Enhancement of mammary carcinogenesis in two rodent models by silymarin dietary supplements

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Silymarin is a mixture of polyphenolic flavonoids isolated from milk thistle (Silybum marianum) with anticancer activities reported for several organ sites. The present study tested the efficacy of dietary silymarin against mammary carcinogenesis in two rodent models. In the Sprague–Dawley rat model, female rats were fed a purified diet supplemented with none, 0.03, 0.1, 0.3 or 1% (w/w) of silymarin from 21 days of age (DOA) and carcinogenesis was initiated by a single i.p. injection of 1-methyl-1-nitrosourea (MNU) at 51 DOA. Mammary tumor (MT) development was followed till 110 days after carcinogen injection. In the MMTV-neu/HER2 transgenic mouse mammary carcinogenesis model, homozygous transgenic females were fed a purified diet supplemented with none or 0.3% silymarin, either from 28 or 120 DOA and MT development was followed to ~300 DOA. The results showed that dietary silymarin increased the plasma concentration of free and total silibinin, a major component of silymarin, in a dose-dependent manner in the rat, but did not decrease either MT incidence or number. Instead silymarin, in a dose-dependent manner in the rat, but did increase the number of MNU-induced MTs in rats. Similarly, silymarin increased MT incidence and multiplicity and non-MTs in the neu-transgenic mice. In cell culture, treatment of human MCF-7 breast cancer cells with serum-achievable concentrations of silymarin in the rodent models stimulated their growth, in part through inhibition, differentiation and/or apoptosis (24–27). Moreover, we have shown that silymarin expresses strong anti-angiogenic activity in cell culture (28). Based on these findings, we hypothesized that silymarin could exert a chemopreventive activity against mammary carcinogenesis in vivo.

We sought to test this hypothesis using two rodent models as follows: 1-methyl-1-nitrosourea (MNU)-induced mammary breast cancers is far from satisfactory. Chemoprevention, i.e. the use of either synthetic or naturally occurring agents to inhibit pre-cancerous events, has become recognized as a plausible, cost-effective and necessary approach to reduce breast morbidity and mortality (2–4). Some classic clinical trials clearly have demonstrated the value of chemoprevention for several human malignancies, notably, a risk reduction of breast cancer by the anti-estrogen tamoxifen (5), and of prostate, colon and lung cancers by selenium (6).

The importance of new agents effective for breast cancer chemoprevention is highlighted by the fact that tamoxifen, the only FDA-approved chemopreventive agent for breast cancer, causes severe adverse effects including an increase of the risk of endometrial cancer (7). Phytochemicals present in fruits, vegetables, tea, wine and some medicinal herbs, especially botanical polyphenolic antioxidants, have in the last decade received increasing attention as potential chemopreventive agents for different human cancers (8). Silymarin, isolated from milk thistle [Silybum marianum (L.) Gaertn], is a mixture of polyphenolic flavonoids with strong antioxidant activity (9–12). Several studies have shown that silymarin protects against hepatotoxicity in rodents (10,11). For several decades silymarin has been used clinically in Europe and Asia for the treatment of liver cirrhosis (13). Recently, it has become popular in the United States as a sole dietary supplement or as a component of various antioxidant mixtures. Studies using different modes of administration to laboratory rodents, rabbits and dogs indicated that silymarin is non-toxic in acute, short- and long-term tests (10,11). Therefore, silymarin could be well suited for continual use as a cancer chemopreventive agent.

Preclinical studies have indicated a potent preventive efficacy of silymarin, regardless of its administration routes, for mouse skin cancer induced either by chemical carcinogens or UV irradiation (14–19). In addition, recent animal studies have shown that silymarin inhibits chemically-induced carcinogenesis of the colon (20), tongue (21) and bladder (22). An increasing body of literature suggests that silymarin may prevent the initiation and/or development of breast cancer. Silymarin was shown to inhibit 7,12-dimethylbenz[a]anthracene-initiated and 12-O-tetradecanoylphorbol-13-acetate promoted formation of mammary lesions in an organoid culture model (23). In cell culture, a direct exposure of breast or other cancer cells to silymarin or its major component silibinin leads to an inhibition of mitogenic signaling molecules, and a selective induction of cyclin-dependent kinase inhibitor Kip1/p27, and ultimately causes cellular G1 arrest, followed by cell growth inhibition, differentiation and/or apoptosis (24–27). Moreover, we have shown that silymarin expresses strong anti-angiogenic activity in cell culture (28). Based on these findings, we hypothesized that silymarin could exert a chemopreventive activity against mammary carcinogenesis in vivo.

Introduction

Breast cancer is the most common malignancy of women in the United States with over 200 000 new cases diagnosed every year (1). It is also the second leading cause of cancer mortality in women, next to lung cancer (1). In spite of the sophistication and advances of modern medicine, treatment for metastatic breast cancers is far from satisfactory. Chemoprevention, i.e. the use of either synthetic or naturally occurring agents to inhibit pre-cancerous events, has become recognized as a plausible, cost-effective and necessary approach to reduce breast morbidity and mortality (2–4). Some classic clinical trials clearly have demonstrated the value of chemoprevention for several human malignancies, notably, a risk reduction of breast cancer by the anti-estrogen tamoxifen (5), and of prostate, colon and lung cancers by selenium (6).

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We sought to test this hypothesis using two rodent models as follows: 1-methyl-1-nitrosourea (MNU)-induced mammary...
carcinogenesis in Sprague–Dawley rats; and MMTV-neu-transgenic mice bearing a human breast cancer proto-oncogene Her2/neu. Owing to the unexpected finding of an enhancing effect of silymarin on mammary carcinogenesis in both rodent models, we also carried out cell culture studies with the estrogen-dependent MCF-7 human breast cancer cells to explore potential mechanisms underlying the risk enhancement.

Materials and methods

Sprague–Dawley rat model study

**Diet.** Silymarin was purchased from Sigma-Aldrich (St Louis, MO) as an extra fine powder. Silymarin was mixed in AIN-93G semipurified diet at 0.03, 0.1, 0.3 and 1% (w/w). The AIN-93G formulation (29) was used to minimize potential dietary interactions between silymarin and other nutritive and non-nutritive ingredients of a rodent chow, which is based mainly on natural feed stuff. Diets (in mesh form) were made every 3 weeks and stored at −20°C in the dark to minimize photo destruction of silymarin. Fresh diets were provided twice a week for the first 2 months, and once weekly thereafter. The animals were housed in a AAALAC-accredited animal facility with 12-h light/dark cycle. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of AMC Cancer Research Center (Lakewood, CO) and was carried out at that institution while the senior author (J.L.) was on its staff.

Mammary carcinogenesis. At 51 DOA, each animal was given a single i.p. injection of MNU (50 mg/kg body wt) to initiate mammary carcinogenesis (30). Starting at 28 days after MNU injection, the rats were palpated twice weekly for mammary tumors (MTs). When a tumor was palpated, its location and date of detection were recorded. The experiment was terminated at 110 days post-carorgen injection, when the tumor incidence and multiplicity data did not support a chemopreventive effect of the dosages of silymarin used.

Necropsy. Rats were euthanized by carbon dioxide inhalation and cervical dislocation. Blood was taken from approximately one-third of the animals in each group for plasma preparation. Plasma samples were stored at −80°C until analyzed for silibinin content. Tumors were dissected and trimmed off capsular materials and weighed. A small piece of each MT was fixed in neutral formalin for histological classification after hematoxylin and eosin staining. Only histologically verified adenocarcinomas were included in cancer incidence and multiplicity data analyses.

Chemical analyses. Free silibinin was directly extracted with ethyl acetate from 200 µl plasma sample. For total silibinin, 100 µl plasma sample was first digested with β-glucuronidase and sulfatase, and then extracted with ethyl acetate. Silibinin content was quantified by HPLC analysis as described in detail recently (31) and was carried out at the Pharmacology Core Facility of University of Colorado School of Pharmacy, Denver, CO.

Transgenic mouse model study

Experimental animals. MMTV-neu-transgenic mice (wild-type) new is a well-established model (32) to study human breast cancer linked to an overexpression of Her2/ERBB2 proto-oncogene (33,34). Experimental animals were obtained by mating homozygous transgenic males and females from line 202 (32). Semiquantitative multiplex PCR was performed to confirm the homozygosity of the neu-transgene in the experimental mice using a modification of a reported procedure (35). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as normalization control for PCR analysis.

**Diet.** The basal control diet consisted of a modified AIN-93M diet with increased fat contents in order to shorten the latency of MTs (35). For that purpose AIN-93M diet was supplemented with soybean oil at the amount of 154.5 g/kg, which translated to 33.5% of calorie intake from fat, approximating the average fat intake of Americans. Silymarin was added to the basal diet at 0.3% w/w. Diets were prepared by Harlan Teklad (Madison, WI) into pellets every 6 months and were kept at −20°C to minimize their oxidation.

Mammary carcinogenesis experiment. Mice were assigned at 28 DOA to one of three groups. Care was taken to equally assign siblings into each group. The first (control) group of animals was fed the basal diet throughout the study. The second (early silymarin) group of mice was fed silymarin-supplemented diet throughout the study. The third (late silymarin) group was first fed the basal diet until 120 DOA, then was fed the silymarin-supplemented diet throughout the rest of the experiment. Four mice were housed per cage. However, along the duration of the experiment some animals were separated owing to fighting. Food and water was given ad libitum. The animal room was maintained at 22°C on a 12-h light/dark cycle and 50% humidity. The mice were observed daily for any changes in overall health and behavior, or for any sign of distress. The mice were weighed every other week to monitor their growth. Starting from 120 DOA, mice were palpated weekly to detect MTs. Mice were euthanized when their tumors were ulcerated or reached the level of ~20 mm in diameter, or when they experienced excessive distress as indicated by loss of body weight >20%, or severely injured. The study was terminated after ~10 months, when the incidence of MTs in the control group reached ~50%. The study was approved by the IACUC of the University of Minnesota, and carried out at the Hormel Institute’s AAALAC-accredited animal facility.

Necropsy and tissue processing. Mice were euthanized by carbon dioxide inhalation. During necropsy MTs and other tumors or organ/tissue abnormalities were identified and collected for histopathological verification. MTs were dissected, trimmed off capsular materials and weighed. A small piece of each MT and other abnormalities was fixed in 10% neutral formalin, and stained with hematoxylin and eosin. Histopathology was examined by an experienced clinical pathologist (J.P.G.) on a ‘blind’ basis to avoid any bias.

MCF-7 cell culture study. Human MCF-7 breast cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in EMEEM medium (ATCC) supplemented with 10 µg/ml insulin and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA). To assess the estrogen receptor (ER) agonist activity, we seeded MCF-7 cells in phenol red-free IMEM medium (Bioscience, Rockville, MD) containing 5% charcoal-stripped FBS (CS-FBS, Atlanta Biologicals) in 6-well plates for 2 days to decrease background ER signaling and treated the cells with increasing levels of silymarin in the absence or presence of 17-β-estradiol (E2, Sigma) for 7 days. The cells were fixed and stained with crystal violet and photographed. The cell number was estimated by the optical density of the dissolved crystal violet dye in 70% ethanol. The pure anti-estrogen compound Faslodex (ICI 182,780, Tocris Bioscience, Avonmouth, UK) was used in some experiments to establish the specificity of the estrogen-like activity of silymarin.

Cell proliferation rate was estimated by bromodeoxyuridine (BrDU, Sigma) incorporation into S-phase cells. In brief, after 2 days of exposure to silymarin, BrDU (18 µg/ml) was added to the cell culture medium for 2 h and the cells were harvested by trypsinization and processed for BrDU immunofluorescence. The cellular DNA was also stained with propidium iodide. BrDU and cellular DNA contents were detected by two color flow cytometry. Immunoblot analyses of the expression of selected proteins associated with mitogenesis and cell cycle regulation were carried out to determine probably molecular mediators/targets for enhancing cell proliferation by silymarin.

Results

The effects of silymarin on MNU-induced MTs in Sprague–Dawley rats

The effect of silymarin feeding on body weight. Before we initiated the current mammary cancer prevention project, all published anticarcinogenesis studies had used topical application of silymarin directly on the mouse skin (15–19). Because there was no information available on the effect of this agent delivered to Sprague–Dawley rats via the dietary route, a pilot experiment was performed on four groups of rats to provide such information. Four rats at 8 weeks of age were assigned into each of the dietary groups and were fed for 2 weeks the AIN-93G diet supplemented with 0, 0.01, 0.1 and 1% of silymarin. The body weight gain was (mean ± SD) 10.1 ± 4.3, 9.5 ± 1.7, 13.8 ± 2.5 and 16.0 ± 3.0 g per rat, respectively, indicating that a short-term silymarin consumption did not inhibit growth of the rats. Based on these results we chose 1% as the highest dose to carry out the carcinogenesis/prevention experiment.

Silymarin intake over the duration of the carcinogenesis study of 140 days had minimal adverse effects on body weight.
At the initial growth stage, i.e. before MNU injection, there was no difference in the average body weights of rats from all dietary groups. After MNU injection, there was a small decrease of the growth rate for rats fed the silymarin-supplemented diets. However, there was no obvious dose–response relationship between silymarin level and the body weight retardation, which at the end of experiment was only 3.2–7.4% of control level. These results further supported the premise of well-tolerated nature of this agent for long-term oral use.

Plasma silibinin. The plasma levels of free and total, i.e. free plus conjugated, silibinin (Figure 1B), the major active component of silymarin, at the termination of the experiment were increased in a concentration-dependent manner. The free and total silibinin reached ~5 and 35 μg/ml, respectively, by the highest dietary dose.

The effect of silymarin supplement on MTs in Sprague–Dawley rats. Figure 2A shows the incidence of MTs, i.e. percentage of rats with at least one MT, as a function of post-carcinogen exposure time. Dietary silymarin neither delayed the time it took to reach 50% incidence of MTs nor decreased the number of tumor-bearing animals at the end of the experiment, even though there appeared to be an initial delay of tumor occurrence in the group fed 0.1% silymarin. Numerically, the number of tumor-bearing rats at the end of the experiment was greater in the silymarin-fed groups than in the control group. Yet, there was no clear dose-dependency on the silymarin content in the diet (Figure 2A).

Similarly, the number of palpable MTs developed per carcinogen-exposed rat (Figure 2B) was not decreased by silymarin consumption. On the contrary, the rats fed silymarin-containing diets developed ~30–40% more tumors than animals in the control group by the end of the study (Figure 2C). There was no apparent dose–response effect of silymarin.

Owing to heavy tumor burden, 3, 5, 3, 5 and 7 rats in the control and 0.03, 0.1, 0.3 and 1% of silymarin groups, respectively, had to be terminated before the end of the experiment. The tumor burden (sum of weight of tumors per tumor-bearing animal) determined at the end of the study for the remaining rats did not differ significantly between the groups (Table I). Taken together, these data suggest that there was no protective effect of silymarin on tumor initiation and growth in Sprague–Dawley rats. Instead, silymarin exerted a modest and statistically significant enhancement effect in this model.

The effects of silymarin on MTs in MMTV-neu-transgenic mice

The unexpected results obtained with the MNU-induced mammary carcinogenesis model raised the question whether the enhancing effect is limited to chemically-induced carcinogenesis only or if this effect is of a more general nature. Therefore, we examined the effect of silymarin on a different model of mammary carcinogenesis in the MMTV-neu-transgenic mice. All female offspring from our in-house breeding colony were genotyped by PCR to ensure the homozygous status for the neu-transgene. Figure 3A shows an example of the detection method capable of distinguishing heterozygous (lane 1) from homozygous mice (lanes 2–6 and 8) and non-transgenic mice (lane 7).

Based on the results obtained from the rat model, we chose only the 0.3% silymarin diet for the mouse experiment. In addition, we wanted to address the question whether the timing of dietary supplementation in relation to mammary gland maturity and tumor lesion development modulate the effect of silymarin on mammary carcinogenesis. Therefore, one group of mice was fed the silymarin-supplemented diet starting at 120 DOA (late silymarin group), after their sexual maturation was well-established and early stage mammary lesions would have developed (32).
The effect of silymarin on mouse body weight. The average body weight at the beginning of the study (28 DOA) was 17.6 (± 1.7 SD), 17.5 (± 1.7) and 17.6 (± 1.6) g for control, early silymarin and late silymarin group, respectively. Initially, 55 or 56 mice were assigned to each dietary group, and then these numbers declined during the progress of the experiment owing to attrition as well as tumor burden. The data on mouse body weight shown in Figure 3B represent the mean obtained on all mice alive at the time of analysis. Silymarin supplement did not alter the growth of mice. In addition, there were no apparent differences among the experimental groups in the appearance or behavior of the mice. These mice were quite active, and any lethargy could have been easily observed. As expected, the results indicated that silymarin dietary supplement given as early as 4 weeks of age did not hamper the mouse growth.

The effect of silymarin on mammary carcinogenesis in MMTV-neu-transgenic mice. The transgenic mice became quite aggressive as they matured. Despite best attempts to separate them, some of the mice were found dead or were badly injured and had to be euthanized. Consequently, there was ~5–10% loss of mice owing to attrition. Because most of these occurred before the onset of MTs, these mice were removed from the study without influencing the conclusions drawn regarding the effects of silymarin.

As shown in Figure 3C, the onset of MTs started at the age of 29 weeks in the late silymarin group, and 32 weeks in the control as well as in the early silymarin group. Half of the mice in the early silymarin and the late silymarin groups developed MTs at 38 and 37 weeks of age, respectively. However, in the control group it took 45 weeks to reach 48% incidence. Feeding from 28 DOA (early silymarin group, immature mammary glands) increased the numbers of mice with MT (incidence) toward the end of the experiment, but did not speed up the initial onset of MT.

Numerically, the MT incidence at the end of experiment was higher by 30 and 35% in the early and late silymarin groups, respectively, as compared with the control group (Table II). The number of MTs from the early silymarin group, immature mammary glands) increased the numbers of mice with MT (incidence) toward the end of the experiment, but did not speed up the initial onset of MT.

Non-mammary abnormalities detected in MMTV-neu-transgenic mice. The transgenic mice developed additional

### Table 1. The effect of dietary silymarin on MNU-induced mammary carcinogenesis in female Sprague–Dawley rats

<table>
<thead>
<tr>
<th>Silymarin</th>
<th>Rats alive at termination(^a)</th>
<th>Tumor-bearing rats at termination</th>
<th>Total numbers of MTs/group</th>
<th>Tumor burden/tumor-bearing rat (g)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>27</td>
<td>19</td>
<td>77</td>
<td>9.9 ± 2.3(^c)</td>
</tr>
<tr>
<td>0.03</td>
<td>25</td>
<td>20</td>
<td>103</td>
<td>6.4 ± 1.1</td>
</tr>
<tr>
<td>0.1</td>
<td>27</td>
<td>24</td>
<td>105</td>
<td>7.3 ± 1.9</td>
</tr>
<tr>
<td>0.3</td>
<td>25</td>
<td>20</td>
<td>94</td>
<td>9.9 ± 3.0</td>
</tr>
<tr>
<td>1</td>
<td>23</td>
<td>21</td>
<td>99</td>
<td>7.8 ± 2.4</td>
</tr>
</tbody>
</table>

\(^a\)Initial number of rats was 30/group. Rats with heavy tumor burden were euthanized per IACUC rule prior to the termination of experiment at 110 days post-injection with carcinogen.

\(^b\)Data obtained on live tumor-bearing rats at the termination of experiment.

\(^c\)Mean ± SEM. ANOVA analyses indicated no significant difference among the group means, \(P > 0.1\).
Silymarin enhances mammary carcinogenesis

Table II. Effect of silymarin-supplemented diet on MTs in female MMTV-neu-transgenic mice

<table>
<thead>
<tr>
<th>Control group</th>
<th>Early silymarin group</th>
<th>Late silymarin group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective number of mice in the study</td>
<td>50</td>
<td>53</td>
</tr>
<tr>
<td>Number of mice with MTs</td>
<td>24</td>
<td>33</td>
</tr>
<tr>
<td>Percentage of mice with MTs (%)</td>
<td>48.0</td>
<td>62.3</td>
</tr>
<tr>
<td>Total number of MTs</td>
<td>43</td>
<td>64</td>
</tr>
<tr>
<td>Number of mice with one MT</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Number of mice with multiple MTs</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>Percentage of mice with multiple MTs (%)</td>
<td>45.8</td>
<td>54.6</td>
</tr>
<tr>
<td>Tumor weight/MT-bearing mouse (g)</td>
<td>1.21</td>
<td>1.08</td>
</tr>
</tbody>
</table>

*aEarly silymarin group was fed continually with 0.3% silymarin-supplemented diet starting at 28 DOA.

*bLate silymarin group was fed control diet from 28 to 120 DOA.

In the late silymarin group there was one case of salivary gland adenocarcinoma, and one mouse developed a high-grade lymphoma/poorly-differentiated carcinoma that spread over the fallopian tubes, spleen, pancreas and liver.

Effects of silymarin on growth of MCF-7 cells in cell culture

The results from both rodent models prompted us to investigate the possible mechanisms for silymarin to enhance breast cancer growth. Since it has been suggested that silymarin may have phytoestrogen activities (36), we focused on the possibility of serum-achievable levels of silymarin might act as an ER agonist in the estrogen-responsive human MCF-7 breast cancer cells. As expected, the addition of 17β-estradiol (E2) significantly increased cell growth (Figure 4A, +0.1 nM E2 control versus −E2 control). Increasing E2 level stimulated cell growth in a curve-linear manner, indicating that 0.1 nM E2 represented a submaximal stimulation because 1 nM E2 promoted an even greater cell accumulation (Figure 4B). In the absence and presence of the submaximal E2, silymarin increased E2 level stimulated cell growth in a curve-linear manner, indicating that 0.1 nM E2 represented a submaximal stimulation because 1 nM E2 promoted an even greater cell accumulation (Figure 4B).

In the late silymarin group there was one case of salivary gland adenocarcinoma, and one mouse developed a high-grade lymphoma/poorly-differentiated carcinoma that spread over the fallopian tubes, spleen, pancreas and liver.

Table III. Non-MTs in female MMTV-neu-transgenic mice

<table>
<thead>
<tr>
<th>Control group</th>
<th>Early silymarin group</th>
<th>Late silymarin group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ovarian teratoma</td>
<td>1 Ovarian cystic neoplasia</td>
<td>3 Ovarian teratomas</td>
</tr>
<tr>
<td>1 Ovarian adenocarcinoma</td>
<td>1 Salivary adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>1 High-grade metastasis</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

*aEarly silymarin group was fed continually with 0.3% silymarin-supplemented diet starting at 28 DOA.

*bLate silymarin group was fed control diet from 28 to 120 DOA, and then with 0.3% silymarin-supplemented diet.

abnormalities (Table III). It is noteworthy that seven out of the eight non-mammary abnormalities occurred in mice fed the silymarin-supplemented diet. Out of 154 mice in the study, six mice developed tumors in their ovaries. Four of the mice (one in control group and three in late silymarin group) had tumors with histological features consistent with teratoma (multiple cystic structures containing squamous epithelium and keratin, foci of cartilage and areas containing primitive epithelium). In the early silymarin group one mouse developed a cystic ovarian neoplasm consisting primarily of keratin-filled cysts, bone and epithelial-lined cystic tissue, and one mouse developed a high-grade ovarian adenocarcinoma.
Faslodex. As shown in Figure 4D, Faslodex decreased cell growth in a concentration-dependent manner in the absence of added E₂ (columns 2 and 3 versus 1). It effectively blocked silymarin-induced (columns 5 and 6 versus 4) as well as E₂-induced cell growth (column 8 versus 7).

Next, we investigated the effect of silymarin on the rate of cell proliferation using BrdU incorporation as a marker (Figure 5A). After the MCF-7 cells had been treated with silymarin for 2 days in the absence of added E₂, BrdU was added for 2 h to pulse-label cells actively engaged in DNA replication. As shown in Figure 5B, within the serum-achievable concentration range, silymarin induced a near-linear stimulatory effect on BrdU-positive cells.

Immunoblot analyses of MCF-7 cells identically treated as in Figure 5A without added E₂ showed a concentration-dependent induction of c-Myc protein (Figure 5C), which is
a known early response gene of estrogen stimulation (37,38 and references therein). Consistent with an increased S-phase population, the abundance of the S-phase-specific cyclin E was increased by silymarin, although the extent of change was about half of that observed for c-Myc. The enhanced proliferative effect was observed without a significant effect on the expression level of the ER-α protein or the early G1-specific cyclin D1. Together, the cell culture results have indicated that serum-achievable levels of silymarin increase ER signaling and cell proliferation.

Discussion

The results from the two rodent models of mammary carcinogenesis were quite unexpected. They were in sharp contrast to the results of several preclinical studies in animal models of carcinogenesis in other organ sites. For example, dietary supplement with silymarin has been shown to decrease the tumor incidence and number in chemically-induced rat models of carcinogenesis in the colon (20), tongue (21) and bladder (22) and to decrease the growth of human prostate cancer and lung cancer xenografts in immunodeficient mice (39,40). These reports were consistent with the potent protective action of silymarin observed in the mouse skin carcinogenesis models (15–19). Instead of the anticipated protective effects, we observed a moderate increase of MNU-induced carcinogenesis in Sprague–Dawley rats (Figure 2B and Table I) and oncogene-driven MTs in MMTV-neu mice (Figure 3C and Table II). It should be noted that in the mammary carcinogenesis models, in particular, adverse effect of agents on the health or physiology of the animals as reflected by reduced body weight is usually associated with a reduced tumor outcome (41,42). Chronic silymarin feeding for ~140 days to rats and 300 days to mice showed very little, if any, adverse effects on body weight gains (Figure 1A and Figure 3B), supporting the well-tolerated nature of this agent. Thus, since we have observed negligible effect of silymarin on body weights in both animal models, it is unlikely that its ‘true’ chemopreventive effect might have been compromised owing to body weight impact.

In view of the anticancer activities in the other organ sites, the unexpected enhancement effects in mammary models support a specific enhancement activity of silymarin in this estrogen-sensitive organ. This notion was further supported by cell culture experiments in which we focused on the estrogen-like activity of silymarin in the estrogen-dependent MCF-7 cell culture model (Figures 4 and 5). The plasma silybinin levels achieved in the rats (up to 5 μg free per ml) (Figure 1B) were in the same range as those reported for mice fed a silymarin-supplemented diet that exerted a chemopreventive effect against prostate cancer (31,39). Because silybinin is the major constituent of silymarin, these values would correspond to ~10 μg/ml free silymarin in cell culture medium. The cell colony growth experiments (Figure 4A and C) revealed biphasic response curves as a function of silymarin concentration. Within the serum-achievable concentration range, silymarin and submaximal E2 produced additive stimulation of cell growth in a concentration-dependent manner (Figure 4A and C). Conversely, the growth stimulation by silymarin as well as by E2 was reversed by blocking ER function with Faslodex (Figure 4D). These results support stimulation of ER signaling by silymarin as a major pathway to stimulate breast cancer cell growth, which was clearly reflected by the linear increase of S-phase cells when the silymarin concentration was elevated (Figure 5A). In further support of this notion, silymarin treatment increased the abundance of c-Myc (Figure 5C), which is a known estrogen-responsive gene product (37,38). Because estrogen is an endogenous promoter for breast cancer and for the rodent mammary carcinogenesis models studied here (42–48), these cell culture results provide a plausible mechanism to account for the enhanced mammary carcinogenesis in the rodent models.

The cell culture data also help to reconcile with growth suppression effects reported in earlier literature with human breast cancer cell lines (24,49). We also observed the strong growth inhibitory effect at 50–100 μg/ml silymarin (Figure 4A). However, such levels may only be achievable through pharmacological means and may not be applicable to chemoprevention through dietary supplement.

Because of the experimental nature of the animal models in our study, the data presented here do not preclude a possible benefit of silymarin for breast cancer prevention in women. They nevertheless add a cautionary note to such an application. The finding of an apparent enhancement of non-MTs by silymarin consumption in the neu-transgenic mice, chiefly in the ovary (Table III), further supports this notion. However, the risk implications of consuming silymarin will be more meaningful in the context of currently used doses in humans. For example, silymarin is used as a dietary supplement for a variety of ailments in the range of 200–600 mg/day. Available human pharmacokinetic studies with silymarin or silybin have shown poor bioavailability (50,51). For example, plasma level peaks at 0.05 μg/ml with a single oral dose of 360 mg of silybin, the major component of silymarin. When silybin is complexed with phosphatidylcholine into phytosomes, the plasma peak concentration can be improved to 0.2–0.3 μg/ml (50). In rodent studies, plasma silybinin levels associated with either preventive effect for prostate cancer (39) or enhancement effect in our studies (Figure 1) are ~5–10 times higher than in human plasma. Therefore, the risk and benefit assessment of silymarin consumption will require careful consideration of dosage as well as differences in metabolism between rodents and men.

Since silymarin is a natural product of four major silybinin compounds, and several minor components (8–12), each of these components may exert different biological activities as have been demonstrated by a recent study of silybinin derivatives in a prostate cancer cell culture model (52). It will be essential and necessary in future studies to identify the silymarin components that confer the estrogen-like and growth stimulatory activities. Studies of the structure and activity relationship of the different silymarin constituents and silybinin derivatives in terms of their growth stimulatory activities may help to identify compounds that lack the enhancement activity on mammary cancer risk or even be chemopreventive for mammary cancer.

In summary, our data did not support the hypothesis regarding a chemopreventive effect of silymarin dietary supplement, delivered from pre-pubescence on the chemical induction of mammary carcinogenesis in the rats, or delivered from pre- and post-pubescence to mice on their oncogene-driven MTs. On the contrary, consumption of silymarin led to modest increases of MTs in both models. We showed that silymarin within the serum-achievable concentration range in the rodents stimulated MCF-7 breast cancer cell growth in cell culture, probably through estrogen-like activities. Given the positive preventive...
activities in animal models of other organ sites (15–22,39), we believe that additional studies are warranted to define the structural features of silymarin compounds that confer the estrogen-like activity and to develop derivatives that eliminate this activity. Before such information is attained, a cautionary note is prudent for women who are on, or intend to use, silymarin-containing dietary supplements for breast cancer prevention purposes.

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References


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