Silibinin Prevents Lung Tumorigenesis in Wild-Type but not in iNOS–/– Mice: Potential of Real-Time Micro-CT in Lung Cancer Chemoprevention Studies

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Abstract

**Purpose:** Sustained nitric oxide (NO) generation positively correlates with lung cancer development and progression. Herein, we genetically confirmed this role of iNOS and evaluated the chemopreventive efficacy of silibinin in carcinogen-treated B6/129 wild-type (WT) and iNOS–/– mice.

**Experimental Design:** Male B6/129-Nos2<sup>tm1Lau</sup> (iNOS–/–) and B6/129PF2 WT mice were injected i.p. with 1 mg/g body weight urethane once weekly for 7 consecutive weeks, followed by silibinin gavage (742 mg/kg body weight) for 5 d/wk for 18 weeks.

**Results:** Quantification of micro-CT data in real-time showed that silibinin significantly decreases urethane-induced tumor number and size in WT mice, consistent with measurements made ex vivo at study termination. Genetic ablation of iNOS decreased urethane-induced tumor multiplicity by 87% (P < 0.001) compared to WT mice. Silibinin decreased tumor multiplicity by 71% (P < 0.01) in WT mice, but did not show any such considerable effect in iNOS–/– mice. Tumors from WT mice expressed more iNOS (P < 0.01) but almost similar eNOS and nNOS than those in silibinin-treated mice. In these tumors, silibinin moderately (P < 0.01) inhibited cell proliferation but strongly (P < 0.01) reduced the number of newly formed nestin-positive microvessels. Silibinin decreased VEGFR2 level, and STAT3 and NF-κB activation in tumors.

**Conclusions:** The lack of effect of silibinin in iNOS–/– mice suggests that silibinin exerts most of its chemopreventive and angiopreventive effects through its inhibition of iNOS expression in lung tumors. Our results support iNOS as a potential target for controlling lung cancer, and demonstrate the value of real-time noninvasive micro-CT imaging modality for evaluating the efficacy of lung cancer chemopreventive agents.

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Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States. Lung cancer patients are usually diagnosed at a late stage when prognosis for most patients remains poor, with a 5-year survival rate of less than 16% (1). There has been little improvement in the efficacy of lung cancer treatments in recent decades, indicating the need for alternative strategies to help control this disease (2, 3). One way to reduce the risk of this disease is by chemoprevention using phytochemicals administration to high-risk populations. A deeper molecular understanding of the events regulating tumor progression in model organisms should enhance development of novel chemopreventive and therapeutic approaches to this problem.

The flavolignan, silibinin, is a major constituent in silymarin, an extract of milk thistle (Silybum marianum). Recently, silibinin received significant attention for its strong chemopreventive and anticancer efficacy. Silibinin inhibited growth in cancer models of skin (4, 5), prostate (6–9), bladder (10), and colon (11). Silibinin inhibits multiple cytokine-induced signaling pathways that regulate inducible nitric oxide synthase (iNOS) expression in A549 cells, and inhibited the in vivo growth of A549 xenografts, and reduced the systemic toxicity of doxorubicin in these studies (12, 13). Dietary silibinin decreased cell proliferation and angiogenesis by targeting iNOS in a urethane-induced lung tumorigenesis model (14). It has also shown chemotherapeutic efficacy in urethane-induced, established lung adenocarcinomas where it also inhibited angiogenesis (15). Silibinin also reduced proliferation of human non-small cell lung carcinoma (NSCLC) H1299, H460, and H322 cells by targeting cell cycle associated proteins (16).

Silibinin antiangiogenic efficacy may be mediated through nitric oxide (NO) signaling, which plays a major
role in tumor angiogenesis. NO is a free radical that regulates diverse physiologic and pathologic events. Sustained and/or excess NO generation occurs during lung cancer development and progression (17). NO is synthesized by three major isoforms of NO synthase (NOS), constitutively expressed endothelial (eNOS), neuronal (nNOS) NOS and inducible NOS (iNOS). Of these isoforms, iNOS produces the most NO (18). More iNOS is expressed in lung tumors than in surrounding normal tissue, and iNOS levels are high in alveolar and tumor-associated macrophages, pulmonary endothelium, airway epithelium, and human AC (19). iNOS expression/activity correlates with angiogenic status and metastatic potential in a wide range of tumors, and iNOS inhibition by natural and synthetic compounds has been efficacious in cancer chemoprevention studies (20, 21). Previously we hypothesized that dietary silibinin mediates its antiangiogenic effects by targeting iNOS in urethane-induced A/J mouse lung tumorigenesis (14).

Herein, we investigate the chemopreventive efficacy of silibinin on urethane-induced lung tumorigenesis in terms of tumor growth and progression in B6/129 wild-type (WT) mice and their iNOS<sup>−/−</sup> counterparts. We hypothesized that if lung cancer development is regulated by NO production, silibinin should, at least in part, exert its chemopreventive effects through iNOS inhibition. The findings in this study supported this hypothesis, and also highlighted, for the first time, the usefulness of microcomputed tomography (micro-CT), a noninvasive imaging technique, to monitor real-time progression of lung tumors in evaluating the efficacy of silibinin.

**Translational Relevance**

Overexpression of inducible nitric oxide synthase (iNOS) and sustained nitric oxide (NO) generation positively correlate with lung cancer development and progression. This study genetically confirmed this role of iNOS using urethane-induced lung tumorigenesis in B6/129-Nos2<sup>tm1Lau</sup> (iNOS<sup>−/−</sup>) and B6/129PF2 WT mice. This study also showed that silibinin, a cancer chemopreventive agent, decreases lung tumor multiplicity by 71% (P < 0.01) in WT mice, but failed to exert such efficacy in iNOS<sup>−/−</sup> mice, suggesting iNOS as a potential chemopreventive target during lung carcinogenesis. The findings also showed that silibinin exerts its chemopreventive and angiopreventive effects mostly through its inhibition of iNOS expression involving downregulation of STAT3 and NF-κB signaling, which otherwise are up-regulated in lung tumors. Overall, these results support iNOS as a potential target for controlling lung cancer by silibinin, and demonstrate the application of real-time noninvasive micro-CT imaging for evaluation of the efficacy of lung cancer chemopreventive agents.

**Materials and Methods**

### Animals

Male B6/129-Nos2<sup>tm1Lau</sup> (iNOS<sup>−/−</sup>) and B6/129PF2 WT mice (5–6 weeks of age), from Jackson Laboratory were injected i.p. with 1 mg/g body weight urethane once weekly for 7 consecutive weeks (22). Eight weeks after the initial urethane injection, mice were randomly divided into two groups and gavaged with either 0.2 mL vehicle [0.5% (w/v) carboxy methyl cellulose and 0.025% Tween 20 in distilled water] or silibinin [742 mg/kg body weight] in vehicle for 5 d/wk for 18 weeks. This dose of silibinin corresponds to 1% (w/w) of silibinin in diet (14) and has been used in our previous study (15). Animal care and experiments were conducted in accordance with an IACUC-approved protocol at University of Colorado Denver. Twenty-five weeks after the first urethane injection, mice were euthanized, tumors enumerated, and tumor diameters measured using digital calipers. Lungs from 4 mice/group were perfused, formalin-fixed, and paraffin-embedded for histologic and immunohistochemical analyses.

### Noninvasive micro-CT imaging

After the final urethane injection, a subset (n = 6) of mice from control and silibinin-treated groups underwent 4 monthly CT scans (at 0, 4, 8, and 12 weeks of silibinin treatment) for longitudinal assessment of number and diameter of lung lesions. Mice were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine mixture (i.p.), placed on a warming pad, and inserted into a Siemens Inveon micro-CT scanner (Siemens Preclinical Solutions). A single 3-dimensional (3D) micro-CT image set was acquired for each mouse using following parameters: 270° rotation; 240 rotation steps; CCD read-out of 2,304/2,048; 4 binnings for matrix size reduction; exposure time of 30 ms with 80 kV voltage and 450 μA current; with a field of view (FOV) of 34.68 × 30.83 mm. The 6-minute acquisition with middle-to-high magnification resulted in effective isotropic resolution of 60 μm (after Shepp-Logan reconstruction algorithm). Animals were monitored during recovery from the anesthesia and returned to their cages. CT image reading was performed by an image specialist (NJS) who was blinded to animal group assignments. All postprocessing analysis was performed using a high-speed CT reconstruction system (COBRA, Siemens Preclinical Solutions) and AsiPro image software for total lesion counts. Maximal diameters of each lesion were also determined for each anatomical direction (transaxial, coronal, and sagittal) and mean diameter determined. Motion artifacts were reduced by restraining anesthetized mice.

### Immunohistochemical analysis and quantification of protein expression

Serial tissue sections (5 μm) were sliced from paraffin-embedded formalin-fixed lungs and immunohistochemical staining performed as described previously (15). Briefly, tissue sections were deparaffinized, hydrated, and
stained using specific primary antibodies, biotin-conjugated secondary antibodies, and horse radish peroxidase (HRP)-conjugated avidin. Specific antibody interactions were detected with the HRP substrate 3, 3′-diaminobenzidine (DAB). Primary antibodies used were mouse monoclonal anti-PCNA (1:250, Dako), nestin (1:100 dilution; Abcam) rabbit polyclonal anti-eNOS (1:100 dilution; Abcam) nNOS (1:100 dilution; Abcam), iNOS (1:100 dilution; Abcam), pSTAT3 (Ser727; 1:200 dilution; Abcam), and p65NF-kB (Ser276; 1:100 dilution; Abcam) followed by appropriate secondary antibodies (Vector Laboratories). PCNA, pSTAT3, and p65/NF-kB-positive cells were quantified by counting the number of brown-stained nuclei/total number of cells in 5 randomly selected 400× magnified fields. eNOS, nNOS, and iNOS were quantified by immunoreactivity (represented by intensity of brown staining) and scored as 0 (no staining), +1 (very weak), +2 (weak), +3 (moderate), and +4 (strong) at 5 randomly selected 400× magnified fields. Newly formed nestin-positive microvessels were quantified as mean number of positive vessels in 5 randomly selected 400× magnified fields per tumor. All microscopic, histologic, and IHC analyses were performed with a Zeiss Axioskop 2 microscope (Carl Zeiss, Inc.), and photomicrographs were captured with a Carl Zeiss AxioCam MRC5 camera.

**Immunoblot analysis**

Randomly selected frozen tumor samples from four individual mice from WT urethane and silibinin-treated groups were homogenized and lysates prepared as reported previously (15). Equal protein per lysate was denatured with 2× sample buffer and resolved on Tris-glycine gels, transferred onto nitrocellulose membrane, and blocked for 1 hour with 5% nonfat dry milk. Membranes were incubated with specific primary antibodies including anti-iNOS (BD BioSciences), anti-nestin and anti-VEGFR2 (Santa Cruz Biotechnology), followed by peroxidase-conjugated appropriate secondary antibody. These antibodies for immunoblot analyses provide better results. Finally, proteins were visualized by enhanced chemiluminescence detection. To confirm equal protein loading, membranes were stripped and reprobed with mouse monoclonal anti-β-actin primary antibody (Sigma) in each case.

**Statistical analysis**

Statistical analyses were performed using SigmaStat software version 3.5 (Jandel Scientific). Quantitative data are presented as mean ± SEM. Statistical significance of differences between control and silibinin-treated groups was determined by an unpaired Student's t-test, with P < 0.05 considered statistically significant.

**Results**

**Imaging analysis of lung tumors**

Imaging technology has been used to monitor lung tumor progression and evaluate the efficacy of preventive/therapeutic approaches. Micro-CT provides 3D representations of high-density tissue structures needed to monitor therapeutic effects over time. This optimizes treatment intervals and reduces the number of animals needed to get reliable results (23). Micro-CT is a preferred imaging modality of choice for lung lesions. Magnetic resonance imaging (MRI) exhibits low-signal intensity and susceptibility artifacts in lung (24). We monitored growth inhibitory effects of silibinin on urethane-induced lung tumorigenesis by subjecting control and silibinin-treated, tumor-bearing WT mice to micro-CT scanning at 4-week intervals. Micro-CT images clearly distinguished lung tumors from surrounding tissue even without any contrast agent, and the reconstructed 3D pulmonary images easily differentiated tumors from blood vessels (Fig. 1A). WT animals developed an average of 5, 7, and 9 tumors at 4, 8, and 12 weeks, respectively, whereas silibinin-treated animals developed only 2, 3, and 3 tumors, respectively, at the same time points (Fig. 1B). Average tumor diameter increased from 0.5 to 2 mm in control animals gradually over 12 weeks. Silibinin treatment significantly reduced tumor diameter by 43% (P < 0.02) and 72% (P < 0.005) at the 8th and 12th weeks compared to corresponding controls, respectively (Fig. 1C). These results directly correlate with our ex vivo tumor data measured at the end of the experiment (Fig. 2B), and confirm the applicability of micro-CT imaging as a noninvasive and quantitative, real-time monitoring tool of lung tumor progression. The calculation of lung lesions for each mouse in each CT session was performed in triplicate by an imaging scientist who was ignorant to the group assignment and histologic findings. The interday variability for CT reads on number of lesions was 8% and on lesion diameters 14%.

**Silibinin inhibits urethane-induced lung tumorigenesis in WT but not iNOS−/− mice**

Tumors were extracted from lungs, and tumor number and diameters compared between WT and iNOS−/− mice. WT mice developed an average of 15 lung tumors/mouse, whereas iNOS−/− animals developed only 2 lung tumors/mouse, an 87% (P < 0.001) reduction in tumor multiplicity (Fig. 2A) in agreement with our earlier studies (25). Silibinin treatment significantly reduced lung tumor multiplicity by 71% (P < 0.01) in WT mice, but there was no statistically significant change in tumor number in control versus silibinin-treated iNOS−/− mice (Fig. 2A). WT mice developed lung tumors with diameters varying from 0.5 to 4.5 mm, whereas all tumors from iNOS−/− mice were <1 mm in diameter, an 82% reduction. Silibinin retarded tumor progression of the <1 mm lesions by 67% (P < 0.01) and of the 1.0 to 1.5 mm lesions by 62%, and completely suppressed the progression to 1.5 to 2.5 mm (P < 0.05) and >2.5 mm diameter lesions in WT mice. However, silibinin did not further reduce tumor size or number in iNOS−/− mice (Fig. 2B). Because of small size and number of iNOS−/− tumors, we were unable to use them for molecular analysis.
Histopathologic characteristics of lung tissue and tumors

Histopathologic examination of lung tissue and tumors in B6/129 mice 16 weeks after the last of 7 weekly urethane injections yielded adenomas that exhibited uniform cellular organization, mild nuclear dysmorphology, and a normal nuclear/cytoplasmic ratio. Airways and alveolar spaces immediately adjacent to the tumors were compressed slightly, and the tumors were surrounded by a small number of macrophages (data not shown). There were no significant differences in histopathology between lesions from silibinin-fed and control groups.
Silibinin selectively reduces the level of iNOS expression in urethane-induced lung tumors

Because in iNOS−/− mice silibinin did not show any considerable effect on urethane-induced lung tumorigenesis, we anticipated that antitumor effect of silibinin could be mostly mediated via iNOS in WT mice. Therefore, we analyzed the levels of all the three forms of NOS in tumors. Immunohistochemical examination of iNOS, eNOS, and nNOS levels in control and silibinin-treated tumor-bearing WT mice showed that silibinin decreased iNOS levels in tumors by 57% \( (P < 0.001) \), which was also confirmed by immunoblot analysis (Fig. 3A). Silibinin did not show any considerable effects on eNOS and nNOS expression levels (Fig. 3B and C). These results provided evidence that silibinin targets iNOS for its antitumor activity in urethane-induced lung tumorigenesis of mouse model.

Silibinin inhibits cell proliferation and neoangiogenesis in urethane-induced lung tumors

Because we observed silibinin-mediated decreases in number and sizes of lung tumors, we next analysed whether silibinin affected cell proliferation and angiogenesis in these tumors. Silibinin decreased expression of the proliferative marker, PCNA, by 18% \( (P < 0.01) \) compared to controls (Fig. 4A). Microscopic examination of nestin, an angiogenic marker for newly formed microvessels, showed numerous nestin-positive microvessels and increased immunoreactivity in tumors of control mice. Silibinin decreased the number of nestin-positive tumor vessels by 61% \( (P < 0.01) \), which was also confirmed by immunoblot analysis (Fig. 4B). We also observed a significant decrease (55%; \( P < 0.01 \)) in the intensity of nestin immunostaining in silibinin-treated versus control tumor-bearing WT mice (data not shown).
The growth of new blood vessels in tumors depends on signaling pathways regulated by vascular endothelial growth factor (VEGF) and its major receptor, VEGFR2 (26). VEGFR2 expression analysis by immunoblotting revealed a significant decrease ($P < 0.01$) in the silibinin-treated group compared to controls (Fig. 4C). These results suggest that silibinin-caused downregulation of VEGFR2 could mediate its anti-neoangiogenesis in lung tumors.

Silibinin inhibits STAT3 and NF-κB activation in urethane-induced lung tumors

The transcription factors, STAT3 and NF-κB, play vital roles in tumor angiogenesis, and the promoter region of iNOS has binding sites for both STAT3 and NF-κB (27). We determined STAT3 and NF-κB activities by examining pSTAT3 (Ser727) and p65NF-κB (Ser276) expression and localization in lung tumors by IHC. Silibinin treatment significantly decreased nuclear pSTAT3 (Ser727)-positive cells by 38% ($P < 0.001$) compared to the control group of tumors (Fig. 5A). Similarly, silibinin also decreased nuclear p65NF-κB (Ser276) positive cells by 31% ($P < 0.001$) as compared to the control group of tumors (Fig. 5B). These results indicate the inhibitory effect of silibinin on STAT3 and NF-κB signaling in lung tumors, which could have mediated its chemopreventive and angiopreventive effects most likely through iNOS regulation.

Discussion

The focus of this study was to assess the chemopreventive potential of oral silibinin on multiple injections of urethane-induced lung tumors in B6/129 wild type and iNOS$^{-/-}$ mice. Previous studies from our laboratory showed that silibinin inhibits tumor formation and the growth of advanced lung tumors in the A/J mouse model (14, 15). We previously observed that silibinin inhibited iNOS and COX-2 expression, the two enzymes which promote lung tumor growth and progression in this model (14). Herein, we demonstrated the usefulness of micro-CT for evaluating the chemopreventive efficacy of silibinin on urethane-induced lung tumorigenesis. Micro-CT images revealed that the number of tumors increased over time after carcinogen exposure, and that silibinin treatment inhibited this growth. We determined that the total number of tumors detected by CT (extrapolated for 18 weeks) was slightly lower than that detected by macroscopic study at 18 weeks, suggesting that micro-CT could not detect all tumors in this study. This may be explained by the differences in spatial resolution of a macroscopic imaging technique in the living animal versus microscopic histologic examination of tissues. However, the trends correlated well and further improvement of the micro-CT imaging protocol is under development. Our results also support recent findings that micro-CT can detect lung cancer lesions as small as 1-mm diameter with high resolution (28–31). Haines et al. (30) longitudinally demonstrated that micro-CT could be used to study lung cancer progression and treatment response to erlotinib in mice genetically engineered to produce mutant KRAS driven lung tumors. Fushiki et al. (29) described a strong correlation between bioluminescence and micro-CT imaging in early lung lesion detection and quantitatively assessed cancer response to cisplatin and gemcitabine therapy in an orthotopic model. Wang and colleagues (31) showed that both MRI and micro-CT can differentiate squamous cell carcinoma from adenocarcinoma induced by N-nitroso-trischloroethylene. We
quantitatively assessed silibinin efficacy in carcinogen-treated, genetically modified mice and demonstrated a strong correlation between histopathologic endpoints and real-time quantitative micro-CT.

To determine whether silibinin chemopreventive activity was working through iNOS, mice lacking the iNOS gene were given a multiple urethane carcinogenesis regimen. We have previously determined that this regimen induces a high multiplicity and incidence of lung tumors in the moderately resistant B6/129 strain (25). This study demonstrates that silibinin is an effective chemopreventive agent for lung tumor formation in strains other than A/J injected once with urethane as compared with a more aggressive carcinogenesis protocol employing multiple injections of urethane. Eighteen weeks of silibinin administration reduced lung tumor multiplicity as well as size, and this was accompanied by significant antiangiogenic effects. B6 resistance alleles present in these chimeric mice did not affect silibinin efficacy, suggesting that silibinin exerts its chemopreventive effects mostly through inhibiting iNOS expression in lung tumors.
To further explore the mechanisms of lung cancer chemoprevention by silibinin, we evaluated the effect of silibinin on cancer cell proliferation. Cell proliferation was assessed by PCNA expression, a cofactor of DNA polymerase required for DNA replication and nucleotide excision repair (32). We observed that lung tumors from silibinin-treated mice had significantly lower PCNA expression than their urethane-treated controls, suggesting that at least part of silibinin’s mechanism of chemoprevention is through inhibition of tumor cell proliferation.

Neangiogenesis facilitates tumor growth, progression, and metastasis. Newly formed microvessels were observed in urethane-induced lung tumors in WT mice, and silibinin treatment significantly decreased the number of these vessels. Because VEGF and its receptors are highly expressed in many tumors (33, 34), iNOS expression is associated with increased VEGF expression, and VEGF expression is decreased in the lung tumors of iNOS-deficient mice (25), we analyzed the expression of angiogenic factors in tumors from control and silibinin-treated mice. VEGFR2 expression level and nestin immunoreactivity were decreased in silibinin-treated mice, implying that angiopreventive mechanisms of silibinin are related to iNOS-dependent suppression of vascular growth in lung tumors.

Next we evaluated the activation state of STAT3 and NF-κB, transcription factors which play major roles in tumorigenesis of a wide variety of cancers including lung cancer (35, 36). STAT3 nuclear translocation and activation is known to increase expression of genes, which influence cell cycle progression (such as cyclin D1) and angiogenesis (such as VEGF) (35). Other studies have demonstrated the importance of NF-κB signaling in chemically induced and genetic models of lung cancer (37, 38). In this study, silibinin strongly inhibited the activation of STAT3 and NF-κB in multiple urethane-induced lung tumors of WT mice. This is further supported by our earlier observations where silibinin inhibited macrophage infiltration as well as the activation of STAT3 and NF-κB in tumors from urethane-treated A/J mice, and has also inhibited doxorubicin-induced NF-κB activation in A549 cells (12, 15).

Nitric oxide production by iNOS is involved in the pathogenesis of many cancers. When we analyzed iNOS expression by immunohistochemistry and immunoblotting in WT mice, we found that silibinin inhibited iNOS expression without affecting that of eNOS and nNOS. High iNOS levels are shown in lung large cell carcinoma and lung adenocarcinoma but not in squamous cell carcinoma of human non–small cell lung cancer (39). iNOS/NO levels are higher in lung tumors from smokers than non-smokers, and in human lung tumors compared to surrounding tissue (17, 40). Inhibition of iNOS activity reduces aberrant crypt foci formation and colon tumor formation (41). Silibinin is shown to inhibit iNOS expression and cytokine-induced activation of STAT and NF-κB in non–small cell lung cancer A549 cells (12). Because the iNOS gene promoter region has binding sites for STAT3 and NF-κB, the inhibition of the activation of these transcription factors by silibinin may account for the observed decrease in iNOS expression (27).

In summary, we demonstrate the chemopreventive efficacy of silibinin in the B6/129 WT lung tumorigenesis model. The key findings are: (a) iNOS+/− mice developed few tumors, indicating the importance of iNOS in lung tumorigenesis; (b) silibinin decreased the number and size of tumors in B6/129 WT but not in iNOS+/− mice; (c) these decreases in tumor number and size correlate with silibinin-mediated inhibition of cell proliferation and angiogenesis in WT mice, most likely through the inhibition of iNOS expression involving down-regulation of STAT and NF-κB signaling; and (d) micro-CT analysis represents a sensitive and reliable imaging modality to longitudinally assess the inhibition of growth of lung tumors by chemopreventive agents including silibinin, and therefore could also be useful in other lung cancer intervention studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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