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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

Paula R. Davis-Searles, Yuka Nakanishi, Nam-Cheol Kim, Tyler N. Graf, Nicholas H. Oberlies, Mansukh C. Wani, Monroe E. Wall, Rajesh Agarwal, and David J. Kroll

Abstract

Extracts from the seeds of milk thistle, *Silybum marianum*, are known commonly as silybinin 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 have been isolated from commercial silymarin extracts. In contrast, silybinin 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 silybinin were shown for the first time to suppress the activity of the DNA topoisomerase Iα 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 silybinin. 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 silybinin 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 evasive, but a number of cancer cell signaling pathways are influenced by these agents that consequently suppress mitogenic and cell survival programming (7). Silibinin and silyimin 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 κB in DU145 cells by reducing p65 and p50 levels, increasing IκBα levels, and decreasing IκBα 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 p21Cip1 and p27Kip1, 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 to reduce mRNA levels of survivin, an apoptotic inhibitor.
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 ERβ but not ERα 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 I (topo I) promoter activity. Topo I, 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 I 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 I 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 the 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. 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.

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 (5 μm, 120 Å, 4.6 × 150 mm; Waters Co., Milford, MA). Signals were monitored at 280 nm using a Varian ProStar 330 PDA detector, and all injections of reference standards, silymarin, and silibinin were of 50 μL 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 ±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 0.05, 0.20, 0.50, 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 R² 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

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assayed and triplicate points averaged and reported with their respective SDs. In initial experiments, a pCMV plasmid encoding β-galactosidase activity (pCMVβ, 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 silybin at final concentrations of 0, 15, 30, 60, and 90 μmol/L. After 24 hours of exposure, the medium was replaced with serum-free medium containing the corresponding flavonolignan or silybin 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 electrophobting. 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 silybin 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 silybin, 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 silidianin in the Indena and Madaus extracts relative to that from Aldrich. As shown below in the cellular growth inhibition assays, silidianin 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 μmol/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 silidianin) 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 μmol/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**

<table>
<thead>
<tr>
<th>Silybin (Sigma)</th>
<th>Silymarin (Aldrich)</th>
<th>Silymarin (Indena)</th>
<th>Silymarin (Madaus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silybin A</td>
<td>46.9</td>
<td>14.8</td>
<td>13.1</td>
</tr>
<tr>
<td>Silybin B</td>
<td>49.6</td>
<td>23.3</td>
<td>21.6</td>
</tr>
<tr>
<td>Iso-silybin A</td>
<td>—</td>
<td>4.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Iso-silybin B</td>
<td>—</td>
<td>2.1</td>
<td>3.9</td>
</tr>
<tr>
<td>Silidianin</td>
<td>—</td>
<td>0.8</td>
<td>9.4</td>
</tr>
<tr>
<td>Silicychristin</td>
<td>—</td>
<td>16.5</td>
<td>15.5</td>
</tr>
<tr>
<td>Iso-silicychristin</td>
<td>—</td>
<td>0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>—</td>
<td>1.7</td>
<td>2.2</td>
</tr>
</tbody>
</table>

**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.
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 μmol/L 4-silybin B, although the rank order of potency remained unchanged.

**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 μmol/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.

NOTE: Results are presented as mean percent survival ± SD (n = 3) relative to vehicle controls. The original number of cells plated exhibited a substrate-dependent LNCaP and PC3 (Table 2), isosilybin B and silybin B exhibited the greatest growth inhibition at 30 μmol/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

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

<table>
<thead>
<tr>
<th>Compound (μmol/L)</th>
<th>LNCaP (%)</th>
<th>DU145 (%)</th>
<th>PC3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>Silybin A</td>
<td>91.0 ± 2.6</td>
<td>75.9 ± 2.4*</td>
<td>55.9 ± 3.1*</td>
</tr>
<tr>
<td>Silybin B</td>
<td>109.9 ± 2.1</td>
<td>88.2 ± 7.0†</td>
<td>32.9 ± 6.3*</td>
</tr>
<tr>
<td>Isosilybin A</td>
<td>107.2 ± 4.4</td>
<td>85.3 ± 5.4†</td>
<td>54.4 ± 10.5*</td>
</tr>
<tr>
<td>Isosilybin B</td>
<td>70.4 ± 3.1*</td>
<td>46.4 ± 3.3*</td>
<td>26.2 ± 4.3*</td>
</tr>
<tr>
<td>Silibinin (Sigma)</td>
<td>90.1 ± 3.7</td>
<td>72.0 ± 3.6*</td>
<td>36.8 ± 11*</td>
</tr>
<tr>
<td>Silimaric (Aldrich)</td>
<td>90.6 ± 2.9</td>
<td>91.4 ± 3.1</td>
<td>47.1 ± 17.6*</td>
</tr>
<tr>
<td>Silimaric (Madaus)</td>
<td>120.0 ± 8.6*</td>
<td>96.0 ± 1.2</td>
<td>71.5 ± 5.0</td>
</tr>
<tr>
<td>Silimaric (Indena)</td>
<td>106.1 ± 2.7</td>
<td>95.8 ± 1.4</td>
<td>58.5 ± 27.5*</td>
</tr>
<tr>
<td>Silychristin</td>
<td>110.4 ± 5.7</td>
<td>99.7 ± 2.4</td>
<td>100.4 ± 3.0</td>
</tr>
<tr>
<td>Isosilychristin</td>
<td>111.7 ± 3.9</td>
<td>102.9 ± 4.2</td>
<td>94.8 ± 1.5</td>
</tr>
<tr>
<td>Silydianin</td>
<td>101.4 ± 4.2</td>
<td>100.4 ± 0.7</td>
<td>99.1 ± 2.4</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>86.8 ± 3.8</td>
<td>57.6 ± 0.7*</td>
<td>30.3 ± 1.4*</td>
</tr>
</tbody>
</table>

*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).

**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 h of exposure to compounds/extracts) is depicted here graphically to illustrate the potency of pure isosilybin B relative to silymarin or silibinin.

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4452 www.aacrjournals.org

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

**Cell cycle effects of milk thistle components.** Silybin is known to cause G1 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 μmol/L (Table 3). At 60 μmol/L, only silybin B exerted a modest but significant G1 arrest on DU145 cells (6-7% increase relative to vehicle control), whereas the other compounds had no appreciable effect. At 90 μmol/L, silybin B led to a greater (11-14%) increase in the G1 population the other compounds had no appreciable effect. At 90 μmol/L, silybin B led to a greater (11-14%) increase in the G1 population (relative to control) followed by isosilybin B and isosilybin A. The slight effect of silybin A on the G1 population at this concentration failed to achieve statistical significance. With silybin treatment, the percent of cells accumulating in G1 increased roughly by the average of that for silybin A and silybin B, consistent with the demonstration that silybin 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 μmol/L (Table 3). At 90 μmol/L, silybin B exerted a modest but significant G1 arrest on DU145 cells (6-7% increase relative to vehicle control), whereas the other compounds had no appreciable effect. At 90 μmol/L, silybin B led to a greater (11-14%) increase in the G1 population (relative to control) followed by isosilybin B and isosilybin A. The slight effect of silybin A on the G1 population at this concentration failed to achieve statistical significance. With silybin treatment, the percent of cells accumulating in G1 increased roughly by the average of that for silybin A and silybin B, consistent with the demonstration that silybin 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 μmol/L. This observation is consistent with a weak apoptotic effect that does not seem shared by silybin B.

**DNA topoisomerase I/IIa 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 I/II, 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 I/IIa promoter-reporter plasmid, -562TOP2LUC, in which a section of the 5’-flanking region of the topo I/IIa 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, silybin, and three preparations of silymarin were compared at 30 and 60 μmol/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 I/IIa gene promoter activity. However, only the suppression with isosilybin A and isosilybin B achieved statistical significance, with isosilybin B the most potent and effective. Silybin and the three silymarin formulations had comparable topo I/IIa 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 I/IIa promoter activity was determined in subsequent experiments with each at 30, 60, 90, or 120 μmol/L. In this case, each of the four compounds significantly suppressed topo I/IIa promoter activity at concentrations as low as 30 μmol/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 I/IIa promoter suppression was comparable: isosilybin B > silybin B > isosilybin A ≈ 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-G2/G1    | G0/G1          | S              | G2-M           |
| DMSO control 60 μmol/L        | 2.2 ± 2.1    | 49.2 ± 1.5     | 19.4 ± 2.1     | 29.4 ± 1.6     |
| Silybin A                     | 1.1 ± 0.3    | 50.2 ± 1.6     | 19.2 ± 0.5     | 29.7 ± 1.9     |
| Silybin B                     | 1.1 ± 0.7    | 56.3 ± 1.8*    | 18.9 ± 0.1     | 23.9 ± 1.0*    |
| Isosilybin A                  | 1.6 ± 1.2    | 52.5 ± 0.4     | 19.9 ± 0.7     | 26.1 ± 1.0     |
| Isosilybin B                  | 1.0 ± 0.0    | 51.5 ± 0.7     | 18.7 ± 0.4     | 28.9 ± 0.4     |
| Silibinin                     | 1.4 ± 0.2    | 52.2 ± 1.0     | 18.1 ± 0.8     | 28.5 ± 0.5     |
| Silybin A 90 μmol/L           | 3.3 ± 1.1    | 51.7 ± 1.3     | 18.6 ± 1.3     | 26.6 ± 0.4     |
| Silybin B 90 μmol/L           | 0.9 ± 1.2    | 62.9 ± 0.5*    | 15.4 ± 0.1     | 20.9 ± 0.7*    |
| Isosilybin A 90 μmol/L        | 3.8 ± 0.5    | 56.0 ± 0.4*    | 16.4 ± 1.9     | 23.0 ± 0.4*    |
| Isosilybin B 90 μmol/L        | 2.5 ± 0.3    | 57.0 ± 0.7*    | 16.4 ± 0.8     | 24.3 ± 1.4*    |
| Silibinin 90 μmol/L           | 4.5 ± 0.1    | 56.5 ± 1.8*    | 16.1 ± 0.5     | 23.1 ± 0.2*    |

NOTE: Exponentially growing DU145 cells were treated with the indicated compounds/mixtures at 60 or 90 μmol/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).
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 μmol/L for any of the compounds. At 30 μmol/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 ≥60 μmol/L. Isosilybin A and isosilybin B reduced PSA secretion to nearly undetectable levels at 90 μmol/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 silybin 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 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
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 IIa 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 IIa 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 IIa promoter at growth inhibitory concentrations. Because the human topo IIa 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...
plasma concentrations of 14 to 27 \(\mu M\) 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 \(\mu M\) 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 flavonoligand isomers should shed greater light on the potential clinical utility of these compounds.

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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.

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References


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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cell line</th>
<th>Flavonolignan rank order of potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth suppression</td>
<td>LNCaP</td>
<td>Iso B &gt; Sily B &gt; Iso A ≈ Sily A</td>
</tr>
<tr>
<td></td>
<td>DU145</td>
<td>Iso B &gt; Iso A &gt; Sily A &gt; Sily B</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>Iso B ≈ Sily B &gt; Iso A ≈ Sily A</td>
</tr>
<tr>
<td>G(1) cell cycle accumulation</td>
<td>DU145</td>
<td>Sily B &gt; Iso B ≈ Iso A &gt; Sily A</td>
</tr>
<tr>
<td>Topo IIα promoter suppression</td>
<td>DU145</td>
<td>Iso B &gt; Sily B &gt; Iso A ≈ Sily A</td>
</tr>
<tr>
<td>PSA suppression</td>
<td>LNCaP</td>
<td>Iso B ≈ Iso A &gt; Sily B ≈ Sily A</td>
</tr>
</tbody>
</table>

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.


