Oral Silibinin Inhibits Lung Tumor Growth in Athymic Nude Mice and Forms a Novel Chemocombination with Doxorubicin Targeting Nuclear Factor κB–Mediated Inducible Chemoresistance

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ABSTRACT

The acute and cumulative dose-related toxicity and drug resistance, mediated via nuclear factor κB (NFκB), of anthracycline anticancer drugs pose a major problem in cancer chemotherapy. Here, we report that oral silibinin (a flavanone) suppresses human non–small-cell lung carcinoma A549 xenograft growth (P = 0.003) and enhances the therapeutic response (P < 0.05) of doxorubicin in athymic BALB/c nu/nu mice together with a strong prevention of doxorubicin-caused adverse health effects. Immunohistochemical analyses of tumors showed that silibinin and doxorubicin decrease (P < 0.001) proliferation index and vasculature and increase (P < 0.001) apoptosis; these effects were further enhanced (P < 0.001) in combination treatment. Pharmacologic dose of silibinin (60 μmol/L) achieved in animal study was biologically effective (P < 0.01 to 0.001, growth inhibition and apoptosis) in vitro in A549 cell culture together with an increased efficacy (P < 0.05 to 0.001) in doxorubicin (25 nmol/L) combination. Furthermore, doxorubicin increased NFκB DNA binding activity as one of the possible mechanisms for chemoresistance in A549 cells, which was inhibited by silibinin in combination treatment. Consistent with this, silibinin inhibited doxorubicin-caused increased translocation of p65 and p50 from cytosol to nucleus. Silibinin also inhibited cyclooxygenase-2, an NFκB target, in doxorubicin combination. These findings suggest that silibinin inhibits in vivo lung tumor growth and reduces systemic toxicity of doxorubicin with an enhanced therapeutic efficacy most likely via an inhibition of doxorubicin-induced chemoresistance involving NFκB signaling.

INTRODUCTION

Due to failure and severe toxicity of cancer chemotherapy, several alternative medicine approaches including herbal phytochemicals are increasingly being claimed to be safe and effective. Is there any scientific merit behind these claims? Because of lack of relevant scientific evidence, this concern represents a common public opinion about potential benefits of herbal and alternative medicines in cancer control. However, current advances in drug development have revealed cancer preventive and curative efficacies of many phytochemicals (1). Several studies have found that consumption of silibinin (Fig. 1A), a major bioactive flavanone in milk thistle, is safe and nontoxic in animals and humans (2–4). Antitumor efficacy of silibinin is shown in prostate, skin, colon, and bladder cancer models (4–6). Here, for the first time we evaluated and observed antitumor effects of silibinin alone and in doxorubicin combination against lung tumor xenograft growth. Doxorubicin possesses a broad spectrum of anticancer activity and is used clinically in chemotherapy of various cancers, including lung (7, 8). Unfortunately, its acute and cumulative dose-related toxicity poses a major problem in therapeutic outcomes (7, 8).

NFκB is a family of inducible dimeric transcription factors that includes p65, p50/p105, RelB, c-Rel, and p52/p100 in mammals. All of the members have a Rel homology domain, which is important for binding with inhibitory κB (IκB) proteins, dimerization, nuclear translocation, and DNA binding (9). NFκB is involved in cell proliferation, apoptosis, differentiation, and immune and inflammatory responses (9). Recent studies have shown that NFκB activation plays an important role in inducible chemoresistance to anthracycline drugs in many cancer cells, including A549 lung cancer cells (10, 11). In addition to toxicity, the development of inducible drug resistance is another paramount problem in cancer chemotherapy, in which patients fail to respond to cancer drugs as in the case of anthracyclines. To overcome the problems of toxicity and inducible drug resistance, apart from combination chemotherapy, chemical modification of doxorubicin also is being explored for enhancing its antitumor efficacy. However, in combination treatment strategy, it is emphasized that a compound having its own antitumor efficacy together with preventive effect on anthracycline drug-caused adverse toxicity could be an ideal situation. If the compound could inhibit anthracycline drug-induced chemoresistance, the therapeutic response to the chemocombination also is bound to increase. Here, we tested this hypothesis using silibinin, a cancer chemopreventive agent, and doxorubicin, an anthracycline anticancer drug, in an A549 lung tumor xenograft model. The selection of A549 cells in the present study was based on the fact that they represent non–small-cell lung carci-

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noma (NSCLC), the most common cause of lung cancer-related deaths, and are studied most extensively as the NSCLC model. Furthermore, it has been shown that doxorubicin chemotherapy activates NFκB in A549 cells, accounting for inducible chemo-resistance.

**MATERIALS AND METHODS**

**In vivo Tumor Xenograft Study.** Athymic (BALB/c, nu/nu) male nude mice were obtained from NCI (Frederick, MD) and fed autoclaved Purina (St. Louis, MO) chow diet and water ad libitum. A549 NSCLC cells were from American Type Culture Collection (Manassas, VA) and grown in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum and 100 units/mL penicillin-streptomycin at standard culture conditions. Doxorubicin and silibinin were from Sigma (St. Louis, MO). For xenografts, ~3 million A549 cells were suspended in 0.1 mL serum-free medium mixed with matrigel (1:1) and subcutaneously injected into right flank of each mouse. The next day (day 1) mice were treated with saline (control) or silibinin (200 mg/kg body weight, 5 d/wk for 33 days) by oral gavage, doxorubicin (4 mg/kg body weight, once a week, on days 1, 8, 15, and 22; total of four doses) by intraperitoneal injection, or combination of silibinin and doxorubicin. Mice were euthanized on day 34, after last dose of silibinin, and tumors were harvested and weighed. A, chemical structure of silibinin, a major bioactive flavanone isolated from milk thistle (Silybum marianum). B, Tumor weight per mouse (g) is presented as mean and SE (bars) of 10 tumors from individual mouse in each group at the end of the study. C, Average diet consumption per mouse per day (g) is shown as a function of week. D, Body weight gain per mouse (g) is calculated as a difference between final and initial body weight of each mouse in each treatment group and presented as mean and SE (bars). The bar below x-axis in doxorubicin treatment represents loss in body weight. Co, saline control; Sb, silibinin 200 mg/kg dose; Dox, doxorubicin 4 mg/kg dose; Sb + Dox, silibinin and doxorubicin treatments together.
total of four doses) by intraperitoneal injection, and combination of oral silibinin and intraperitoneal doxorubicin under exact same treatment regimens as for individual agent. Mice were euthanized on day 34 after last dose of silibinin. Food consumption and animal body weight were monitored twice weekly throughout the study. Once xenografts started growing, their sizes were measured twice weekly in two dimensions. At the termination of the study, tumors were excised, and weights were recorded. Plasma and lung samples also were harvested for the estimation of silibinin levels by high-performance liquid chromatography as published recently (4).

Proliferation Cell Nuclear Antigen, Platelet Endothelial Cell Adhesion Molecule, and Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End-Labeling Staining in Tumors. Part of tumors were fixed in 10% formalin for 12 hours and processed conventionally for immunohistochemical analysis. Paraffin-embedded tumor sections (5 μm) were processed for desired staining, and proliferation and apoptotic indices and microvesSEL density (MVD) were quantified as published recently (12).

A549 Cell Growth and Apoptosis Using Pharmacologically Achievable Doses of Silibinin in Combination with Doxorubicin. Pharmacologically achievable silibinin concentration (in plasma) from animal study was ~60 μmol/L. A549 cells at ~30% confluence were treated with DMSO (0.1%, v/v) or 60 μmol/L silibinin and/or 25 mmol/L doxorubicin for 48 hours and then counted with hemocytometer for cell growth analysis. For apoptotic assay, after similar treatments, cells were harvested and stained with DNA binding dye Hoechst 33342 and propidium iodide, followed by quantification of apoptotic cell death as published recently (13).

Electrophoretic Mobility Shift Assay and Immunoblot Analysis. Cells were treated with DMSO or 60 μmol/L silibinin and/or 25 mmol/L doxorubicin for 48 hours, and whole cell, nuclear, and cytosolic extracts then were prepared as published recently (14). For electrophoretic mobility shift assay (EMSA), NFκB-specific oligonucleotide (3.5 pmol) was end-labeled with γ-32P]ATP (3000 Ci/μmol at 10 mCi/mL) using T4 polynucleotide kinase in 10X kinase buffer as per manufacturer’s protocol (Promega, Madison, WI). Labeled double-stranded oligo probe was separated from free γ-[32P]ATP using G-25 Sephadex column. Consensus sequences of oligonucleotide were 5′-AGT TGA GGC TAT CCC AGG C-3′ and 3′-TCA ACT CCC CTG AAA GGG TCC G-5′. Eight micrograms protein from nuclear extracts were incubated with 5× gel shift binding buffer [20% glycerol, 5 mmol/L MgCl2, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl, and 0.25 mg/mL poly(deoxyinosinic-deoxycytidylic acid)] and then with [32P]end-labeled NFκB consensus oligo nucleotide for 20 minutes at 37°C. In supershift and competition assays, either unlabeled wild-type oligo or mutant oligo was coincubated with labeled oligo or incubated with anti-p65 or anti-p50 antibody before addition of [32P]end-labeled NFκB oligo. DNA protein or DNA protein-antibody complexes thus formed were resolved on 6% DNA retardation gels (In-vitrogen, Carlsbad, CA). The gel was dried, and bands were visualized by autoradiography.

To assess cytoplasmic levels of p65 and p50 and cyclooxygenase-2 (COX-2) level in whole cell lysate, 50 to 80 μg protein per sample were resolved on 12% gels, followed by immunoblot analysis, and the membranes were probed with appropriate primary and secondary antibodies (anti-p65 and anti-p50 from Cell Signaling Technology, Beverly, MA; COX-2 from Santa Cruz Biotechnology, Santa Cruz, CA), followed by enhanced chemiluminescence (Amersham, Piscataway, NJ) detection as published recently (3, 14). Membranes were stripped and reporbed for β-actin (anti-β-actin from Sigma) as loading control.

Statistical, Immunohistochemical, and Densitometry Analyses. Data were analyzed using SigmaStat 2.03 software (San Rafael, CA). For all of the measurements, one-way ANOVA followed by paired t-test were used to assess statistical significance of difference between different treatment groups. A statistically significant difference was considered to be present at P < 0.05. All of the microscopic examinations were done by Zeiss Axioskop 2 microscope (Carl Zeiss Inc, Jena, Germany). Images were taken at 400× magnification by Kodak DC290 zoom digital camera and processed by Windows Millennium DC290 Kodak macroscopic documentation system (Eastman Kodak Company, Rochester, NY). Densitometry analysis of bands was done by Scion image program (NIH, Bethesda, MD).

RESULTS
Oral Feeding of Silibinin Inhibits A549 Tumor Xenograft Growth in Athymic Nude Mice Together with an Enhanced Efficacy in Doxorubicin Combination. Using preclinical tumor xenograft model, we observed that oral feeding of silibinin (200 mg/kg, 5 d/wk for 33 days) significantly inhibits human NSCLC A549 tumor xenograft growth in nude mice in a time-dependent manner (data not shown), which accounted for 58% (P = 0.003) decrease in tumor weight per mouse at the end of the study (Fig. 1B). Doxorubicin treatment (4 mg/kg, intraperitoneally once a week on days 1, 8, 15, and 22; total of four doses) showed 61% (P = 0.005) decrease in tumor weight per mouse as compared with control. In silibinin-doxorubicin combination, 76% (P = 0.002, versus control) decrease in tumor weight per mouse was observed that was significantly different from either treatment alone (P = 0.02, versus silibinin; P = 0.01, versus doxorubicin; Fig. 1B).

Oral Feeding of Silibinin Prevents Doxorubicin-Caused Systemic Toxicity in Mice. There is no report regarding adverse toxicity of silibinin (2–4). Consistent with this, silibinin feeding did not show any adverse health effect in mice as monitored by diet consumption (Fig. 1C), body weight gain (Fig. 1D), and posture and behavioral changes during the entire study. Conversely, doxorubicin showed a gradual decline in the health of mice as observed by reduced diet consumption (Fig. 1C), decline in body weight (P < 0.001, versus control; Fig. 1D), and hunchback posture and reduced activity. In combination treatment, silibinin strongly reduced doxorubicin-caused adverse health effects. Body weight gain was significantly higher (P < 0.001) as compared with doxorubicin alone group, and there also was no loss in initial body weight of mice in combination treatment (Fig. 1D).

Silibinin Inhibits In vivo Tumor Cell Proliferation and Induces Apoptosis Together with an Enhanced Efficacy in Doxorubicin Combination. Enhanced aberrant cell proliferation and resistance to apoptosis are hallmark of almost all of
the cancer cells (15). Therefore, we next analyzed tumors for possible antiproliferative and apoptotic effects of silibinin in relation to its antitumor efficacy (Fig. 2A). Quantification of proliferation cell nuclear antigen (PCNA) staining showed 40 and 50% ($P < 0.001$) inhibition in proliferation index by silibinin and doxorubicin, respectively, which further increased to 57% inhibition in combination treatment ($P < 0.001$, versus all of the other treatments; Fig. 2B). In apoptosis analysis, silibinin and doxorubicin showed 2.7- and 3.2-fold ($P < 0.001$) increase in apoptotic index as compared with control, respectively, which also increased to 3.8-fold in combination treatment ($P < 0.001$, versus all of the other treatments; Fig. 2C). These findings suggest that anticancer efficacy of silibinin and its potentiating activity with doxorubicin against in vivo lung tumor growth involve inhibition of cell proliferation and apoptosis induction.

**Silibinin Inhibits Tumor Angiogenesis and Significantly Enhances Antiangiogenic Efficacy of Doxorubicin.** Because tumor angiogenesis is suggested as an attractive therapeutic target and a prognostic biomarker in cancer therapy (16), we also analyzed tumors for CD31 (platelet endothelial cell adhesion molecule) staining to assess the MVD (Fig. 2A). Silibinin and doxorubicin inhibited tumor MVD by 52 and 58% ($P < 0.001$, versus all of the other treatments). The combination of silibinin and doxorubicin inhibited tumor MVD by 67% ($P < 0.001$, versus all of the other treatments).

**Fig. 2** Effect of silibinin, doxorubicin, and their combination on in vivo proliferation, apoptosis, and angiogenesis in lung tumors. A, Representative immunohistochemical staining of paraffin-embedded 5-$\mu$m-thick tumor sections for PCNA, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL), and CD31 at 400× of original magnification. Quantification of immunostained tumor sections for B, proliferation index by PCNA staining ($n = 8$ to 10); C, apoptotic index (AI) by TUNEL staining ($n = 5$ to 8); and D, MVD/400× microscopic field by CD31 staining ($n = 8$ to 10). All of the data presented are mean and SE (bars). Co, saline control; Sb, silibinin 200 mg/kg dose; Dox, doxorubicin 4 mg/kg dose; Sb+Dox, silibinin and doxorubicin treatments together. *$P < 0.001$ versus control; †$P < 0.001$ combination versus silibinin or doxorubicin treatment alone.
were treated with 60 combination studies with silibinin in cell culture. A549 cells growth inhibitory effect on A549 cells (data not shown) for doxorubicin increased apoptotic cell population by 17-fold (P < 0.001). In similar treatments, silibinin and \( \text{H11005} \) in combination treatment (\( \text{H11021} \)) respectively, which enhanced strongly to 70% inhibition significantly higher than each agent alone (\( \text{P} < 0.001 \)), respectively (Fig. 3B). The combination of both agents showed 85% cell growth inhibition, which was significantly higher than each agent alone (\( \text{P} < 0.001 \), versus other treatment groups; Fig. 3B). In similar treatments, silibinin and doxorubicin increased apoptotic cell population by 17-fold (\( \text{P} = 0.034 \)) and 29-fold (\( \text{P} = 0.008 \)), which further increased to 37-fold (\( \text{P} = 0.006 \)) in combination treatment (Fig. 3C).

**Silibinin Decreases COX-2 Levels in Doxorubicin Combination.** There are many target genes for NFκB, which are activated to mediate inducible drug resistance in cancer cells. COX-2 also is regulated by NFκB and is implicated in lung cancer growth and development (18). In similar treatments described previously, we observed a high level of COX-2 protein in control and doxorubicin-treated cells, which decreased by silibinin alone treatment of the cells (Fig. 4D). However, combination treatment showed a strong decrease in COX-2 level as compared with doxorubicin alone (Fig. 4D, top). Membrane was reprobed with β-actin antibody to confirm the equal protein loading (Fig. 4D, bottom). The densitometry analysis of bands after loading correction with the level of β-actin showed 38 and 53% decrease in band intensity of COX-2 in silibinin-
silibinin-doxorubicin–treated cells as compared with control, respectively (Fig. 4E). This result suggests that doxorubicin-induced NFκB activation may be responsible for maintaining the threshold/constitutive level of COX-2 contributing to tumor growth and progression and acquired chemoresistance in A549 cells. Therefore, the inhibition of COX-2 by silibinin may be involved in inhibiting tumor growth and doxorubicin-induced chemoresistance in A549 tumor xenograft.

**DISCUSSION**

Nude mouse tumor xenograft is a widely accepted model to evaluate antitumor efficacy of a test agent and associated toxicity (4). Proliferation, apoptosis, and angiogenesis are the most reliable and common biomarkers to assess efficacy of a compound on tumor growth and progression (12). Furthermore, bioavailability of test agent is another important criterion required for dosing and translation of antitumor effects and associated mechanisms from *in vivo* to *in vitro* condition and vice versa (4). In accord with these standards, we observed that oral feeding of silibinin (a) inhibits human NSCLC A549 tumor xenograft growth in nude mice; (b) significantly enhances therapeutic efficacy of doxorubicin toward inhibition of tumor growth; (c) is devoid of any apparent toxicity in nude mice and profoundly and significantly (body weight gain) prevents doxorubicin-caused adverse health effects; (d) inhibits tumor cell proliferation and angiogenesis and induces apoptosis and further significantly enhances these antitumor effects in combination with doxorubicin; and (e) is bioavailable in plasma and lung of mice. Furthermore, pharmacologic dose of silibinin achieved in tumor xenograft study inhibits cell proliferation and induces...
apoptosis in A549 cells and significantly enhances these effects in doxorubicin combination. Silibinin also almost completely inhibited doxorubicin-induced NFκB activation accompanied by inhibition of translocation of NFκB subunits from cytosol to nucleus. Silibinin also caused a strong decrease in COX-2 protein levels in combination with doxorubicin.

Dose-accumulated toxicity of chemotherapeutic drugs is a major obstacle in getting desired outcomes in cancer treatment. Doxorubicin is used clinically for the treatment of many cancers, including adenocarcinomas, melanomas, sarcomas, lymphomas, and leukemia; however, dose-related cardiotoxocities and neurotoxicities limit the success of therapy (7). In this regard, it has been hypothesized that a compound inhibiting doxorubicin-induced toxicity could form an ideal combination for therapeutic success. It recently was reported that silibinin inhibits doxorubicin-induced cardiotoxicity in mice (19). In the present study, we also observed that silibinin strongly prevents doxorubicin-induced adverse health effect such as loss in body weight, decrease in diet consumption, and hunchback posture and reduced activity. In another recent study, we observed that silibinin feeding up to 1 g/kg body weight/d for 16 days does not result in any adverse health effects in mice (3). Silibinin is already in human use as a hepatoprotective drug and as a dietary supplement (milk thistle extract), and to date no major toxicity and side effects are observed with this agent (2). Collectively, these reports and present finding suggest that silibinin is nontoxic and has potential to prevent doxorubicin-induced adverse health effects in addition to enhancing therapeutic responses of doxorubicin.

Most of the chemotherapeutic drugs are developed for clinical uses based on their antiproliferative and apoptotic responses; however, after a certain period of treatment, tumor recurrence is common in patients with a chemotherapy regimen (7, 15). Not only anthracine analogs (doxorubicin and daunomycin) but also a wide range of antineoplastic agents, such as paclitaxel, Vinca alkaloids (vincristine and vinblastine), interferon-γ, and tumor necrosis factor α, induce chemoresistance via activation of NFκB in A549 and other cancer cells (10). These agents can activate IkB kinase, which phosphorylates IkB for its ubiquitination and proteosomal degradation, making p65 and p50 free for nuclear translocation and transcriptional activation of genes responsible for indulcible chemoresistance (9, 11). It has been observed that dominant negative IkB protein potentiates the efficacy of chemotherapy and radiotherapy of cancer (20). Our results showed almost complete inhibition of doxorubicin-induced NFκB DNA binding activity together with retention of p65 and p50 in cytosol by silibinin. Furthermore, one of the NFκB targets, COX-2 (inducible cyclooxygenase), which is known to be associated with lung tumor growth and progression, was decreased by silibinin and silibinin-doxorubicin combination. Therefore, NFκB-mediated inhibition of COX-2 by silibinin could be involved in suppressing tumor growth and doxorubicin-induced chemoresistance in A549 tumor xenograft.

Collectively, these data indicate that silibinin is a potential agent for lung tumor growth inhibition, alone and in combination chemotherapy with antineoplastic agents including anthracycline drugs. Present findings together with earlier reports support the preventive effect of silibinin against doxorubicin-caused systemic toxicity; however, more studies in different animal models are needed to further justify silibinin as a novel agent in combination chemotherapy against lung and other cancers.

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