium at antitumor concentrations. Most studies with milk thistle extracts have used either the crude extract, silymarin, or a semipurified fraction, silibinin. Biologists have long regarded silibinin as a pure compound, but evidence existed in the chemistry literature as early as 1979 to indicate that it was a diastereomeric mixture (30). It is now appreciated that silibinin is roughly a 1:1 mixture of two flavonolignans, silybin A, and silybin B. This mixture clearly possesses *in vitro* and *in vivo* anticancer activity (14, 24). However, the crude extract silymarin has, in some cases, been reported to be more effective than silibinin against cell proliferation or biochemical end points (34). Our recent demonstration that silymarin contains five other distinct flavonolignans in addition to the two in silibinin (28) provides an opportunity to determine the relative contribution of each component to the anticancer activity described for both silibinin and silymarin.

The current studies were initiated to determine whether individual milk thistle components possessed distinct biochemical actions that act in concert in the botanical mixture, or whether each compound exhibited similar activities with variable potency. With this initial report, it seems that the latter scenario is operating for milk thistle flavonolignans toward prostate carcinoma cells *in vitro*. The four major flavonolignans, when given at concentrations high enough, were all capable of arresting the growth of androgen-dependent and androgen-independent prostate carcinoma cells *in vitro*. A similar conclusion can be made for cell cycle arrest, *topo II α* gene promoter suppression, and inhibition of PSA secretion. However, the summary table (Table 4) reveals some notable trends.

First, isosilybin B ranked as the most potent flavonolignan for nearly all of the end points evaluated in these human prostate carcinoma cell lines. The value of isosilybin B is of interest on several fronts in that it is absent from silibinin and composes only 2.1% to 4.4% of the silymarin extracts examined (Table 1). Therefore, this low-abundance compound might be the most useful single isomer for *in vivo* studies or in combination experiments using isosilybin B-enriched milk thistle extracts. Second, silybin A was most often the least potent of the four major compounds. It composes half of silibinin and is generally the second most abundant naturally occurring constituent of crude silymarin extracts. Although silybin A was capable of causing growth arrest at higher concentrations, its presence and high abundance may be one reason that *in vivo* experiments with milk thistle extracts require such relatively high doses. Third, silybin B exhibited the most erratic and perplexing behavior. Silybin B was the least effective in inhibiting DU145 cell growth but was the most effective in causing G₁ cell cycle accumulation. One possible explanation for this discrepancy is that the other three related flavonolignans may have caused apoptosis preferentially over growth arrest in the cell growth inhibition experiments. This scenario is potentially

Figure 4. Isosilybin B is the most potent of the four major flavonolignans in suppressing human *topo II α* gene promoter activity in DU145 prostate carcinoma cells. DU145 PCA cells were transiently transfected with -562TOP2LUC as described in Materials and Methods. Each replicate set was treated with 30, 60, 90, or 120 $\mu\text{mol/L}$ purified silybin A, silybin B, isosilybin A, or isosilybin B for 24 hours and processed for luciferase activity determinations. Relative to mean reporter expression of DMSO vehicle control-treated cells ($2.53 \pm 0.20 \times 10^6$ relative light units). Untreated and DMSO controls as means and SD of six replicates; experimental samples as means and SD of three replicates. All samples, except untreated controls, contained 0.12% (v/v) DMSO. In this case, each flavonolignan treatment group mean was significantly different from the DMSO control ($P < 0.001$, ANOVA, Bonferroni's correction) at all four concentrations tested. This experiment was performed twice with similar results.



supported by the trend toward increased subdiploid DNA distribution observed with the other three major flavonolignans (Table 3). Although these changes did not achieve statistical significance, the compounds should be compared in more sensitive and specific biochemical assays for their potential proapoptotic effects. Silybin B also displayed the greatest interexperiment variability in suppressing *topo II α* gene promoter activity (compare Fig. 3A with Fig. 4). Part of this discrepancy could be attributed to the more variable nature of transient transfection assays relative to others used in this study, yet it was robust enough to reproducibly

observe isosilybin B as the most potent suppressor of *topo II α* gene promoter activity. Finally, isosilybin A was sometimes as potent as isosilybin B (i.e., in PSA suppression) and was at least of intermediate potency on other end points. It is worthy to note that isosilybin A is the least water soluble among these four major compounds and special care must be exercised when adding it to any aqueous medium.

The anticancer effects of most naturally occurring plant extracts can often be attributed to one compound, such as taxol (paclitaxel) in *Taxus brevifolia* (36), or few compounds, such as camptothecin and 10-hydroxycamptothecin in *Camptotheca acuminata* (37). The current studies were initiated to determine which compounds are responsible for documented antiproliferative effects of milk thistle extracts. Interestingly, none of the four most closely related milk thistle compounds was devoid of activity on any of the end points tested thus far. Several other compounds, such as taxifolin and silychristin, also possessed antiproliferative activity when used at higher exposure concentrations. This is perhaps a reflection that each end point (cell proliferation, PSA secretion) results from upstream contributions by a number of discrete molecular events. The question remaining is whether each milk thistle compound possesses unique effects on these more discrete events (such as CDK activity, CDKI levels and localization, cyclin levels), or whether each of the compounds exhibit some range of graded responses on common biochemical targets. Some distinctions are already apparent which suggest that some compounds may have effects distinct from others; for example, whereas silychristin and taxifolin inhibited DU145 cell growth, neither compound suppressed activity of the *topo II α* promoter at growth inhibitory concentrations. Because the human *topo II α* gene is subject to regulation by the CDKI, p21^{Cip1} (38), it is possible that only a subset of growth-inhibitory milk thistle compounds can trigger p21 induction.

The potential use of plant-derived chemopreventive agents is clearly influenced by the extent to which the active compounds are bioavailable. The primary challenge is whether concentrations observed to be effective *in vitro* can be achieved *in vivo*. Singh et al. (14) have shown in a dietary feeding study of silibinin to SCID mice harboring DU145 xenografts that antitumor effects correlated with

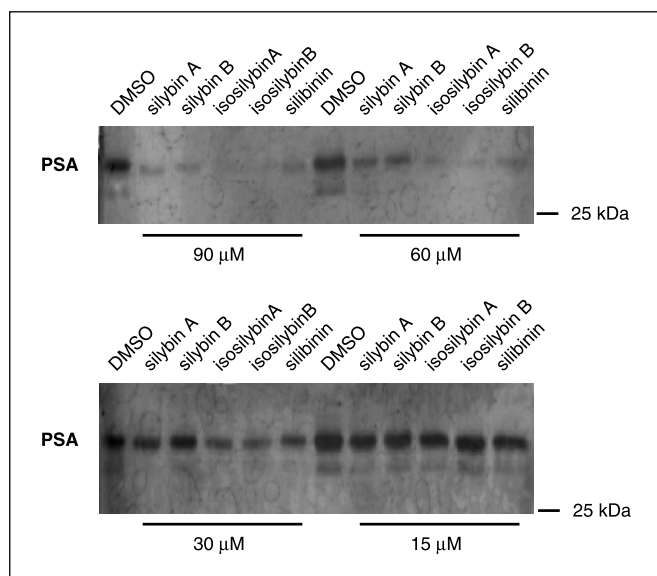


Figure 5. The four major milk thistle flavonolignans suppress PSA secretion by LNCaP prostate carcinoma cells. LNCaP cells grown in complete RPMI 1640 (10% FCS) were treated with 0, 15, 30, 60, or 90 $\mu\text{mol/L}$ of each pure flavonolignan for 24 hours, and medium replaced with serum-free medium containing the same respective concentration of each compound. After 48 hours of further incubation, 60 μL of medium from each culture were resolved by SDS-PAGE and processed for anti-PSA immunoblotting as described in the Materials and Methods.

Table 4. Summary of flavonolignan rank order of potency in this study

Variable	Cell line	Flavonolignan rank order of potency
Growth suppression	LNCaP DU145 PC3	Iso B > Sily B > Iso A ≈ Sily A Iso B > Iso A > Sily A > Sily B Iso B ≈ Sily B > Iso A ≈ Sily A
G ₁ cell cycle accumulation	DU145	Sily B > Iso B ≈ Iso A > Sily A
<i>Topo IIα</i> promoter suppression	DU145	Iso B > Sily B > Iso A ≈ Sily A
PSA suppression	LNCaP	Iso B ≈ Iso A > Sily B ≈ Sily A

NOTE: Isosilybin B is shown in bold for orientation.

Abbreviations: Sily A, Silybin A; Sily B, Silybin B; Iso A, Isosilybin A; Iso B, Isosilybin B.

plasma concentrations of 14 to 27 μmol/L total silibinin. In that study, the authors also reported that the silibinin consumption in the two dosage groups ranged from 1.8 to 3.5 mg/d. With typical allometric scaling to a 70-kg human, this consumption correlates roughly to a daily dose of 650 to 1,300 mg silibinin. However, direct human pharmacokinetic studies with milk thistle extracts are few and difficult to interpret because the formulation used can dramatically affect bioavailability (39, 40), and only one study (41) has attempted to follow individual concentrations of pure stereoisomers. For instance, in a study of nine healthy male volunteers given a phosphatidylcholine-silibinin formulation (IdB 1016; corresponding to 120 mg silibinin, bid) for 8 days, median peak plasma levels of unconjugated total silibinin ranged from 0.14 to 7.8 μmol/L across individuals (42). The safety profile of milk thistle extracts is suggestive that higher doses can be given that will produce plasma concentrations consistent with the effects observed in this report. Moreover, identification of isosilybin B as

the most potent prostate carcinoma growth inhibitor indicates that any subsequent preclinical or phase I trials should include extracts enriched for this component and that each isomer should be monitored separately. Continued study of the biological effects of milk thistle flavonolignan isomers should shed greater light on the potential clinical utility of these compounds.

Acknowledgments

Received 12/31/2004; revised 2/24/2005; accepted 3/8/2005.

Grant support: NIH grants R01 CA76201 (D.J. Kroll) and R01 CA104286 (D.J. Kroll and R. Agarwal), and NIH contract N01 ES55386.

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We dedicate this article to the memory of our coauthor, Dr. Monroe E. Wall (1916-2002), in recognition of over four decades of his contributions to cancer research, anticancer drug discovery, and the initial direction of this study, his final project.

We thank Dr. Aglaia Pappa for critical review of the article and graphical assistance.

References

- Jemal A, Murray T, Samuels A, et al. Cancer statistics. *CA Cancer J Clin* 2003;53:5-26.
- Demark-Wahnefried W, Robertson CN, Walther PJ, et al. Pilot study to explore effects of low-fat, flaxseed-supplemented diet on proliferation of benign prostatic epithelium and prostate-specific antigen. *Urology* 2004; 63:900-4.
- Nelson WG, DeMarzo AM, Isaacs WB. Prostate cancer. *N Engl J Med* 2003;349:366-81.
- Singh DK, Lippman SM. Cancer chemoprevention. Part 2. Hormones, nonclassic antioxidant natural agents, NSAIDs, and other agents. *Oncology (Huntingt)* 1998;12:1787-800.
- Fotsis T, Pepper MS, Aktas E, et al. Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. *Cancer Res* 1997;57:2916-21.
- Goodman GE. The clinical evaluation of cancer prevention agents. *Proc Soc Exp Biol Med* 1997;216: 253-9.
- Agarwal R. Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents. *Biochem Pharmacol* 2000;60:1051-9.
- Le Marchand L. Cancer preventive effects of flavonoids: a review. *Biomed Pharmacother* 2002;56:296-301.
- Hahn G, Lehmann HD, Kürten M, et al. *Silybum marianum* (L.) Gaertn. *Arzneimittelforschung* 1968;18: 698-704.
- Singh RP, Agarwal R. Flavonoid antioxidant silymarin and skin cancer. *Antioxid Redox Signal* 2002;4:655-63.
- Flora K, Hahn M, Rosen H, Benner K. Milk thistle (*Silybum marianum*) for the therapy of liver disease. *Am J Gastroenterol* 1998;93:139-43.
- Agarwal R, Katiyar SK, Lundgren DW, Mukhtar H. Inhibitory effect of silymarin, an anti-hepatotoxic flavonoid, on 12-*O*-tetradecanoylphorbol-13-acetate-induced epidermal ornithine decarboxylase activity and mRNA in SENCAR mice. *Carcinogenesis* 1994;15: 1099-103.
- Tyagi A, Bhatia N, Condon M, et al. Antiproliferative and apoptotic effects of silibinin in rat prostate cancer cells. *Prostate* 2002;53:211-7.
- Singh RP, Dhanalakshmi S, Tyagi AK, et al. Dietary feeding of silibinin inhibits advance human prostate carcinoma growth in athymic nude mice and increases plasma insulin-like growth factor-binding protein-3 levels. *Cancer Res* 2002;62:3063-9.
- Singh RP, Tyagi AK, Zhao J, Agarwal R. Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice via modulation of mitogen-activated protein kinases and induction of apoptosis. *Carcinogenesis* 2002;23:499-510.
- Giacomelli S, Gallo D, Apollonio P, et al. Silybin and its bioavailable phospholipid complex (IdB 1016) potentiate *in vitro* and *in vivo* the activity of cisplatin. *Life Sci* 2002;70:1447-59.
- Dhanalakshmi S, Agarwal P, Glode LM, Agarwal R. Silibinin sensitizes human prostate carcinoma DU145 cells to cisplatin- and carboplatin-induced growth inhibition and apoptotic death. *Int J Cancer* 2003;106: 699-705.
- Liu YQ, Kyle E, Patel S, et al. Prostate cancer chemoprevention agents exhibit selective activity against early stage prostate cancer cells. *Prostate Cancer Prostatic Dis* 2001;4:81-91.
- Sharma Y, Agarwal C, Singh AK, Agarwal R. Inhibitory effect of silibinin on ligand binding to erbB1 and associated mitogenic signaling, growth, and DNA synthesis in advanced human prostate carcinoma cells. *Mol Carcinog* 2001;30:224-36.
- Dhanalakshmi S, Singh RP, Agarwal C, Agarwal R. Silibinin inhibits constitutive and TNF α -induced activation of NF- κ B and sensitizes human prostate carcinoma DU145 cells to TNF α -induced apoptosis. *Oncogene* 2002;21:1759-67.
- Zi X, Zhang J, Agarwal R, Pollak M. Silibinin up-regulates insulin-like growth factor-binding protein 3 expression and inhibits proliferation of androgen-independent prostate cancer cells. *Cancer Res* 2000; 60:5617-20.
- Tyagi A, Agarwal C, Agarwal R. The cancer preventive flavonoid silibinin causes hypophosphorylation of Rb/p107 and Rb2/p130 via modulation of cell cycle regulators in human prostate carcinoma DU145 cells. *Cell Cycle* 2002;1:137-42.
- Tyagi A, Agarwal C, Agarwal R. Inhibition of retinoblastoma protein (Rb) phosphorylation at serine sites and an increase in Rb-E2F complex formation by silibinin in androgen-dependent human prostate carcinoma LNCaP cells: role in prostate cancer prevention. *Mol Cancer Ther* 2002;1:525-32.

24. Zi X, Agarwal R. Silibinin decreases prostate-specific antigen with cell growth inhibition via G₁ arrest, leading to differentiation of prostate carcinoma cells: implications for prostate cancer intervention. *Proc Natl Acad Sci U S A* 1999;96:7490–5.
25. Tyagi AK, Agarwal C, Singh RP, et al. Silibinin down-regulates survivin protein and mRNA expression and causes caspases activation and apoptosis in human bladder transitional-cell papilloma RT4 cells. *Biochem Biophys Res Commun* 2003;312:1178–84.
26. Seidlova-Wuttke D, Becker T, Christoffel V, Jarry H, Wuttke W. Silymarin is a selective estrogen receptor β (ER β) agonist and has estrogenic effects in the metaphysis of the femur but no or antiestrogenic effects in the uterus of ovariectomized (ovx) rats. *J Steroid Biochem Mol Biol* 2003;86:179–88.
27. Šimánek V, Kren V, Ulrichová J, Vicar J, Cvak L. Silymarin: what is in the name? An appeal for a change of editorial policy. *Hepatology* 2000;32:442–4.
28. Kim NC, Graf TN, Sparacino CM, Wani MC, Wall ME. Complete isolation and characterization of silybins and isosilybins from milk thistle (*Silybum marianum*). *Org Biomol Chem* 2003;1:1684–9.
29. Lee DY, Liu Y. Molecular structure and stereochemistry of silybin A, silybin B, isosilybin A, and isosilybin B, isolated from *Silybum marianum* (milk thistle). *J Nat Prod* 2003;66:1171–4.
30. Arnone A, Merlini L, Zanarotti A. Constituents of *Silybum marianum*. Structure of isosilybin and stereochemistry of silybin. *J Chem Soc Chem Comm* 1979; 16:696–7.
31. Holm C, Stearns T, Botstein D. DNA topoisomerase II must act at mitosis to prevent nondisjunction and chromosome breakage. *Mol Cell Biol* 1989;9:159–68.
32. Rhodes DR, Yu J, Shanker K, et al. Large-scale meta-analysis of cancer microarray data identifies common transcriptional profiles of neoplastic transformation and progression. *Proc Natl Acad Sci U S A* 2004;101: 9309–14.
33. Zi X, Feyes DK, Agarwal R. Anticarcinogenic effect of a flavonoid antioxidant, silymarin, in human breast cancer cells MDA-MB 468: induction of G₁ arrest through an increase in Cip1/p21 concomitant with a decrease in kinase activity of cyclin-dependent kinases and associated cyclins. *Clin Cancer Res* 1998;4:1055–64.
34. Zhu W, Zhang J-S, Young CYF. Silymarin inhibits function of the androgen receptor by reducing nuclear localization of the receptor in the human prostate cancer cell line LNCaP. *Carcinogenesis* 2001;22:1399–403.
35. Fraser DJ, Brandt TL, Kroll DJ. Topoisomerase II α promoter *trans*-activation early in monocytic differentiation of HL-60 human leukemia cells. *Mol Pharmacol* 1995;47:696–706.
36. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc* 1971;93:2325–7.
37. Wall ME, Wani MC. History and future prospects of camptothecin and taxol. In: Cordell G. editors. *The alkaloids*. Vol. 50. San Diego: Academic Press; 1998. p. 509–31.
38. Zhu H, Chang BD, Uchiumi T, Roninson IB. Identification of promoter elements responsible for transcriptional inhibition of polo-like kinase 1 and topoisomerase II α genes by p21(WAF1/CIP1/SDI1). *Cell Cycle* 2002;1:59–66.
39. Schulz HU, Schurer M, Krumbiegel G, et al. [The solubility and bioequivalence of silymarin preparations]. *Arzneimittelforschung* 1995;45:61–4.
40. Kim YC, Kim EJ, Lee ED, et al. Comparative bioavailability of silibinin in healthy male volunteers. *Int J Clin Pharmacol Ther* 2003;41:593–6.
41. Weyhenmeyer R, Mascher H, Birkmayer J. Study on dose-linearity of the pharmacokinetics of silibinin diastereomers using a new stereospecific assay. *Int J Clin Pharmacol Ther Toxicol* 1992;30:134–8.
42. Barzaghi N, Crema F, Gatti G, Pifferi G, Perucca E. Pharmacokinetic studies on IdB 1016, a silybin-phosphatidylcholine complex, in healthy human subjects. *Eur J Drug Metab Pharmacokinet* 1990;15:333–8.

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Milk Thistle and Prostate Cancer: Differential Effects of Pure Flavonolignans from *Silybum marianum* on Antiproliferative End Points in Human Prostate Carcinoma Cells

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