Growth Inhibition and Regression of Lung Tumors by Silibinin: Modulation of Angiogenesis by Macrophage-Associated Cytokines and Nuclear Factor-κB and Signal Transducers and Activators of Transcription 3

Alpna Tyagi,1 Rana P. Singh,1,3 Kumaraguruparan Ramasamy,1 Komal Raina,1 Elizabeth F. Redente,1 Lori D. Dwyer-Nield,1 Richard A. Radcliffe,1 Alvin M. Malkinson1,2 and Rajesh Agarwal1,2

Abstract The latency period for lung tumor progression offers a window of opportunity for therapeutic intervention. Herein, we studied the effect of oral silibinin (742 mg/kg body weight, 5 d/wk for 10 weeks) on the growth and progression of established lung adenocarcinomas in A/J mice. Silibinin strongly decreased both tumor number and tumor size, an antitumor effect that correlates with reduced antiangiogenic activity. Silibinin reduced microvessel size (50%, P < 0.01) with no change in the number of tumor microvessels and reduced (by 30%, P < 0.05) the formation of nestin-positive microvessels in tumors. Analysis of several proteins involved in new blood vessel formation showed that silibinin decreased the tumor expression of interleukin-13 (47%) and tumor necrosis factor-α (47%), and increased tissue inhibitor of metalloproteinase-1 (2-fold) and tissue inhibitor of metalloproteinase-2 (7-fold) expression, without significant changes in vascular endothelial growth factor levels. Hypoxia-inducible factor-1α expression and nuclear localization were also decreased by silibinin treatment. Cytokines secreted by tumor cells and tumor-associated macrophages regulate angiogenesis by activating nuclear factor-κB (NF-κB) and signal transducers and activators of transcription (STAT). Silibinin decreased the phosphorylation of p65NF-κB (ser276, 38%; P < 0.01) and STAT-3 (ser727, 16%; P < 0.01) in tumor cells and decreased the lung macrophage population. Angiopoietin-2 (Ang-2) and Ang-receptor tyrosine kinase (Tie-2) expression were increased by silibinin. Therapeutic efficacy of silibinin in lung tumor growth inhibition and regression by antiangiogenic mechanisms seem to be mediated by decreased tumor-associated macrophages and cytokines, inhibition of hypoxia-inducible factor-1α, NF-κB, and STAT-3 activation, and up-regulation of the angiogenic inhibitors, Ang-2 and Tie-2.

Lung cancer is the leading cause of cancer death in both men and women in the United States, with an estimated 213,380 new lung cancer cases and 160,390 associated deaths in 2007 (1). The 5-year survival rate of 14% has shown little improvement over the last 30 years, even with the development of molecularly targeted therapies such as epidermal growth factor receptor inhibitors. Tobacco exposure has been implicated in 90% of lung carcinomas; compared with never smokers, smokers have a 20-fold greater risk of developing lung cancer (2). Because smoking is the major risk factor for developing lung cancer and most smokers have small pulmonary nodules, strategies for inducing nodule regression or preventing their further growth should decrease the number of patients diagnosed with advanced malignant disease.

Efforts are being made towards identifying dietary supplements to prevent and treat lung cancer. One such agent, silibinin, inhibits the growth of various cancer cell lines and primary tumors in several chemically induced rodent models, including mouse lung (3–7). Silibinin is a flavonolignan, a major component in the silymarin complex of flavonolignans and polyphenols present in milk thistle (Silybum marianum) seeds. Silymarin has been extensively used in patients with liver disease for decades (8). Silibinin has shown strong anti-cancer efficacy against SHP-77 and A549 lung cancer cells, in which it inhibits cell growth and induces cell cycle arrest.

Lung cancer is the leading cause of cancer death in both men and women in the United States, with an estimated 213,380 new lung cancer cases and 160,390 associated deaths in 2007 (1). The 5-year survival rate of 14% has shown little improvement over the last 30 years, even with the development of molecularly targeted therapies such as epidermal growth factor receptor inhibitors. Tobacco exposure has been implicated in 90% of lung carcinomas; compared with never smokers, smokers have a 20-fold greater risk of developing lung cancer (2). Because smoking is the major risk factor for developing lung cancer and most smokers have small pulmonary nodules, strategies for inducing nodule regression or preventing their further growth should decrease the number of patients diagnosed with advanced malignant disease.

Efforts are being made towards identifying dietary supplements to prevent and treat lung cancer. One such agent, silibinin, inhibits the growth of various cancer cell lines and primary tumors in several chemically induced rodent models, including mouse lung (3–7). Silibinin is a flavonolignan, a major component in the silymarin complex of flavonolignans and polyphenols present in milk thistle (Silybum marianum) seeds. Silymarin has been extensively used in patients with liver disease for decades (8). Silibinin has shown strong anti-cancer efficacy against SHP-77 and A549 lung cancer cells, in which it inhibits cell growth and induces cell cycle arrest.

Lung cancer is the leading cause of cancer death in both men and women in the United States, with an estimated 213,380 new lung cancer cases and 160,390 associated deaths in 2007 (1). The 5-year survival rate of 14% has shown little improvement over the last 30 years, even with the development of molecularly targeted therapies such as epidermal growth factor receptor inhibitors. Tobacco exposure has been implicated in 90% of lung carcinomas; compared with never smokers, smokers have a 20-fold greater risk of developing lung cancer (2). Because smoking is the major risk factor for developing lung cancer and most smokers have small pulmonary nodules, strategies for inducing nodule regression or preventing their further growth should decrease the number of patients diagnosed with advanced malignant disease.

Efforts are being made towards identifying dietary supplements to prevent and treat lung cancer. One such agent, silibinin, inhibits the growth of various cancer cell lines and primary tumors in several chemically induced rodent models, including mouse lung (3–7). Silibinin is a flavonolignan, a major component in the silymarin complex of flavonolignans and polyphenols present in milk thistle (Silybum marianum) seeds. Silymarin has been extensively used in patients with liver disease for decades (8). Silibinin has shown strong anti-cancer efficacy against SHP-77 and A549 lung cancer cells, in which it inhibits cell growth and induces cell cycle arrest.
Angiogenesis, the formation of new blood vessels, is required early on in tumor development. It is essential for tumor expansion, progression, and metastasis (13). Angiogenesis is a complex process that involves degradation of the basement membrane and invasion of the stroma by endothelial cells, which then proliferate, migrate, and become organized into capillary structures (14). Tumor-associated macrophages (TAM) constitute an important interface between tumor cells and the immune system, and influence neoplastic growth and progression in several ways (15). Macrophage infiltration affects angiogenesis by influencing the production of angiogenic cytokines and growth factors including interleukins, tumor necrosis factor-α (TNF-α), and IFN-γ (16). Therefore, modulation of TAMs may be critical in nascent vessel formation in tumors, and could be targeted to inhibit tumor angiogenesis.

Recently, we showed that dietary silibinin markedly reduced the growth and progression of urethane-induced primary lung tumors in A/J mice. In a previous study, silibinin was fed to mice 2 weeks after carcinogen administration to model a chemopreventive treatment regimen (7). We now examine the chemotherapeutic effect of silibinin on established advanced lung tumors in this animal model. Tumors at this stage are adenocarcinomas with malignant characteristics such as invasiveness, nuclear dysmorphology, and cellular heterogeneity. Our findings are novel and significant because this chemopreventive agent, which is known to inhibit cell proliferation and induce apoptosis, did not effect these biological activities in a therapeutic setting. Instead, silibinin targeted vessel size and nascent tumor microvessel formation via its effects on TAMs, known modulators of angiogenesis.

**Materials and Methods**

**Chemicals**
Urethane (ethyl carbamate) and silibinin were purchased from Sigma. The purity (>98%) of silibinin was confirmed by high-performance liquid chromatography, as previously described (17). Mice were fed AIN-76A rodent diet pellets (Dyets, Inc.).

**Urethane-induced lung tumorigenesis experimental design**
A/J male mice (4-6 weeks of age) were purchased from the Jackson Laboratory and housed under standard laboratory conditions in the Center of Laboratory Animal Care at the University of Colorado Denver. Animal care was performed in accordance with institutional guidelines, and all animal treatments were done under an institutionally approved animal protocol. Mice (n = 25; 8 weeks of age) were given a single i.p. injection of 1 mg/g body weight of urethane in saline, as previously described (7). Urethane-induced lung tumors harbor a mutation in codon 61 of the Kras proto-oncogene (18). Thirty-two weeks after urethane injection, five mice were sacrificed and their lung tumors dissected, counted, and diameters measured with digital calipers, and pooled tumors weighed to determine total tumor burden/mouse. The remaining 20 mice were randomly divided into two groups (n = 10 mice per group) and gavaged with normal saline (control group) or silibinin (742 mg/kg body weight) for 5 d/wk for 10 weeks. This dose of silibinin was extrapolated from the dietary consumption of A/J mice exposed to 1% of silibinin (w/w) and fed AIN-76A diet as in our previous study (7). At 43 weeks, mice were sacrificed by i.p. pentobarbital injection, lung tumors harvested, and the variables mentioned above were recorded from five mice in each group. Lungs from the remaining five mice from each group were perfused, formalin-fixed, and paraffin-embedded for histologic and immunohistochemical analyses. Plasma samples from all mice were collected by cardiac puncture into heparinized tubes and stored at −80°C.

**Immunohistochemistry**
Fixed lung samples were sectioned (5 μm), deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval in citrate buffer (pH 6.0) for 30 min at 90°C. Nonspecific binding sites were blocked with serum-free blocking reagent (Dako), and antibodies against proliferating cell nuclear antigen (PCNA; 1:400, mouse monoclonal; Dako), COX-2 (1:200, goat polyclonal; Santa Cruz Biotechnology), COX-2 (1:200, goat polyclonal; Santa Cruz Biotechnology), p65NF-κB (ser276; 1:200 rabbit polyclonal; Cell Signaling), hypoxia-inducible factor-1α (HIF-1α; 1:200, mouse monoclonal; Novus), and the macrophage-specific marker, F4/80 (1:100; Caltag Laboratories) were applied overnight at 4°C followed by appropriate secondary antibodies. Proteins were visualized using 3,3′-diaminobenzidine as previously described (7). Sections were counterstained with hematoxylin. Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using Dead End Colorometric TUNEL system (Promega Corp.). The PCNA, TUNEL, p65NF-κB (ser276), and pSTAT3 (ser727)-positive cells (brown) were quantified by counting the number of stained cells compared with the total number of cells in five randomly selected tumor fields at 400× magnification. HIF-1α nuclear staining was visualized at ×1,000 magnification using an oil immersion lens. Blood vessels (CD31-positive) and newly formed vessels (nestin-positive) were quantified as the mean number of positive vessels per 400× field in five randomly selected tumor fields. CD31-positive blood vessel diameter of circle-shaped vessel cross-sections was measured using Spot Advanced software on an Olympus BX-41 microscope equipped with an Olympus U-TVX1-1 camera. The intensity of COX-2 and iNOS immunoreactivities were scored as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), or 4+ (very strong staining) as recently published (7). Because most epithelial cells express these proteins basally, the intensity of staining described increases in protein expression more clearly than did ratios of expressing cells to nonexpressing cells.

**Immunoblots analysis**
Lung tumor lysates from three to four individual mice from each group were prepared and analyzed by immunoblotting as previously described (7). Primary antibodies against PCNA, cyclin D1, vascular endothelial growth factor (VEGF), tissue inhibitor of metalloproteinase-1 (TIMP-1), and angiopoietin receptor tyrosine kinase (Tie-2; Santa Cruz Biotechnology); p65NF-κB (ser276), total p65NF-κB (ser536), pSTAT3 (ser727), total STAT3, and phosphorylated Tie-2 receptor (Ty992; Cell Signaling); HIF-1α (Novus Biologicals, Inc); nestin, angiopoietin-2 (2-2; 2630; Sigma-Aldrich); and secondary antibodies against anti-rabbit IgG (Cell Signaling) or anti-mouse IgG (GE Healthcare) were used. Equal protein loading was determined by stripping and reprobing membranes with anti-β-actin primary antibody (Sigma Aldrich). Protein bands were visualized by enhanced chemiluminescence detection.
Mouse angiogenesis antibody array

Two randomly selected lung tumor lysates from each group were applied to a Mouse Angiogenesis Antibody Array (RayBiotech, Inc.) to analyze the expression of angiogenesis-related molecules according to the manufacturer’s protocol. The expression of each protein was represented in duplicate on the membrane. Duplicate dots identifying each protein were scanned using Adobe Photoshop software and quantified by ScionImage Program. The mean intensity of these dots (arbitrary units) was determined for intergroup comparisons.

ELISA assay for IL-13

Plasma samples from the control and silibinin-fed groups were applied to a mouse IL-13 ELISA kit, following the vendor’s protocol (RayBiotech, Inc.) to quantify secreted IL-13. The assay is based on quantitative sandwich enzyme immunoassay using an antibody specific for mouse IL-13 coated onto a microplate for solid phase ELISA. Briefly, 100 µL plasma samples (collected from four to five individual mice/group) were assayed, and absorbance of the developing color was determined by microplate reader at 450 nm wavelength. IL-13 concentration was extrapolated from a recombinant mouse IL-13 standard curve.

Statistical and microscopic analyses

Statistical analyses were carried out by using SigmaStat software version 2.03 (Jandel Scientific). All statistical tests were two-sided and \( P < 0.05 \) was considered statistically significant. The differences between controls (urethane-treated group) and silibinin-fed groups were analyzed by unpaired two-tailed Student’s \( t \) test and one-way ANOVA followed by a Bonferroni \( t \) test for pairwise multiple comparisons. Densitometric analysis of immunoblots was done using the Scion Image program (NIH). Immunohistochemical staining was visualized with a Zeiss Axioscope 2 microscope (Carl Zeiss, Inc.), and photographs were captured with a Carl Zeiss AxioCam MrC5 camera at 400× or 1,000× magnification.

Results

Silibinin inhibits urethane-induced lung tumor progression in A/J mice

A/J mice given a single 1 mg/g body weight i.p. injection of urethane developed microadenomas (<0.3 mm) within 3 weeks, macroscopic adenomas (tumors <1.5 mm with uniform nuclei that aren’t invasive) within 16 weeks, and adenocarcinomas (invasive tumors with nuclear dysmorphology and cellular heterogeneity) within 30 weeks (19, 20). Metastasis is rare in this model as the mice die from oxygen deprivation before tumors progress to that stage. To determine whether silibinin could act as a chemotherapeutic agent in this model, we examined the effect of oral silibinin (742 mg/kg body weight for 5 d/wk for 10 wk and sacrificed) on mice with 32-week established lung tumors; also, a group of mice were sacrificed 32 weeks after urethane injection as a reference control (Fig. 1A). Mice receiving silibinin had 33% \( (P < 0.05) \) fewer tumors than age-matched 43-week-old controls; this control group developed slightly more tumors compared with the 32-week-old control group (Fig. 1B). From 32 to 43 weeks, a high tumor growth rate \( (P < 0.001) \) was observed. Silibinin decreased tumor burden (pooled tumor weight/mouse) by 39% \( (P < 0.05) \) compared with mice receiving vehicle (Fig. 1C). When tumors were categorized according to size, smaller tumors (<1.5 mm) progressed to a larger size \((≥1.5 \text{ mm})\) from 32 to 43 weeks \( (P < 0.05-0.001; \text{Fig. 1D}) \). Silibinin treatment significantly decreased the number of larger tumors \( (>2.5 \text{ mm}) \) by 37% \( (P < 0.01) \), and caused a 50\% \( (P < 0.01) \) decrease in the number of tumors between 1.5 and 2.5 mm in diameter compared with the 43-week-old control group (Fig. 1D). The decreased number of tumors in the silibinin group indicates regression, whereas the decreased number of larger tumors in the silibinin group indicates its growth-inhibitory effects. For molecular analysis, we used samples from the 43-week-old control and silibinin-treated groups.

Histopathologic characteristics of lung tissue and tumors

Histopathologic examination of lung tissue and tumors in A/J mice at 32 and 43 weeks after treatment with a single
urethane injection showed well-vascularized adenocarcinomas that exhibited invasiveness, nuclear dysmorphology, decreased cytoplasm/nuclear ratio, and undifferentiated cellular organization (data not shown). In the 43-week-old groups, most tumors were large (>1.5-2.5 mm in size), and the surrounding alveolar spaces contained numerous large foamy macrophages. Airways and alveolar spaces immediately adjacent to the tumors were compressed. Bronchi-associated lymphatic tissue was present adjacent to large tumors. When sections from untreated tumor-bearing mice were compared with those from mice given silibinin for 10 weeks prior to harvest, few differences besides the reduction in tumor size were evident, but large alveoli with characteristic “emphysematous” hooked alveolar walls appeared in the silibinin-treated samples. Because silibinin decreased tumor number, these large open spaces may indicate areas of tumor regression. To determine whether tumor regression left fibrotic deposits, as described in other tumor models, pentachrome stains were done on lung sections from control and silibinin-treated mice. However, no differences in staining for collagen I, mucin, ground substance, muscle, or elastic fibers were observed between the two groups (data not shown).

Effect of silibinin on lung tumor cell proliferation and apoptosis

Because we observed tumor growth inhibition by silibinin, tumors from both control and silibinin-treated groups were analyzed for PCNA and TUNEL immunohistochemical staining. No significant difference in PCNA immunoreactivity (Supplementary Fig. S1A) was observed in the silibinin-fed group (39 ± 1.97%) as compared with controls (43 ± 2.34%). To confirm our immunohistochemical results, tumor lysates from control and silibinin groups were analyzed by Western immunoblotting for PCNA and cyclin D1. Little change in PCNA and cyclin D1 expression was observed in silibinin-fed group of tumors as compared with control tumors (Supplementary Fig. S1B). TUNEL staining, done to assess the apoptotic effect of silibinin in tumors, showed a similar number of TUNEL-positive cells in both silibinin and control groups ( Supplementary Fig. S1C). Additionally, we did not observe any considerable effect of silibinin on ERK1/2 and Akt phosphorylation in lung tumors (data not shown). These results suggest that silibinin does not considerably affect cell proliferation and apoptosis in established lung tumors, although a trend toward lower proliferative and higher apoptotic rates was observed. Because small differences in tumor proliferative rates could amount to big differences in tumor size over 10 weeks, the significance of these slight changes cannot be accurately assessed.

Silibinin inhibits angiogenesis in urethane-induced lung tumors

Because significant antiproliferative and apoptotic effects of silibinin on lung tumors were not detected, we assessed the effect of silibinin on tumor angiogenesis using CD31 staining, a marker for endothelial cells (both established and nascent). Image analysis followed by quantitative counting of CD31-positive vessels did not reveal significant differences in tumor microvessel density between control and silibinin-fed groups (data not shown). However, the vessel cross-sectional area in the silibinin-fed group decreased by 50% (P < 0.01; Fig. 2A). Nestin, a stem cell marker, is expressed on newly formed microvessels in advanced tumors but is lost upon endothelial cell maturation (21). Tumors in mice gavaged with silibinin showed fewer nestin-positive microvessels (30% decrease, P < 0.05) compared with control tumors (Fig. 2B). Western immunoblotting for nestin in tumor lysates confirmed that less nestin was expressed in mice treated with silibinin (Fig. 2C). Overall, silibinin had little significant effect on tumor cell proliferation and apoptosis but may suppress tumor angiogenesis by inhibiting increased microvessel size and the formation of new microvessels.

Silibinin modulates angiogenesis-related cytokines in tumors and decreases TAMs

Mechanisms of inhibition of angiogenesis by silibinin in lung tumors were explored by performing protein array analysis for biomolecules that regulate angiogenesis in tumor lysates. Silibinin treatment decreased levels of several interleukins and cytokines including IL-1α (34%), IL-6 (44%), IL-9 (29%), IL-13 (47%), and IL-16 (44%), as well as IFN-γ (16%) and TNF-α (47%) in tumors compared with controls (Fig. 3A). Expression levels of TIMP-1 and TIMP-2 were increased 2-fold and 7-fold, respectively, by silibinin (Fig. 3A). Results for the most strongly effected proteins, IL-13, TNF-α, TIMP-1, and TIMP-2, were confirmed by Western blot analysis, which showed changes similar to those observed in the antibody arrays (Fig. 3B). Densitometry confirmed decreases of 14% and 31% in IL-13 and TNF-α, respectively, and increases of 1.5-fold and 3-fold in TIMP-1 and TIMP-2, respectively.

IL-13 plays an important role in regulating angiogenesis, and circulating IL-13 could functionally affect tumor angiogenesis. To test this, we measured levels of IL-13 in mouse plasma by ELISA, and observed that the silibinin-treated group had fewer circulating IL-13 than controls (317.8 ± 43.4 pg/mL in the control group versus 137.5 ± 10.6 pg/mL in the silibinin group; 57% decrease, P < 0.01; Fig. 3C). Silibinin treatment did not influence VEGF or Fas ligand expression either in the protein array or Western blot analyses (Fig. 3B). Because macrophages critically modulate cytokine secretion (22), and several cytokines decreased upon silibinin treatment, we used F4/80 staining of macrophages to examine whether silibinin affected the number of macrophages in tumor-bearing lungs. In sections from control mice, macrophages were more populous around tumors compared with sections from the silibinin-treated group. Quantification of these macrophages showed a 38% (P < 0.05) decrease in TAM number following silibinin feeding (Fig. 3D). Overall, these results suggest that silibinin targets TAMs to suppress the angiogenic tumor microenvironment.

Effect of silibinin on HIF-1α, iNOS, and COX-2 expression in lung tumors

HIF-1α, iNOS and COX-2 can promote tumor angiogenesis (23, 24). To examine whether the antiangiogenic effects of silibinin were mediated at least in part by HIF-1α, iNOS, and COX-2, we did an immunohistochemical analysis of these proteins in lung tumors. Lung tumors from mice in the control group displayed more cells containing HIF-1α-positive nuclei [8 ± 1.5% in the control group compared with 2 ± 0.5% (P < 0.01) in the silibinin-treated group; Fig. 4A]. The effect of silibinin on
HIF-1α expression was also analyzed by immunoblotting tumor lysates, which exhibited <25% HIF-1α protein in the silibinin-treated group of tumors (Fig. 4A). Our previous studies showed that urethane-induced mouse lung tumors usually express high levels of iNOS and COX-2 enzymes (25, 26), but no effects of silibinin on iNOS and COX-2 immunoreactivity by either immunohistochemical or immunoblot analysis were noted in these samples (data not shown). These results suggest a selective effect of silibinin on HIF-1α rather than on iNOS or COX-2 in these advanced tumors.

**Silibinin inhibits the activation of p65NF-κB in urethane-induced lung tumors**

In addition to HIF-1α, the NF-κB transcription factor also regulates the expression of many genes that control angiogenesis (27, 28). High levels of NF-κB expression have been reported in non–small cell lung cancer cell lines compared with normal bronchial epithelial cell lines (29). In order to examine the status of NF-κB activity in urethane-induced lung tumors, immunohistochemical staining for phosphorylated p65NF-κB (ser276) was done. Thirty-eight percent less nuclear immunoreactivity was observed in tumors from silibinin-treated mice (Fig. 4B). When phosphorylated p65NF-κB (ser276 and ser536) levels were examined by Western blot in tumors lysates, the silibinin group had lower expression of both p65 (ser276, 15%) and p65 (ser536, 40%) than controls, with no effect on total p65 in tumors (Fig. 4B). These results indicate that silibinin inhibits the activity of the angiogenic cytokine/NF-κB loop in urethane-induced lung tumors.

**Silibinin inhibits the activation of STAT3 in urethane-induced lung tumors**

Because we observed a down-regulation of cytokine expression by silibinin treatment in urethane-induced tumors, inhibited STAT signaling was anticipated. STAT3 was originally discovered as a mediator of cytokine signaling pathways that play an active role in oncogenesis, inflammation, and angiogenesis (30). Accordingly, we analyzed STAT3 activity in lung tumors by assessing phosphorylated STAT3 (ser727) by immunohistochemistry. Fifty-eight percent of the tumor cells had...
nuclear staining of phosphorylated STAT3 in the silibinin-treated group as compared with 70% (P < 0.01) in the control group (Fig. 4C). Western immunoblot analysis of phosphorylated STAT3 (ser727) and total STAT3 in tumor lysates showed that, consistent with the immunohistochemical data, control tumor samples had more phosphorylated STAT3 (ser727) than the silibinin-treated group with no change in total STAT3 protein levels (Fig. 4C). These results revealed inhibition by silibinin of STAT3 (ser727) phosphorylation reduced its nuclear translocation and, thus, its transcriptional activity in lung tumors.

Silibinin enhances Ang-2 and Tie-2 levels in urethane-induced lung tumors

Ang-2 is the ligand for the Tie-2 (tyrosine kinase) receptor in endothelial as well as immune cells. Ang-2 can act as both an antagonist and agonist after binding to the Tie-2 receptor (31). Transgenic overexpression of Ang-2 impairs angiogenesis as well as vasculogenesis (31). In the present study, lung tumors from silibinin-treated mice expressed higher levels of Ang-2 (1.5-fold increase, P < 0.05) protein, compared with control tumors (Fig. 5). We did not observe any considerable change in phosphorylated Tie-2 (tyr992) levels, adjusted with total Tie-2 levels following silibinin treatment (Fig. 5). Consistent with immunoblot data, we also observed increased Tie-2 protein in lung tumor lysates in silibinin-treated mice using a Kinexus assay, a global protein kinase antibody–based assay system (data not shown). Concurrently, we observed enhanced Ang-2 expression (1.6-fold increase, P < 0.01) in lung tumors from silibinin-treated mice as compared with the controls (Fig. 5). These results suggest that silibinin increased Ang-2 and Tie-2 levels as well as enhancing Ang-2–activated Tie-2 receptor signaling, which may in part be responsible for destabilizing and regressing tumor vasculature.

Discussion

The focus of the present study was to assess the chemotherapeutic potential and related mechanisms of oral silibinin on urethane-induced advanced lung tumors in A/J mice, a model of human adenocarcinoma. Previously, we showed that silibinin was a potent chemopreventive agent in preventing tumor formation and adenoma to adenocarcinoma prevention in this model (7). Our novel findings are that silibinin reduced tumor number, tumor burden, and progression of adenocarcinomas without adverse effects on mice. Tumor angiogenesis, as opposed to tumor cell proliferation or survival was targeted by silibinin. Silibinin did not exert any significant effect on tumor microvessel density, but rather inhibited increases in vessel size and inhibited new microvessel growth. This antiangiogenic effect of silibinin was accompanied by a decrease in the number of TAMs as well as reduced levels of cytokines.
known to promote inflammation and angiogenesis (Fig. 6). Enzymes important for inhibiting metalloproteinases such as TIMP-1 and TIMP-2 were up-regulated by silibinin treatment of mice bearing advanced lung tumors. Activation of the transcription factors HIF-1α, NF-κB, and STAT3 was also inhibited by silibinin. Finally, a higher content of Ang-2 and Tie-2 proteins were observed; sustained levels of these proteins impair microvessel development (31).

In this study, A/J mice were given urethane, and after 33 weeks, treated with oral silibinin for 10 weeks. Lung tumors at this stage were mainly adenocarcinomas, i.e., invasive tumors with nuclear dysmorphology and cellular heterogeneity. Silibinin feeding for 10 weeks reduced lung adenocarcinoma multiplicity as well as tumor burden, and this was accompanied by significant antiangiogenic effects. In a recently completed chemoprevention study in this urethane-induced lung tumorigenesis model, we observed that in early lung lesions, silibinin inhibits tumor growth and progression by strongly inhibiting tumor microvessel density and tumor cell proliferation (7). However, in the present therapeutic experimental design, in which tumors were adenocarcinomas with established tumor vasculature prior to silibinin treatment,

![Image]

**Fig. 4.** Silibinin inhibits the activation of HIF-1α, p65NF-κB, and STAT3 in urethane-induced lung tumor cell nuclei. Tumor-bearing lungs harvested from A/J mice after 43 wk of urethane (n = 5 mice/group) and analyzed by using immunohistochemistry for HIF-1α (A), p65NF-κB (ser276; B), and pSTAT3 (ser727; C), as detailed in Materials and Methods (left). HIF-1α, p65NF-κB (ser276), and pSTAT3 (ser727) immunoreactivities in tumors were quantified by counting (brown) positive cells in five randomly selected fields at ×400 magnification from each of five different samples in both groups. The percentage of positive cells was determined as the number of positive-stained cells × 100 / total number of cells counted. Columns, mean; bars, SE (right). Final magnification, ×1,000 (HIF-1α) and ×400 (phosphorylated p65NF-κB and phosphorylated STAT3; A–C, right), three lung tumor samples were randomly taken from each group and analyzed for HIF-1α, p65NF-κB (ser276), p65NF-κB (ser536), pSTAT3 (ser727), and STAT3 protein levels by immunoblotting total cell lysates. Bands were visualized by enhanced chemiluminescence detection. Membranes were stripped and reprobed with β-actin as loading control. SB, silibinin.
silibinin suppressed the growth of existing tumor vasculature as well as microvessel size. Slight but not significant differences were seen in tumor cell proliferation and apoptosis. We also did not observe any considerable effect of silibinin on ERK1/2 and Akt phosphorylation. It seems that at later stages of lung tumor progression, silibinin has no effect on mitogenic and survival pathways. Increased cellular heterogeneity and further mutations in key growth and cell death regulatory pathway components may render cells in these advanced tumors resistant to the effects of silibinin seen in our previous chemoprevention study. These observations suggest that silibinin can target tumors at different stages of progression through its antiangiogenic effects.

Another novel finding is that silibinin inhibited the infiltration of macrophages into tumor-bearing lungs; macrophages were sparse around the tumors from silibinin-treated mice as compared with those in untreated controls. This observation suggested that silibinin may target TAMs as part of its mechanism in blocking angiogenesis. Numerous studies showed macrophage involvement in promoting tumor angiogenesis (15). Other agents can also influence these macrophages to down-modulate angiogenesis, such as linoamide- and thiol-containing compounds (32, 33). Activated macrophages create a proangiogenic microenvironment by secreting high levels of cytokines, but tumor cells at advanced stages of neoplasia also express high levels of angiogenic cytokines, possibly in response to TAMs (16, 34). Silibinin treatment decreased tumor expression of many inflammatory and angiogenic cytokines, including IL-1α, IL-6, IL-9, IL-13, IL-16, IFN-γ, and TNF-α, which may represent a combined effect of silibinin on macrophages as well as tumor cells. Metalloprotease activity is required for paving a path for growing microvessels, which can be regulated by TIMPs (35). Silibinin treatment increased the expression of inhibitors of metalloproteases, TIMP-1 and TIMP-2, providing support for another mechanistic explanation of the inhibition of tumor microvessel growth and size by silibinin.

Angiogenesis is a highly orchestrated process involving the sprouting of new capillary-like structures from the existing vasculature that mature into new blood vessels (36). It can be triggered and modified by many factors, including cytokines, growth factors and their receptors, chemokines, and extracellular matrix macromolecules (36). These factors regulate many transcription factors, including HIF-1α, NF-κB, and STAT (23, 24). Hypoxia within tumors stimulates the production of HIF-1α, among other proangiogenic molecules. HIF-1α levels are low in normal cells, but reach high intracellular concentrations in many cancers, and these concentrations strongly correlate with poor prognosis and resistance to therapy (37). Silibinin significantly decreased the number of HIF-1α-positive nuclei, indicating the inhibition of its transcriptional activity. Therefore, it is likely that down-regulation of HIF-1α, in part, plays a role in the antiangiogenic effect of silibinin on advanced lung tumors.

Recently, activated macrophages have been linked to NF-κB activation in urethane-induced lung carcinogenesis (34). To our knowledge, our finding of inhibition of STAT3 activation is the first report showing STAT3 signaling in urethane-induced lung tumors in mice. Pulmonary macrophages can produce various cytokines, such as TNF-α, interleukins, and IFN-γ (as observed in the present study), which in turn activate NF-κB and STAT3 signaling in tumors. As anticipated, we

Fig. 5. Silibinin enhances Ang-2 and Tie-2 levels in urethane-induced lung tumors. Three lung tumor samples were randomly taken from each group and analyzed for pTie-2(yl992), Tie-2, and Ang-2 protein levels by immunoblotting. Bands were visualized by enhanced chemiluminescence detection. Membranes were stripped and reprobed with β-actin as a loading control. Densitometric analysis of band intensity for each protein was adjusted with β-actin. Columns, mean intensity of three bands; bars, SE. SB, silibinin.
observed the activation of both NF-κB and STAT3 in tumors, as indicated by increased levels of their phosphorylated forms, i.e., p65 NF-κB (ser276 and ser536) and pSTAT3 (ser727). Silibinin treatment inhibited macrophage infiltration in tumor-bearing lungs and inhibited the activation of both NF-κB and STAT3 in tumors, which correlates with the diminished size of microvessels as well as a decrease in newly formed, nestin-positive microvessels.

We were surprised to note that even after inhibition of HIF-1α, NF-κB, and STAT3 signaling by silibinin in tumors, we observed no effect on expression of VEGF, iNOS, and COX-2, potential targets of these transcription factors that usually play important roles in tumor angiogenesis. This is relevant because in our previous chemoprevention study, silibinin decreased the expression of all three proteins (VEGF, iNOS, and COX-2) as well as angiogenesis in developing lung tumors (7). This suggests that lung tumor progression in this animal model recruits different angiogenic mediators in early stages of tumor development (microadenoma to adenoma) than late adenocarcinoma stages. Furthermore, we observed that silibinin enhances the expression of Ang-2 and Tie-2 receptor tyrosine kinase factors which regulate vessel stabilization and angiogenesis (31). Ang-1, the ligand for the Tie-2 receptor, promotes angiogenesis and recruits pericytes to stabilize vessels (38). However, Ang-2 is a conditional antagonist and agonist for Tie-2 receptor, whose systemic overexpression leads to tumor vessel regression without concomitant inhibition of VEGF (31). Similar results were observed with silibinin treatment; we saw no change in VEGF levels but observed more Ang-2 and Tie-2 levels. This could be a potential antiangiogenic mechanism of silibinin on established lung adenocarcinoma.

In summary, oral silibinin showed antitumor effects in urethane-induced and established lung adenocarcinomas, most likely by decreasing microvessel size and inhibiting newly formed microvessel growth in tumors. The decrease in TAM infiltration into lungs as well as the lower levels of angiogenic cytokines, and greater TIMP-1 and TIMP-2 concentrations, along with the inhibition of HIF-1α, NF-κB, and STAT3 activation, could account for the antiangiogenic effects of silibinin. Additionally, elevating levels of Ang-2 and Tie-2 without changing VEGF amounts could have led to microvessel regression in tumors by silibinin. Overall, our findings here, together with our earlier studies (7), suggest that silibinin is a promising agent for intervention in human lung cancer oncogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


Growth Inhibition and Regression of Lung Tumors by Silibinin: Modulation of Angiogenesis by Macrophage-Associated Cytokines and Nuclear Factor-κB and Signal Transducers and Activators of Transcription 3

Alpna Tyagi, Rana P. Singh, Kumaraguruparan Ramasamy, et al.