Effect of Silibinin on the Growth and Progression of Primary Lung Tumors in Mice

Rana P. Singh, Gagan Deep, Manesh Chittezhath, Manjinder Kaur; Lori D. Dwyer-Nield, Alvin M. Malkinson, Rajesh Agarwal

Background: Silibinin, a flavanone from milk thistle, inhibits the growth of tumors in several rodent models. We examined the effects of dietary silibinin on the growth, progression, and angiogenesis of urethane-induced lung tumors in mice.

Methods: A/J mice (15 per group) were injected with urethane (1 mg/g body weight) or saline alone and fed normal diets for 2 weeks, after which they were fed diets containing different doses of silibinin (0%–1% wt/wt silibinin) for 18 or 27 weeks. Immunohistochemistry and Western blot analysis were used to examine angiogenesis and enzymatic markers of inflammation, proliferation, and apoptosis. All statistical tests were two-sided.

Results: Urethane-injected mice exposed to silibinin had statistically significantly lower lung tumor multiplicities than urethane-injected mice fed the control diet lacking silibinin (i.e., control mice). Mice that received urethane and 1% (wt/wt) dietary silibinin for 18 weeks had 93% fewer large (i.e., 1.5–2.5-mm-diameter) lung tumors than control mice (mean number of tumors/mouse: 27 in the urethane group versus 2 in the urethane + 1% silibinin group, difference = 25 tumors/mouse, 95% confidence interval [CI] = 13 to 37 tumors/mouse, P = .005). Lung tumors of silibinin-fed mice had 41%–74% fewer cells positive for the cell proliferation markers proliferating cell nuclear antigen and cyclin D1 than lung tumors of control mice. Tumor microvessels density was reduced by up to 89% with silibinin treatment (e.g., 56 microvessels/400× field in tumors from control mice versus 6 microvessels/400× field in tumors from urethane + 1% silibinin-treated mice [difference = 50 microvessels/400× field, 95% CI = 46 to 54 microvessels/400× field; P < .001]). Silibinin decreased lung tumor expression of vascular endothelial growth factor (VEGF) and of inducible nitric oxide synthase and cyclooxygenase-2, two enzymes that promote lung tumor growth and progression by inducing VEGF expression.

and, in its absence, chemically-induced lung tumor development is decreased by more than 80% (23). Thus, agents that inhibit expression of iNOS might prevent lung cancer progression. We examined the effect of dietary administration of silibinin on angiogenesis in a mouse model of urethane-induced primary lung tumor growth and progression. We assessed the effects of silibinin on expression of proliferating cell nuclear antigen (PCNA), an enzyme crucial to DNA synthesis, and on cyclin D1, a necessary component for cell cycle transit and a marker of lung cell proliferation. We examined angiogenesis at a morphologic level and at a molecular level, examining the expression of CD31, iNOS, the cyclooxygenase enzyme, COX2, and vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF), two essential growth factors for endothelial cells.

**MATeRIALS AND METHODS**

**Chemicals and Diets**

Urethane (ethyl carbamate) and silibinin were purchased from Sigma (St. Louis, MO). We confirmed that the purity of silibinin used in this study was 100% by high-performance liquid chromatography, as previously described (24). Our ongoing laboratory studies have established that the pure compound is stable for many years at −20 °C (Authors, 1st 6 to contribute to this observation: unpublished observations). We observed that approximately 100% of the silibinin added to animal diets stored at 4 °C was recovered after about 6 months (12). We used AIN-76A rodent diet pellets without silibinin (control) and containing 0.033% (wt/wt), 0.1% (wt/wt), 0.33% (wt/wt), and 1% (wt/wt) silibinin. The diets were prepared by Dyets Inc. (Bethlehem, PA) and stored at 4 °C.

**Mouse Model of Urethane-Induced Lung Tumorigenesis**

Six-week-old A/J male mice (The Jackson Laboratory, Bar Harbor, ME) were housed under standard laboratory conditions in the Center of Laboratory Animal Care at the University of Colorado Health Sciences Center. Animal care was in accordance with institutional guidelines, and all animal treatments were done under an institutional protocol that was approved by the animal care and use committee. Mice were given a single intraperitoneal injection of urethane (1 mg/g body weight) freshly dissolved in 0.9% saline or of saline only, as previously described (12). We used AIN-76A rodent diet pellets without silibinin (control) and containing 0.033% (wt/wt), 0.1% (wt/wt), 0.33% (wt/wt), and 1% (wt/wt) silibinin. The diets were prepared by Dyets Inc. (Bethlehem, PA) and stored at 4 °C.

**Experimental Design**

Ninety mice were randomly assigned to six groups (15 mice/group). The mice in five groups were injected intraperitoneally with urethane; mice in the remaining group received saline. All mice were fed AIN-76A (control diet) for 2 weeks after urethane or saline injection. The five groups of mice that received urethane injections were then randomly assigned to the control diet (AIN-76A diet) or to one of four treatment diets (pelleted AIN-76A diet containing 0.033%, 0.10%, 0.33%, or 1.0% [wt/wt] silibinin) ad libitum for the remainder of the experiment. The control group received the control diet. Treatment groups were as follows: group I, control diet (0.9% saline); group II, urethane + control diet; group III, urethane + 0.033% silibinin diet; group IV, urethane + 0.10% silibinin diet; group V, urethane + 0.33% silibinin diet; and group VI, urethane + 1.0% silibinin diet. Ten mice from each group were killed by ketamine injection at 20 weeks, the time when lung adenomas are in early stage of the growth involving neo-angiogenesis, after urethane injection or saline injection. Lung tumors from five randomly chosen mice per group were removed under a dissecting microscope, counted, and measured with the use of digital calipers. Whole lungs from another five randomly chosen mice per group were removed and fixed in formalin for immunohistochemical analysis. The remaining five mice in each group were killed by ketamine injection at 29 weeks after urethane or saline injection, and their lung tumors were harvested, counted, measured, and stored at −80 °C, the time when lung adenomas are in late phase of the growth and start progressing to adenocarcinoma involving angiogenesis. Plasma samples from all mice in the 29-week study were prepared from blood collected by cardiac puncture and placed into heparinized tubes. No mice used in this study died prematurely, and none had to be killed because of the morbidity criteria specified in the institutional protocol.

**Immunohistochemistry**

Formalin-fixed lungs were embedded in paraffin and sectioned (5 μm thick). Sections were stained using antibodies against proliferating cell nuclear antigen (PCNA), cyclin D1, CD31 (platelet-derived endothelial cell adhesion molecule), vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) protein expression by 3,3′-diaminobenzidine staining, as previously described (17). The primary antibodies used were mouse monoclonal anti-PCNA antibody (1:400 dilution; Dako, Carpinteria, CA), rabbit polyclonal anti-cyclin D1 antibody (1:300 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-CD31 antibody (1:200 dilution; Santa Cruz Biotechnology), rabbit polyclonal anti-VEGF antibody (1:200 dilution; Santa Cruz Biotechnology), rabbit polyclonal anti-iNOS antibody (1:100 dilution; BD Transduction Laboratory, San Diego, CA), and rabbit polyclonal anti-COX-2 antibody (1:100 dilution; Santa Cruz Biotechnology). The biotinylated secondary immunoglobulin antibodies used were anti-mouse (1:200 dilution; Dako), anti-rabbit (1:400 dilution; Santa Cruz Biotechnology), and anti-goat (1:600 dilution; Santa Cruz Biotechnology). The proliferation index and microvessel density were quantified in five randomly selected individual tumor fields (at 400× magnification) per lung sample. The proliferation index was the total number of PCNA-positive cells × 100 divided by the total number of cells counted. Microvessel density was defined as the mean number of CD31-positive cells per 400× field. Cyclin D1, VEGF, iNOS, and COX-2 immunoreactivities (represented by brown staining) were analyzed in five individual tumors for each lung sample and were scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), or 4+ (very strong staining) by subjective assessment in an unblinded fashion. Positivity indicates average immunoreactivity score of 1 or higher. The mean immunoreactivity...
scores from 20–25 individual tumors, four or five tumors from each of five mice per group, were used for the analysis of cyclin D1, VEGF, iNOS, and Cox-2 expression levels. All immunohistochemical analyses were performed using a Zeiss Axioscope 2 microscope. Microscopic images were captured with a Kodak DC290 camera and processed by the Windows Millennium DC290 Kodak microscopy documentation system.

In Situ Detection of Apoptosis by TUNEL Staining

We used a terminal deoxynucleotidyl transferase-mediated dUDP-nick-end labeling (TUNEL)-based TumorTACS In Situ Apoptosis Detection Kit (R & D Systems, Inc., Minneapolis, MN) to identify apoptotic cells in formalin-fixed, paraffin-embedded lung sections, as previously described (17). Apoptotic cells within lung tumors were visualized at 400× magnification and scored by counting positive (i.e., brown-stained) cells in five individual tumors (or field) for each lung sample.

Mouse Angiogenesis Antibody Array

Plasma samples collected at the end of the 29-week study were used to analyze the expression of circulating angiogenesis-related molecules using a Mouse Angiogenesis Antibody Array (RayBiotech, Inc., Norcross, GA). A randomly selected plasma sample (100 μL/sample/assay) from each treatment group was analyzed according to the manufacturer’s protocol. Expression of each protein was represented by a pair of dots (duplex dots) on the membrane. Duplex dots identifying each protein were scanned using Adobe Photoshop software and quantitated by ScionImage program. The mean intensity of the two dots (arbitrary units) was determined for intergroup comparisons.

Western Blot Analysis of Tumor Lysates

All lung tumors from each mouse were pooled and homogenized in lysis buffer (20 mM HEPES [pH 7.4], 10% [vol/vol] glycerol, 2 mM EDTA, 2 mM EGTA, 10 μM leupeptin, 5 μg/mL aprotinin). Protein concentration in lysates was determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Seventy μg protein/lysat was loaded on each lane for Western blot analysis, as previously described (23). Membranes were probed with rabbit polyclonal anti-iNOS (1:1000 dilution; BD Transduction Laboratory), rabbit polyclonal anti-NOS3 (1:1000 dilution; Santa Cruz Biotechnology), and rabbit polyclonal anti-COX-2 (1:1000 dilution; Santa Cruz Biotechnology) antibodies followed by peroxidase-conjugated anti-rabbit secondary antibody (1:1000 dilution; Cell Signaling Technology, Beverly, MA). Bound antibodies were visualized by enhanced chemiluminescence detection (Amersham Corp., Piscataway, NJ). To confirm equal protein loading per lane, the membranes were stripped and reprobed with mouse monoclonal anti-β-actin antibody (1:3000 dilution; Sigma).

Statistical Analysis

Tumor number data are expressed as mean values with 95% confidence intervals (CIs) for five mice. We randomly selected 20–25 tumors—four or five tumors from each of five mice in each treatment group—for all of the immunohistochemical analyses. Statistical significance of differences in all measurements between the urethane/control diet group and the urethane + silibinin treatment groups was determined by one-way analysis of variance followed by a Tukey’s procedure for all pairwise multiple comparisons, excluding the saline/control diet group. Student’s paired t test was used for pairwise between-group comparisons, as needed. All statistical tests were two-sided, and P < 0.05 was considered statistically significant. Relationships between different study parameters were measured by using Pearson’s correlation coefficient (r) and P values determined for each analysis. Mean values for each variable for the urethane/control diet group and each urethane + silibinin treatment group were included in the correlation analysis.

RESULTS

Effect of Silibinin on Urethane-Induced Lung Tumorigenesis in A/J Mice

We first examined the effect of silibinin on urethane-induced lung tumorigenesis (Fig. 1, A). Urethane-injected mice exposed to 18 weeks of any of the diets containing silibinin had statistically significantly lower lung tumor multiplicities (i.e., mean number of tumors/mouse) than urethane-injected mice fed the control diet (52 tumors/mouse in the urethane group versus 32 tumors/mouse in the urethane + 0.033% silibinin group, difference = 20 tumors/mouse, 95% CI = 18 to 22 tumors/mouse, P < 0.001; 31 tumors/mouse in the urethane + 0.1% silibinin group, difference = 21 tumors/mouse, 95% CI = 19 to 23 tumors/mouse, P < 0.001; 28 tumors/mouse in the urethane + 0.33% silibinin group, difference = 24 tumors/mouse, 95% CI = 23 to 25 tumors/mouse, P < 0.001; 30 tumors/mouse in the urethane + 1% silibinin group, difference = 22 tumors/mouse, 95% CI = 21 to 23 tumors/mouse, P < 0.001) (Fig. 1, B, left panel). Similarly, in the 29-week study, urethane-injected mice exposed to silibinin had statistically significantly lower lung tumor multiplicities than urethane-injected mice fed the control diet lacking silibinin (44 tumors/mouse in the urethane group versus 31 tumors/mouse in the urethane + 0.033% silibinin group, difference = 13 tumors/mouse, 95% CI = 6 to 15 tumors/mouse, P = 0.041; 33 tumors/mouse in the urethane + 0.1% silibinin group, difference = 11 tumors/mouse, 95% CI = 6 to 15 tumors/mouse, P = 0.003; 26 tumors/mouse in the urethane + 0.33% silibinin group, difference = 18 tumors/mouse, 95% CI = 13 to 23 tumors/mouse, P < 0.001; 28 tumors/mouse in the urethane + 1% silibinin group, difference = 16 tumors/mouse, 95% CI = 10 to 21 tumors/mouse, P < 0.002) (Fig. 1, B, right panel).

In both studies, we monitored the body weights of the mice and their food intake to assess any systemic toxicity that might be associated with the presence of silibinin in the diet. Consistent with our earlier reports (12, 14), we observed no decreases in body weight gains (Fig. 1, C) or in diet consumption (data not shown) among mice that received silibinin-supplemented diets. We therefore concluded that chronic consumption of up to 1.0% (wt/wt) silibinin as a dietary supplement caused no apparent toxicity or adverse health effects. The unaltered weight gains and dietary consumption among the silibinin-fed mice also indicate that the observed decreases in tumor multiplicity were not due to caloric restriction.

Effect of Silibinin on Urethane-Induced Lung Tumor Progression

To study the effects of silibinin on chemically induced lung tumor progression, we harvested lung tumors from mice in the two
urethane + 0.1% silibinin group, difference = 19 tumors/mouse, 95% CI = 11 to 27 tumors/mouse, \( P = .002 \); 3 tumors/mouse in the urethane + 0.33% silibinin group, difference = 24 tumors/mouse, 95% CI = 13 to 35 tumors/mouse, \( P = .004 \); 2 tumors/mouse in the urethane + 1% silibinin group, difference = 25 tumors/mouse, 95% CI = 13 to 37 tumors/mouse, \( P = .005 \) (Fig. 2, A). We also observed statistically significant differences in the mean number of large 1.5–2.5-mm-diameter tumors between the following groups of mice: urethane + 0.033% silibinin versus urethane + 0.33% silibinin (33 tumors/mouse versus 28 tumors/mouse, difference = 5 tumors/mouse, 95% CI = 1 to 7 tumors/mouse, \( P = .022 \)), urethane + 0.033% silibinin versus urethane + 1% silibinin (33 tumors/mouse versus 30 tumors/mouse, difference = 3 tumors/mouse, 95% CI = 1 to 4 tumors/mouse, \( P = .024 \)), urethane + 0.1% silibinin versus urethane + 0.33% silibinin (31 tumors/mouse versus 28 tumors/mouse, difference = 3 tumors/mouse, 95% CI = 0 to 6 tumors/mouse, \( P = .03 \)), and urethane + 0.1% silibinin versus urethane + 1% silibinin (31 tumors/mouse versus 30 tumors/mouse, difference = 1 tumor/mouse, 95% CI = 0 to 2 tumors/mouse, \( P = .033 \)). These findings indicate a dose–response effect of silibinin on the numbers of large tumors.

As expected, we found that all groups of mice had a greater number of large tumors at 29 weeks after urethane injection than at 20 weeks after urethane injection (Fig. 2, B). For example, among urethane-injected mice that did not receive silibinin, the mean number of small (i.e., less than 1.5 mm) tumors/mouse was 4 (95% CI = 2 to 5 tumors/mouse) whereas the mean number of large (i.e., \( > 1.5 \)–3.5 mm) tumors/mouse was 40 (95% CI = 39 to 42 tumors/mouse). The number of large tumors/mouse decreased by 50%–83% as the dose of dietary silibinin increased, compared with numbers in urethane-treated (i.e., no silibinin) mice (40 tumors/mouse in the urethane group versus 20 tumors/mouse in the urethane + 0.033% silibinin group, difference = 20 tumors/mouse, 95% CI = 10 to 29 tumors/mouse, \( P = .004 \); 18 tumors/mouse in the urethane + 0.1% silibinin group, difference = 22 tumors/mouse, 95% CI = 18 to 27 tumors/mouse, \( P = .001 \); 11 tumors/mouse in the urethane + 0.33% silibinin group, difference = 29 tumors/mouse, 95% CI = 26 to 32 tumors/mouse, \( P < .001 \); 7 tumors/mouse in the urethane + 1% silibinin group, difference = 33 tumors/mouse, 95% CI = 30 to 36 tumors/mouse, \( P < .001 \) (Fig. 2, B). We also observed a statistically significant difference in the mean number of large tumors between the urethane + 0.1% silibinin and urethane + 0.33% silibinin groups (difference = 7 tumors/mouse, 95% CI = 4 to 10 tumors/mouse, \( P = .003 \)), between the urethane + 0.033% silibinin and urethane + 1% silibinin groups (difference = 13 tumors/mouse, 95% CI = 6 to 21 tumors/mouse, \( P = .008 \)), and between the urethane + 0.1% silibinin and urethane + 1% silibinin groups (difference = 11 tumors/mouse, 95% CI = 6 to 16 tumors/mouse, \( P = .04 \)). We observed statistically significantly more small tumors (<1.5 mm) as the dose of silibinin was increased (4 tumors/mouse in the urethane group versus 16 tumors/mouse in the urethane + 0.1% silibinin group, difference = 12 tumors/mouse, 95% CI = 6 to 18 tumors/mouse, \( P = .005 \); 14 tumors/mouse in the urethane + 0.33% silibinin group, difference = 10 tumors/mouse, 95% CI = 9 to 12 tumors/mouse, \( P < .001 \); 22 tumors/mouse in the urethane + 1% silibinin group, difference = 18 tumors/mouse, 95% CI = 13 to 23 tumors/mouse, \( P < .001 \), paired \( t \) test), whereas the total number of tumors (i.e., small plus large) was similar in each silibinin dose group and the number of larger tumors decreased in a dose-dependent fashion.
Effect of Silibinin on Urethane-Induced Lung Tumor Cell Proliferation

We previously documented that epithelial cell proliferation in normal and neoplastic lungs can be assessed by quantifying the percentage of tumor cells that are positive for PCNA (27) or cyclin D1 (28). To evaluate possible mechanisms by which silibinin reduced the number of urethane-induced lung tumors, we first analyzed the effect of silibinin on tumor cell proliferation. The percentage of PCNA-positive cells was 41%–74% lower in lung tumors of silibinin-fed mice than in the lung tumors of the urethane-only mice. The mean percentages of PCNA-positive cells were 20.8 in the urethane-only group, 12.2 in the urethane + 0.033% silibinin group (difference = 8.6, 95% CI = 5.2 to 11.8; P<.001), 7.8 in the urethane + 0.1% silibinin group (difference = 13, 95% CI = 10.2 to 15.6; P<.001), 5.5 in the urethane + 0.33% silibinin group (difference = 15.3, 95% CI = 11.8 to 18.7; P<.001), and 8.6 in the urethane + 1% silibinin group (difference = 12.2, 95% CI = 8.7 to 15.6) (Fig. 3, A).

Similarly, the mean number of cyclin D1-positive cells (i.e., those with an average immunoreactivity score of 1 or higher) decreased by 50%–71% with increased silibinin doses (Fig. 3, B and C). Silibinin decreased cyclin D1 mean immunoreactivity scores from 2.4 in the urethane group to 1.2 in the urethane + 0.033% silibinin group (difference = 1.2, 95% CI = 1.1 to 1.5, P<.001), to 1.1 in the urethane + 0.1% silibinin group (difference = 1.3, 95% CI = 1.1 to 1.5, P<.001), to 1.0 in the urethane + 0.33% silibinin group (difference = 1.4, 95% CI = 1.2 to 1.6, P<.001), and to 0.7 in the urethane + 1% silibinin group (difference = 1.7, 95% CI = 1.5 to 1.9, P<.001) (Fig. 3, C). The effect of silibinin on tumor multiplicity was statistically significantly positively correlated with the effect on the percentage of PCNA-positive or cyclin D1-positive tumor cells (tumor number in a 20-week study versus PCNA positivity: r = .97, P = .007; tumor number in a 20-week study versus cyclin D1 positivity: r = .97, P = .006).

Because tumor size reflects the effects of both proliferation and apoptosis and silibinin reduced tumor size and proliferation, we also evaluated the effects of silibinin on apoptosis. We used TUNEL staining to assess the effect of silibinin on apoptosis within the lung tumor parenchyma. The number of apoptotic cells was negligible in all groups of mice, and silibinin only slightly increased the number of TUNEL-positive cells (data not shown). Overall, these results indicate that the antiproliferative effects of silibinin (rather than any effects on apoptosis) are likely to be responsible for the observed decrease in lung tumor multiplicity.

Effect of Silibinin on Angiogenesis in Urethane-Induced Lung Tumors

The growth and progression of cancers from focal to invasive carcinomas is dependent on angiogenesis. Tumor microvessel density is an important prognostic marker for many solid tumors, including lung cancer that independently predicts pathologic stages of tumor progression, including malignant potential (29,30). Tumor microvessel density can also be used to estimate tumor growth rate (29). To examine whether the inhibition of lung tumor growth by silibinin was accompanied in vivo by effects on angiogenesis, we measured intratumoral microvessel density by using immunohistochemical analysis of the endothelial cell marker CD31 (Fig. 4, A). We observed numerous CD31-positive cells in tumors from urethane-treated mice (Fig. 4, A, panel a). Among tumors from silibinin-treated mice, the number of CD31-positive cells decreased as silibinin dose increased (Fig. 4, A, panels b–e). Mean microvessel density was 56 microvessels/400× field in tumors from urethane-treated mice compared with 33 microvessels/400× field in tumors from urethane + 0.033% silibinin-treated mice (difference = 23 microvessels/400× field, 95% CI = 20 to 25 microvessels/400× field; P<.001), 25 microvessels/400× field in tumors from urethane + 0.1% silibinin-treated mice (difference = 31 microvessels/400× field, 95% CI = 29 to 32 microvessels/400× field; P<.001), 6 microvessels/400× field in tumors from urethane + 0.33% silibinin-treated mice.
Fig. 3. Effect of silibinin on urethane-induced lung tumor cell proliferation. Tumor-bearing lungs harvested from mice in the 20-week study (n = 5 mice/group) were fixed and immunohistochemically analyzed for cell proliferation by staining with antibodies against (A) proliferating cell nuclear antigen (PCNA) and (B, C) cyclin D1. In each case, quantitative data for 20–25 randomly selected tumors from each group (four or five tumors per mouse) are presented with 95% confidence intervals (CIs). The upper boundary of the box represents the 75th percentile of the number of tumors per mouse, the lower boundary of the box represents the 25th percentile of the data distribution, the horizontal line within the box represents the median value, and the error bars represent the 95% confidence intervals. A) *P < .001, urethane (U) versus each U + silibinin (SB) group; †P < .001, U + 0.033% SB versus U + 0.1% SB group; ‡P = .043, U + 0.033% SB versus U + 0.33% SB group, Tukey test. The closed circles above and below the bars represent out-of-range values. B) Brown color indicates cyclin D1 immunoreactivity in lung tumors. Images that best represent the staining patterns in tumors from U-treated mice (panel a) and from U + 0.33% SB-treated mice (panel b) are shown. Scale bar = 50 μm. C) Cyclin D1 immunoreactivity was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), or 4+ (very strong staining), and data are presented as immunoreactivity scores with 95% CIs in the box plot. *P < .001, U versus each U + SB group, Tukey test.

(diffference = 50 microvessels/400× field, 95% CI = 46 to 54 microvessels/400× field; P < .001), and 6 microvessels/400× field in tumors from urethane + 1% silibinin-treated mice (difference = 50 microvessels/400× field, 95% CI = 46 to 54 microvessels/400× field; P < .001) (Fig. 4, A, panel f). This effect of silibinin at the two higher doses was statistically significantly different from that of its two lower doses (urethane + 0.033% silibinin-treated mice versus urethane + 0.33% silibinin-treated mice, difference = 27 microvessels/400× field, 95% CI = 22 to 33 microvessels/400× field, P < .001, and urethane + 1% silibinin-treated mice, difference = 27 microvessels/400× field, 95% CI = 22 to 33 microvessels/400× field, P < .001; and urethane + 0.1% silibinin-treated mice versus urethane + 0.33% silibinin-treated mice, difference = 19 microvessels/400× field, 95% CI = 15 to 23 microvessels/400× field, P < .001, and urethane + 1% silibinin-treated mice, difference = 19 microvessels/400× field, 95% CI = 16 to 23 microvessels/400× field, P < .001). Mice that received the two highest doses of silibinin had displayed an 89% reduction in tumor microvessel density compared with mice that received urethane only. Consistent with tumor growth inhibition resulting in lower number of vascular tumors, silibinin decreased tumor microvessel density as observed by CD31 immunostaining of tumors (number of vascular tumors in 20-week study versus tumor microvessel density, r = .93, P = .024).

**Tumor Expression and Secretion of VEGF**

Tumor cells produce and secrete several angiogenic factors, including VEGF, a potent endothelial cell mitogen and downstream mediator of the angiogenic effects of iNOS (31). We found that lung tumors from mice in the urethane-only group had high expression of VEGF in most cells as displayed by strong staining intensity with an antibody to VEGF, whereas the staining of lung tumors from mice in the urethane + silibinin groups weakened as the dose of silibinin increased (Fig. 4, B). To assess the effect of silibinin on circulating angiogenic factors, we collected plasma samples from the mice at the end of the 29-week study and assayed them for the levels of modulators of angiogenesis, including VEGF and basic fibroblast growth factor (bFGF), using a mouse angiogenesis antibody array. Circulating levels of VEGF and bFGF in plasma of tumor-bearing mice in the urethane-only group were approximately three-fold higher than the circulating levels of these proteins in plasma of control mice that received neither urethane nor silibinin (data not shown). Plasma levels of both proteins in all silibinin-treated mice were similar to the levels in control urethane-treated mice (data not shown).

**Effect of Silibinin on iNOS and COX-2 Expression in Urethane-Induced Lung Tumors**

To examine whether the antiangiogenic effects of silibinin were mediated by iNOS, we next analyzed iNOS expression in urethane-induced mouse lung tumors. Lung tumors from mice in the urethane-only group displayed strong immunoreactivity scores with an antibody to iNOS (Fig. 5, A, panel a), whereas the staining of lung tumors from mice in the urethane + silibinin groups decreased as the dose of silibinin increased (Fig. 5, A, panels b–e). Silibinin decreased mean iNOS immunoreactivity scores from 2.7 in the urethane group to 1.7 in the urethane + 0.033% silibinin group (difference = 1, 95% CI = 0.3 to 1.7, P = .005), 1 in the urethane + 0.1% silibinin group (difference = 1.7,
95% CI = 1.2 to 2.3, \( P < .001 \), 0.9 in the urethane + 0.33% silibinin group (difference = 1.8, 95% CI = 1.3 to 2.3, \( P < .001 \)), and 0.8 in the urethane + 1% silibinin group (difference = 1.9, 95% CI = 1.3 to 2.5, \( P < .001 \)) (Fig. 5, A, panel f).

To further study the effects of silibinin on iNOS expression, we subjected tumor-protein lysates to Western blot analysis for iNOS. Tumor levels of iNOS decreased as the dose of silibinin increased (Fig. 5, B). Silibinin appeared to act specifically on iNOS because we observed no such decrease in the protein levels of endothelial cell-specific NOS3 (Fig. 5, B) or of NOS1 (data not shown).

Like iNOS, which stimulates angiogenesis and whose expression increases during mouse lung tumorigenesis, COX-2 also stimulates angiogenesis (32), and COX-2 protein levels are higher in urethane-induced mouse lung tumors than in lungs in naive mice (33). We therefore examined the effect of silibinin on COX-2 levels in lung tumors. In both Western blot (Fig. 5, B) and immunohistochemical (Fig. 5, C) analyses, we found that lung tumors of mice in the urethane + silibinin groups had lower levels of COX-2 than mice in the urethane-only group. Silibinin decreased mean COX-2 immunoreactivity scores from 3 in the urethane group to 2.5 in the urethane + 0.033% silibinin group (difference = 0.5, 95% CI = 0.01 to 1.1, \( P = .439 \)), 2.3 in the urethane + 0.1% silibinin group (difference = 0.7, 95% CI = 0.2 to 1.4, \( P = .219 \)), 2 in the urethane + 0.33% silibinin group (difference = 1, 95% CI = 0.4 to 1.6, \( P = .044 \)), and 1.7 in the urethane + 1% silibinin group (difference = 1.3, 95% CI = 0.6 to 2.1, \( P = .001 \)) (Fig. 5, A panel f).

The extent of the effect of silibinin on COX-2 was weaker than that on iNOS and was observed only at higher silibinin doses. We observed no silibinin-induced change in COX-1 levels (data not shown).

**Discussion**

Herein, we report that chronic oral consumption of silibinin, a naturally occurring flavanone, administered to A/J mice exposed to the carcinogen urethane inhibited the development and growth of primary lung tumors. Although the mechanisms by which silibinin interferes with lung tumor growth in preclinical models remain to be explored, these results raise the possibility that silibinin may have chemopreventive activity against lung tumor growth and progression in humans.

Long-term (i.e., 18–27 weeks) oral consumption of silibinin, even at a dose of 1.0% (wt/wt), posed no apparent adverse health effects for the mice, as monitored by body weight gain and diet consumption. This apparent lack of toxicity is consistent with findings from earlier reports (12,14). In addition, the parent flavonolignan silymarin has been in clinical use for more than three decades in Europe as a hepatoprotective agent, and no serious side effects of its use have been reported (11). Silibinin protects against hepatotoxicity, cardiotoxicity, and nephrotoxicity (11,34–36) and is sold as a dietary supplement in western countries (11). These reports, together with our present findings, substantiate the nontoxic nature of silibinin and its acceptability for long-term human consumption.
Our most clinically relevant observation was that silibinin prevented tumors from growing beyond a small size in a dose-dependent fashion. We believe that this inhibition is due, at least in part, to the inhibitory effect of silibinin on angiogenesis. From 16 to 30 weeks after urethane injection is injected into A/J mice, induced benign tumors expand in size and then begin to undergo progression to malignancy (26). We observed inhibition of the increase in tumor size by silibinin most strikingly in analyses that were conducted 29 weeks after urethane injection. Silibinin was associated with decreased cell proliferation, as detected by PCNA and cyclin D1 immunostaining, which may account for the silibinin-associated decrease in lung tumor multiplicity. Silibinin also inhibited the progression of tumors from avascular to vascular stages, as assayed by tumor size. In the 20-week study, the number of tumors smaller than 1.5 mm was similar across all silibinin-treated groups compared to those in the urethane-only group, whereas the number of tumors 1.5 mm or larger decreased in a dose-dependent fashion up to 93%. In the 29-week study, the number of small tumors (<1.5 mm size) increased concurrently with the decrease in the number of larger tumors in silibinin-treated mice.

The effect of silibinin on tumor size indicated that tumor angiogenesis is a potential biologic target that contributes to the efficacy of silibinin against lung tumorigenesis. We therefore sought to identify molecular targets that might account for this antiangiogenic effect. One potential regulatory target is iNOS, which mediates production of nitric oxide (NO), thereby enhancing tumor vascularization, growth, and blood flow (19–21). COX-2, VEGF, and bFGF are the other potential targets for antiangiogenic effect; however, these may also be regulated by iNOS. The angiogenic role of iNOS is consistent with the involvement of NO in lung carcinogenesis (20–23). For example, iNOS is highly expressed in human lung adenocarcinoma, and lung cancer patients exhale more NO than healthy individuals (37). Genetic ablation of the gene encoding iNOS inhibits urethane-induced lung tumorigenesis and decreases VEGF expression (23). iNOS regulates expression of another proangiogenic and proinflammatory enzyme, COX-2 in some systems, but genetic ablation of iNOS does not affect COX-2 expression (23). iNOS has profound proinflammatory effects, but this iNOS deficiency may not exert its antitumor effects through inhibiting the inflammatory response associated with mouse lung tumorigenesis but probably acts instead by inhibiting angiogenesis. Silibinin inhibited expression of iNOS and COX-2 but not expression of NOS1, NOS3, or COX-1. Because COX-2 stimulates
angiogenesis, COX-2 may also be a target for the inhibitory effect of silibinin on angiogenesis.

Many proteins mediate angiogenesis, and two of the most well studied are VEGF and bFGF. Circulating levels of VEGF and bFGF are statistically significantly higher in non—small—cell lung cancer patients than in healthy control subjects, and the extent of the response to chemotherapy is associated with the level of VEGF (38). NO-mediated angiogenesis is accompanied by increased expression of bFGF in coronary endothelial cells (39). Consistent with these data, we observed that silibinin inhibited expression of these two proangiogenic factors (VEGF and bFGF) in the plasma of mice bearing lung tumors. Overall, these findings suggest that silibinin may target lung tumor—associated iNOS and/or COX-2 expression to inhibit expression of proangiogenic factors and tumor angiogenesis.

Our study has several limitations. First, immunoreactivity assessments were done in an unblinded fashion. Second, our data concern an animal model of human disease, and as such, are similar but do not mirror human lung cancer. Thus, the usual data concern an animal model of human disease, and as such, and/or COX-2 expression to inhibit expression of proangiogenic factors and tumor angiogenesis.

Our study has several limitations. First, immunoreactivity assessments were done in an unblinded fashion. Second, our data concern an animal model of human disease, and as such, are similar but do not mirror human lung cancer. Thus, the usual data concern an animal model of human disease, and as such, and/or COX-2 expression to inhibit expression of proangiogenic factors and tumor angiogenesis.

References

(27) Aminz NJ, Goussia AC, Batistato A, Stefanou D. Tumor markers in lung cancer patients. An update of their prognostic signifi-
(30) Bauer AK, Dwyer-Nield LD, Malkinson AM. High cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) contents in mouse lung tumors.


NOTES

The work was supported in part by RO1 grants CA113876, CA33497, and CA96361. The study sponsor had no role in the design of the study, collection, analysis, or interpretation of the data; writing of the manuscript; or the decision to submit the manuscript for publication.

Manuscript received October 10, 2005; revised April 11, 2006; accepted April 27, 2006.