Silibinin Strongly Synergizes Human Prostate Carcinoma DU145 Cells to Doxorubicin-induced Growth Inhibition, G_2-M Arrest, and Apoptosis[1]

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ABSTRACT

Purpose: **We recently demonstrated the strong anticancer efficacy of silibinin, an active constituent of a widely consumed dietary supplement milk thistle extract, against human prostate cancer cells in culture and nude mice xenografts. We also observed that pharmacologically achievable concentrations of silibinin in animal studies were in the range of 25–100 μM, depending on the dose regimen, which did not show any apparent toxicity to the animals. In this study, we assessed whether silibinin synergizes the therapeutic potential of the chemotherapeutic drug doxorubicin against prostate cancer, the effectiveness of which is limited because of high systemic toxicity.**

Experimental Design: **Prostate cancer cells were treated with silibinin and doxorubicin, either alone or in combination, and cell growth was determined by manual cell counting. Cell cycle progression was assessed by saponin/propidium iodide staining and fluorescence-activated cell sorter analysis. Protein levels of cell cycle regulators were determined by Western blotting, and cdc2/p34 kinase activity was analyzed by in-beads kinase assay. Apoptosis was quantified by annexin V/propidium iodide staining and fluorescence-activated cell sorter analysis.**

Results: **Silibinin strongly synergized the growth-inhibitory effect of doxorubicin in prostate carcinoma DU145 cells (combination index, 0.235–0.587), which was associated with a strong G_2-M arrest in cell cycle progression, showing 88% cells in G_2-M phase by this combination compared with 19 and 41% of cells in silibinin and doxorubicin treatment alone, respectively. The underlying mechanism of G_2-M arrest showed a strong inhibitory effect of combination on cdc25C, cdc2/p34, and cyclin B1 protein expression and cdc2/p34 kinase activity. More importantly, this combination caused 41% apoptotic cell death compared with 15% by either agent alone. Silibinin and doxorubicin alone as well as in combination were also effective in inhibiting the growth of androgen-dependent prostate carcinoma LNCaP cells.**

Conclusion: **These findings suggest a need for *in vivo* studies with this combination in preclinical prostate cancer models. Positive outcomes might be relevant for a clinical application in prostate cancer patients.**

INTRODUCTION

PCA[3] is the leading cause of death from cancer in older men and the most commonly diagnosed cancer in men overall (1). For confined disease of low to moderately differentiated PCA, surgical or radiotherapy techniques are curative in the majority of individuals (2, 3). Unfortunately, there is no effective cure for PCA once the cancer has spread beyond the pelvis; these patients are estimated to account for 30,200 deaths in 2002 (1). Cytotoxic chemotherapy is being used to control and treat PCA at this stage but remains relatively nonselective and highly toxic to normal tissues. Doxorubicin is one of the clinical chemotherapy agents, which possesses a broad spectrum of therapeutic activity against various cancers including PCA (4, 5). However, the success of doxorubicin chemotherapy in PCA patient is limited, because its sufficient concentration could not be achieved without systemic toxicity (4). The reduction in doxorubicin-caused systemic toxicity poses a major challenge in maximizing the beneficial outcome of its therapy in PCA patients. Because of these limitations, there has been intense public and scientific interest in the use of other approaches to control the growth of PCA as well as its treatment. In an effort to develop effective strategies that increase the therapeutic potential of cytotoxic anticancer drugs with less systemic toxicity in recent years, more efforts are being directed toward combination chemotherapy (6, 7). In this regard, dietary supplements as well as phytotherapeutic agents with high anticancer efficacy and least toxicity to normal tissues are suggested as possible candidates to be investigated for their synergistic efficacy in combination with anticancer drugs (8–11).

In recent studies, we have reported cancer chemopreventive and therapeutic efficacy of silibinin, an antioxidant flavonoid, present in a widely consumed dietary supplement milk

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[3] The abbreviations used are: PCA, prostate cancer; CI, combination index; PI, propidium iodide.

thistle (*Silybum marianum* L.) extract, against different cancers of epithelial origin, including PCA (11–17). More recently, we observed the therapeutic efficacy of dietary feeding of silibinin on a human prostate carcinoma DU145 tumor xenograft in athymic nude mice (18). As a therapeutic agent as well as a dietary supplement, silibinin, silymarin (a crude form of silibinin with other constituents), and milk thistle extract are well tolerated and are largely free of any adverse side effects in humans (19). In the studies on mice, rats, rabbits, and dogs with different doses and modes of administration, silymarin and silibinin have been shown to be nontoxic in acute, subchronic, and chronic tests, even at large doses, and do not show any side effects; there is no known LD_{50} for silymarin and silibinin in laboratory animals (20, 21). This is also supported by our animal studies, where we did not observe any apparent signs of toxicity with silibinin (18).

Because, after an initial hormone-sensitive period, hormone-insensitive PCA remains our greatest therapeutic challenge, here we asked the question whether a nontoxic dietary supplement agent, silibinin, which is also effective against PCA, could synergize the therapeutic efficacy of doxorubicin in advanced human prostate carcinoma DU145 cells. The results obtained show clearly that, indeed, silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G_2-M arrest, and apoptosis. These results suggest the first rationale for the use of lower doses of doxorubicin in combination with silibinin or an increase in the efficacy of standard dose regimens in PCA patients.

MATERIALS AND METHODS

Cell Lines and Reagents. Human prostate carcinoma DU145 and LNCaP cells were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO_2 atmosphere under 90–95% humidity. Silibinin was from Sigma Chemical Co. (St. Louis, MO), and its purity was checked as 100% by high-performance liquid chromatography analysis (13). Doxorubicin was obtained from Cetus Oncology (Emeryville, CA). Antibodies to Chk1, Chk2, cdc25C, cdc2/p34, and cyclin B1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was from Sigma Chemical Co.

Cell Growth Assay. Cells were plated at 5000 cells/cm^2 density in 60-mm dishes under the standard culture conditions as described above. One day after initial seeding, cells were treated with either DMSO alone (0.1% v/v, control), different doses of silibinin (10–100 μM) dissolved in DMSO or doxorubicin (10–100 nM) alone, or both agents in different combinations. Each treatment and time point had three plates. After 48 h of these treatments, both attached and floating cells were collected by trypsinization and counted using a hemocytometer. On the basis of the results of these experiments, combinations of 100 μM silibinin with 25 nM doxorubicin were further studied, where cells were treated with silibinin or doxorubicin either alone or first with silibinin and 24 h later with doxorubicin, or *vice versa*, or both agents together for a total of 48 h, and cell growth and viability were determined as described above. The combined growth-inhibitory effect was analyzed with CI/isobologram method (22).

Determination of CI. The CI was calculated by the Chou-Talalay equation (22), which takes into account both the potency (D_m or IC_{50}) and the shape of the dose-effect curve. The general equation for the classic isobologram (CI = 1) is given by:

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 \quad \text{(A)}$$

where $(D_x)_1$ and $(D_x)_2$ in the denominators are the doses (or concentrations) of D_1 (drug #1, for example, silibinin) and D_2 (drug #2, for example, doxorubicin) alone that gives *x*% inhibition, whereas $(D)_1$ and $(D)_2$ in the numerators are the doses of D1 and D2 in combination that also inhibits *x*% (*i.e.*, isoeffective). The $(D_x)_1$ and $(D_x)_2$ can be readily calculated from the Meridian-effect equation of Chou *et al.* (23):

$$D_x = D_m [f_a/(1 - f_a)]^{1/m} \quad \text{(B)}$$

where D_x is the median-effect dose that is obtained from the anti-log of the X-intercept of the median-effect plot, X-log (*D*) *versus*, $Y = \log [f_a/(1 - f_a)]$, or $D_m = 10\text{-}^{(Y\text{-intercept})/m}$, f_a is the fraction affected by dose *D* (*e.g.*, 0.5 if cell growth is inhibited by 50%) and *m* is the slope of the median-effect plot. From $(D_m)_1$, and $(D_x)_2$, and $D_1 + D_2$, it becomes easy to construct an isobologram based on Eq. A: CI <1 indicates synergism; CI = 1 indicates additive effect; and CI >1 indicates antagonism.

For conservative mutually nonexclusive isobolograms of two agents, a third term,

$$(D)_1(D)_2/(D_x)_1(D_x)_2$$

is added to Eq. A. For simplicity, the third term is usually omitted, and thus the mutually exclusive assumption or classic isobologram is indicated. In this study, the CI values obtained from classic (mutually exclusive) calculation are given.

Cell Cycle Analysis. DU145 cells at 60% confluency were treated with silibinin and doxorubicin either alone or in combination for 48 h. At the end of treatment, cells were processed and incubated in saponin/PI solution [0.3% saponin (w/v), 25 μg/ml PI (w/v), 0.1 mM EDTA, and 10 μg/ml RNase in PBS] at 4°C for 24 h in dark. Cell cycle distribution was then analyzed by flow cytometry analysis of the fluorescence-activated cell sorter analysis core service of the University of Colorado Cancer Center.

Western Blotting. For cell cycle regulatory molecules, at the end of treatment, attached cells were washed twice with PBS, and cell lysates prepared in nondenaturing lysis buffer as described earlier (16). For immunoblot analysis, 70 μg of protein lysates/sample were denatured in 2× SDS-PAGE sample buffer and subjected to SDS-PAGE on 12% Tris-glycine gel. The separated proteins were transferred on to nitrocellulose membrane followed by blocking with 5% nonfat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature or over night at 4°C. Membranes were probed for the protein levels of Chk1, Chk2, cdc25C, cdc2/p34, and cyclin B1 using specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibody, and visualized by the ECL detection system.

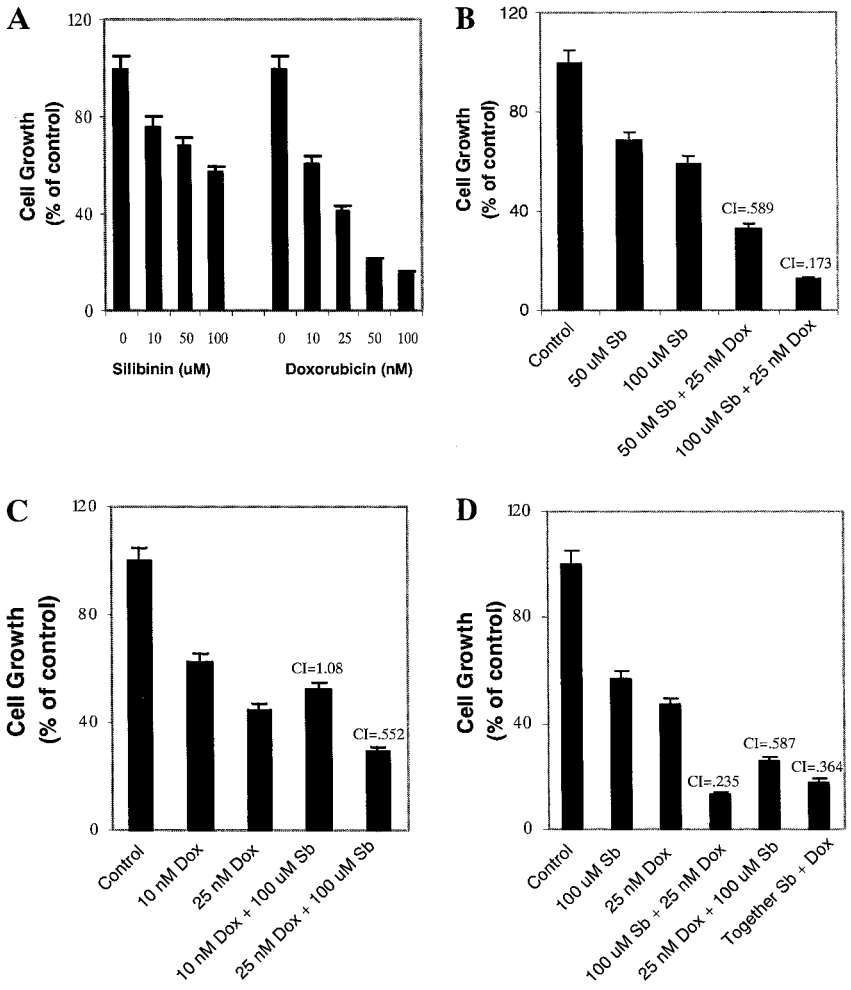

Fig. 1 Effect of silibinin and doxorubicin alone and in combination on DU145 cell growth. Cells were cultured as described in "Materials and Methods" and treated with either DMSO or silibinin (*Sb*) or doxorubicin (*Dox*) alone or the combination of Sb and Dox, and cell number was determined at the end of a total 48 h of treatment. *A*, cells were treated with 0 (DMSO control), 10, 50, and 100 μM Sb or 10, 25, 50, and 100 nM Dox alone for 48 h. *B*, control (DMSO), 50 and 100 μM Sb alone, and in combination with 25 nM Dox, which was added for the last 24 h. *C*, control (DMSO), 10, and 25 nM Dox alone and in combination with 100 μM Sb, which was added for the last 24 h. *D*, in this combination experiment, cells were treated with either DMSO (control), 100 μM silibinin, 25 nM doxorubicin alone, or 100 μM silibinin, and 24 h later, 25 nM doxorubicin or *vice versa*, or a combination of both agents simultaneously for a total of 48 h. The data presented are the percentage of control as means of triplicate samples for each treatment; *bars*, SE. The CI was calculated as detailed in "Materials and Methods." Experiments were repeated three times with similar results.

Cdc2/p34 Kinase Assay. Cdc2/p34-associated H1 histone kinase activity was determined as described by us recently (16) with some modifications. Briefly, 200 μg of protein lysates from each sample were precleared with Protein A/G-plus agarose beads, and cdc2/p34 protein was immunoprecipitated using anti-cdc2/p34 antibody (2 μg) and Protein A/G-plus agarose beads. Beads were washed three times with lysis buffer and finally once with kinase assay buffer [50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 1 mM DTT]. Phosphorylation of histone H1 was measured by incubating the beads with 30 μl of "hot" kinase solution [0.25 μl (2.5 μg) of histone H1, 0.5 μl of [γ-^{32}P]ATP (specific activity, 3000 μCi/mmol), 0.5 μl of 0.1 mM ATP, and 28.75 μl of kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography.

Apoptotic Cell Death Assay. To quantify apoptosis, DU145 cells were stained with annexin V and PI using Vybrant Apoptosis Assay Kit2 from Molecular Probes, Inc. (Eugene, OR) following the step-by-step protocol as provided by the manufacturer and analyzed by flow cytometry. Briefly, at the end of treatment with 100 μM silibinin and 25 nM doxorubicin either alone or in combination for 48 h, both floating and attached cells were collected, washed twice with PBS, and subjected to annexin V (conjugated with fluorophores and Alexa fluro 488 dye) and PI staining. Flow cytometry was performed within 30 min with a 488-nm line of an argon-ion laser for excitation.

Statistical and Densitometric Analyses. The data were analyzed using SigmaStat 2.03 software. Statistical significance of difference was assessed by Student's *t* test. A statistically significant difference was considered to be present at $P < 0.05$. Autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA) and adjusted for brightness and contrast for minimum background. The mean density of each band was analyzed by the Scanimage program (NIH, Bethesda, MD). Densitometric data presented below the bands are fold change as compared with control.

RESULTS

Synergistic Effect of Silibinin-Doxorubicin Combination on DU145 Cell Growth Inhibition. To assess the effect of silibinin and doxorubicin on DU145 cell growth, cells in the

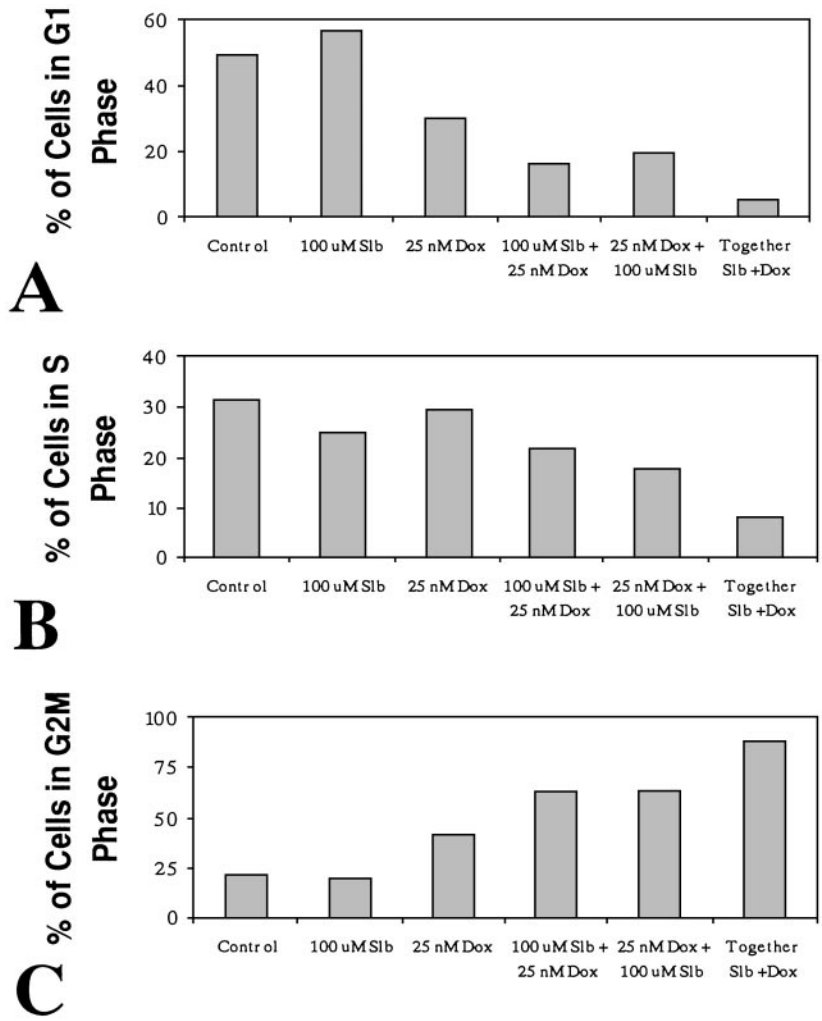

Fig. 2 Effect of silibinin and doxorubicin alone and in combination on cell cycle progression of DU145 cells. Cells were cultured as described in "Materials and Methods" and treated with either DMSO alone (*Control*); 100 μM silibinin (*Slb*); 25 nM doxorubicin (*Dox*); 100 μM Slb and 24 h later 25 nM Dox; 25 nM Dox and 24 h later 100 μM Slb; or a combination of both 100 μM Slb and 25 nM Dox for a total of 48 h. At the end of treatments, cells were harvested and stained with saponin and PI as detailed in "Materials and Methods," followed by flow cytometry analysis. The data shown for the percentage of cells in G_1 (*A*), S (*B*), or G_2-M (*C*) phase of the cell cycle are means of two independent samples in each treatment, which were reproducible in three independent experiments.

exponential growth phase were treated with different doses of silibinin (10, 50, and 100 μM) and doxorubicin (10, 25, 50, and 100 nM) for 48 h. At the end of the treatment, the determination of cell number showed that both of these agents inhibited cell growth in a dose-dependent manner, accounting for 24–43 and 39–85% inhibition, respectively (Fig. 1*A*). On the basis of these results, in the first combination experiment, we used 50 and 100 μM doses of silibinin (for 48 h) in combination with 25 nM dose of doxorubicin (added for the last 24 h), which resulted in a strong synergistic growth inhibition of DU145 cells with CIs 0.589 and 0.173, respectively (Fig. 1*B*), where CI $<$ 1 means synergism, CI = 1 means additive effects, and CI $>$ 1 means antagonism. In the second combination experiment, we used 10 and 25 nM doses of doxorubicin (for 48 h) in combination with a 100 μM dose of silibinin (added for last 24 h), which also showed synergistic growth inhibition (CI = 0.552) at 25 nM doxorubicin and 100 μM silibinin combination treatment (Fig. 1*C*). On the basis of these two combination studies, we selected the effective silibinin (100 μM) and doxorubicin (25 nM) doses for the further studies in DU145 cells.

In the third combination study, 100 μM silibinin and 25 nM doxorubicin doses were used which alone showed 43 and 53% growth inhibition, respectively (Fig. 1*D*). In combination treatment, as shown in Fig. 1*D*, the addition of both agents together for 48 h resulted in a strong synergistic effect on cell growth inhibition (82%; CI = 0.364). Interestingly, treatment with either of the agents for 24 h, followed by cotreatment with the other agent for another 24 h, resulted in a similar strong synergistic effect on cell growth inhibition. As shown in Fig. 1*D*, when cells were first treated with silibinin and after 24 h with doxorubicin for a total of 48 h, a strong synergistic effect was observed on cell growth inhibition (87%; CI = 0.235; fourth column). Next, we investigated the effect of doxorubicin treatment for 48 h, where silibinin was added for last 24 h. This study also showed synergistic effect on cell growth inhibition (74%; CI = 0.587; fifth column), although the effect was less profound compared with the treatment designs where cells were either pretreated (fourth column) or cotreated (sixth column) with silibinin in combination with doxorubicin (Fig. 1*D*). Overall, these results showed that silibinin strongly synergizes the doxorubicin-caused, growth-inhibitory effect in human prostate carcinoma DU145 cells.

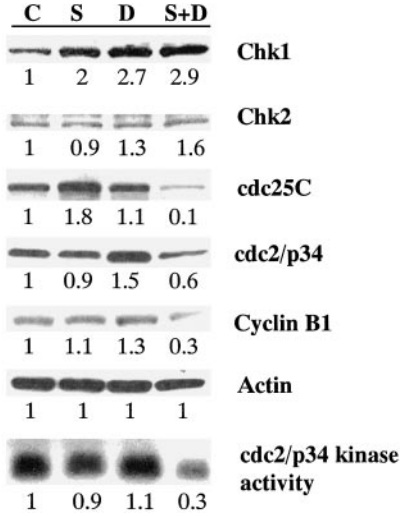

Fig. 3 Effect of silibinin and doxorubicin alone and in combination on G_2-M cell cycle regulators in DU145 cells. Cells were cultured as described in "Materials and Methods" and treated with DMSO alone (*C*, control), 100 μM silibinin (*S*), 25 nM doxorubicin (*D*), or combination (*S+D*) of both agents together for 48 h. At the end of treatments, total cell lysates were prepared, and an equal amount of protein was subjected to SDS-PAGE, followed by Western immunoblotting. Membranes were probed with anti-Chk1, Chk2, cdc25C, cdc2/p34, cyclin B1, and β-actin antibodies, followed by peroxidase-conjugated appropriate secondary antibodies, and visualized by the ECL detection system. cdc2/p34 kinase activity was determined by in-bead histone H1 kinase assay as described in "Materials and Methods." After the assay, the labeled substrate was subjected to SDS-PAGE, and the gel was dried and exposed to X-ray film. The numbers shown below each *band* indicate the fold change in band intensity compared with DMSO control. The experiment was repeated with similar results.

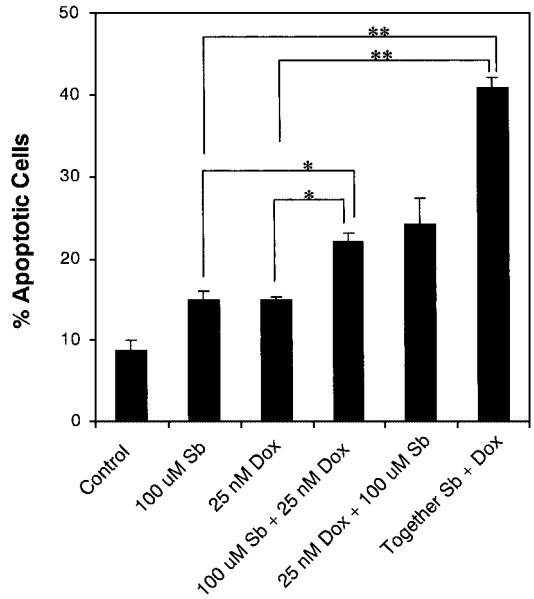

Fig. 4 Effect of silibinin and doxorubicin alone and in combination on apoptotic death in DU145 cells. Cells were treated with either DMSO (*Control*), 100 μM silibinin (*Sb*), 25 nM doxorubicin (*Dox*) alone, or 100 μM silibinin and 24 h later 25 nM doxorubicin, or *vice versa*, or both agents together for a total of 48 h. At the end of treatments, cells were harvested and stained with annexin V and PI as detailed in "Materials and Methods." The data presented are the percentage of apoptotic cells as means of duplicate samples; bars, *SE*. The experiment was repeated with similar results. *, $P < 0.05$; **, $P < 0.01$.

Strong Synergistic Effect of Silibinin-Doxorubicin Combination on Selective G_2-M Arrest in DU145 Cell Cycle Progression. On the basis of the synergistic effect of silibinin-doxorubicin combination on cell growth inhibition, we next examined the effect of similar doses (100 μM silibinin and 25 nM doxorubicin, either alone or in combination, as used in the third combination study) of both the compounds on cell cycle progression in DU145 cells. As shown in Fig. 2, silibinin treatment showed G_1 arrest (56% *versus* 49% in control), whereas doxorubicin caused G_2-M arrest (41% *versus* 20% in control) after 48 h of treatment. The effect of silibinin on G_1 arrest was largely accompanied by a decrease in S-phase cells, whereas doxorubicin-caused G_2-M arrest was associated with a decrease in the G_1 population (Fig. 2). Interestingly, doxorubicin treatment after silibinin switched the silibinin-induced G_1 arrest to a strong G_2-M arrest (63% *versus* 20% in control; Fig. 2*C*). Similarly, treatment of doxorubicin-treated cells with silibinin led to a further increase in G_2-M arrest (64%) to that caused by doxorubicin alone (41%; Fig. 2*C*). When cells were simultaneously treated with both the agents for 48 h, a much stronger G_2-M arrest (88%) was evident at the expense of both G_1 and S-phase populations (Fig. 2).

In other studies, when cells were treated with 50 or 100 μM dose of silibinin alone for 48 h, they showed strong G_1 arrest; however, a cotreatment with 25 nM doxorubicin during the last 24 h of treatment caused a much stronger G_2-M arrest compared with doxorubicin alone also showing G_2-M arrest (data not shown). Similarly, compared with doxorubicin alone showing G_2-M arrest at the 10 and 25 nM dose treatment for 48 h, a cotreatment with silibinin at the 100 μM dose during the last 24 h of treatment caused a much stronger G_2-M arrest (data not shown). Together, these results suggest that no matter how, a combination of silibinin and doxorubicin causes a very strong G_2-M arrest compared with these agents alone showing G_1 and G_2-M arrests, respectively, and that DU145 cell growth inhibition in silibinin-doxorubicin combination, could be, in part, attributable to a stronger arrest in cell cycle progression at the G_2-M check point.

Effect of Silibinin-Doxorubicin Combination on G2-M Cell Cycle Regulators. On the basis of a strong synergistic effect of silibinin-doxorubicin combination on G2-M arrest, next we investigated their effect on the modulation of molecular events associated with the G2-M phase of cell cycle progression in DU145 cells. Immunoblot analysis showed that 48 h treatment of cells with a combination of 100 μM silibinin and 25 nM doxorubicin results in a very strong decrease in cdc25C, cdc2/p34, and cyclin B1 protein levels compared with either compound alone or control group (Fig. 3). Consistent with these results, this drug combination also inhibited cdc2/p34 kinase activity assayed for histone H1 as substrate (Fig. 3). We also analyzed the upstream kinases (Chk1/2) associated with cdc25C-cyclin B1-cdc2/p34. Combination of these two agents also moderately increased the protein levels of the upstream

kinases Chk1 and Chk2 compared with each agent alone (Fig. 3). Together, these results suggest that down-regulation of the Chk1/2-cdc25C-cyclin B1-cdc2/p34 pathway could be one of the possible underlying molecular events associated with the strong synergistic effect of the silibinin-doxorubicin combination on G2-M arrest, leading to cell growth inhibition and possible apoptotic death.

Effect of Silibinin-Doxorubicin Combination on Apoptotic Death of DU145 Cells. By use of a quantitative apoptotic cell death assay, we next assessed whether the observed synergistic cell growth inhibition by silibinin-doxorubicin combination is accompanied by a stronger apoptotic death. The doses of silibinin and doxorubicin and treatment design were the same as that for other studies. As shown in Fig. 4, the apoptotic cell population increased from 8% in control to 15% after 100 μM silibinin or 25 nM doxorubicin treatment for 48 h. Silibinin treatment followed by doxorubicin caused 22% apoptotic cell death, which was significantly ($P < 0.05$) higher than that caused by either of the agent alone (Fig. 4). However, when cells were treated *vice versa*, apoptotic cell death was 24%, but the increase was not significant ($P = 0.11$) when compared with either agent alone. Furthermore, when cells were treated simultaneously with the silibinin-doxorubicin combination for 48 h, it resulted in 41% apoptotic cell death, an approximate 3-fold increase ($P < 0.01$) in apoptotic response as compared with that caused by either agent alone (Fig. 4). Together, these results suggest that apoptosis could be a major contributor in the silibinin-doxorubicin combination-caused biological efficacy in DU145 cells.

Silibinin-Doxorubicin Combination Inhibits Growth of Prostate Carcinoma LNCaP Cells. On the basis of the above findings, we next studied whether the observed synergistic effect of the silibinin-doxorubicin combination on DU145 cells is specific or whether other human prostate carcinoma cells also behave similarly to these treatments. The effect of the silibinin-doxorubicin combination was studied on androgen-dependent human prostate carcinoma LNCaP cell growth. To evaluate the effect of silibinin and doxorubicin on LNCaP cell growth, cells were treated with different doses of silibinin (25, 50, and 100 μM) and doxorubicin (5, 10, 15, 20, and 25 nM) for 48 h, which showed a highly significant growth inhibition in a dose-dependent manner (Fig. 5*A*). Compared with DMSO control, 48 h of silibinin treatment at 25, 50, and 100 μM doses resulted in 38, 68, and 87% inhibition, respectively, whereas doxorubicin treatment at 5, 10, 15, 20, and 25 nM resulted in 12, 29, 39, 50, and 61% inhibition, respectively (Fig. 5*A*).

In combination study, cells were treated with the 25 μM dose of silibinin and 15 nM dose of doxorubicin either alone or first treated with 25 μM silibinin and 24 h later with 15 nM doxorubicin or *vice versa*, or both the agents were added together for a total of 48 h. In combination, 62–69% growth inhibition was observed compared with the single agent showing 43 and 42% inhibition, respectively (Fig. 5*B*). However, a moderate synergistic effect (CI = 0.929), if any, of the combination was evident only when cells were pretreated with silibinin and followed by doxorubicin treatment (Fig. 5*B*). Overall, these results suggest that the synergistic effect of the silibinin-doxorubicin combination is more profound in advanced androgen-independent DU145 cells as compared with androgen-dependent LNCaP cells.

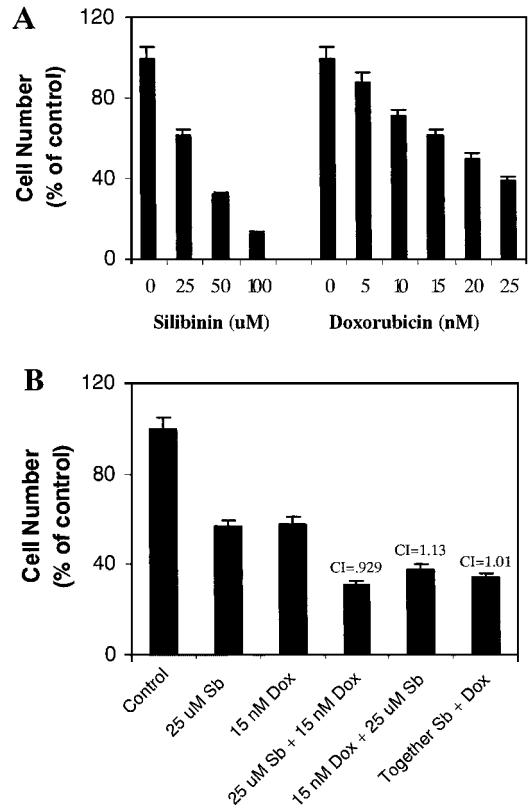

Fig. 5 Effect of silibinin and doxorubicin alone and in combination on LNCaP cell growth. Cells were cultured as described in "Materials and Methods" and treated with DMSO (*Control*), silibinin (*Sb*), doxorubicin (*Dox*) alone, or the combination of Sb and Dox; and cell number was determined at the end of a total 48 h of treatment. *A*, cells were treated with 0 (DMSO control), 25, 50, and 100 μM Sb or 5, 10, 15, 20, and 25 nM Dox alone for 48 h. *B*, in this combination experiment, cells were treated with either DMSO (*Control*), 25 μM silibinin, 15 nM doxorubicin alone, or 25 μM silibinin and 24 h later 15 nM doxorubicin or *vice versa*, or a combination of both agents simultaneously for a total of 48 h. The data presented are the percentages of control as means of triplicate samples for each treatment; *bars*, SE. The CI was calculated as detailed in "Materials and Methods." Experiments were repeated twice with similar results.

DISCUSSION

The central finding of present study is that silibinin strongly synergizes the therapeutic effect of doxorubicin in advanced human prostate carcinoma DU145 cells, via an induction of G2-M arrest and apoptotic cell death. This finding is significant because doxorubicin as well as other anthracycline chemotherapy drugs cause high toxicity to normal tissues during treatment of an advanced stage of PCA as well as other cancers. Their adverse health effects, such as immunosuppression and cardiomyopathy, which severely increases in a dose-dependent manner, as well as development of primary or secondary drug resistance in tumor cells, limit their clinical success in cancer chemotherapy (4, 5).

The increase in systemic toxicity and drug resistance, the major drawbacks of cancer chemotherapeutic agents, have led to a new challenge in the field of cancer research. To overcome this problem, extensive research has been directed toward reducing systemic toxicity and increasing drug activity in cancer therapy (4–6). In this regard, combination chemotherapy has received more attention for the purpose of finding compounds with a known mechanism of action that could increase the therapeutic index of clinical anticancer drugs (24). Because most of the dietary supplements generally have health-improving effects, mostly via strengthening the cellular antioxidant system in the body, they provide a reasonably strong rationale for their role in combination chemotherapy. Additionally, those dietary supplements that also possess anticancer activity apart from their other biological efficacy have an added advantage in combination chemotherapy. Our completed studies show that silibinin is one such agent, which is nontoxic, consumed widely as a dietary supplement, and possesses strong anticancer activity against different epithelial cancers including PCA (11–18).

On the basis of the above findings and the fact that reduction in doxorubicin-caused systemic toxicity poses a major challenge in maximizing the beneficial outcome of doxorubicin therapy in PCA patients, we hypothesized that using a combination approach of silibinin with doxorubicin could produce synergistic efficacy. The results of our present study, showing a strong synergistic therapeutic effect of doxorubicin in combination with silibinin in PCA DU145 cells, support this hypothesis. We found that *in vitro* therapeutic effect of doxorubicin in terms of cell growth inhibition at the 100 nM dose could be achieved at its one-fourth concentration (25 nM dose used in present study) in combination with the 100 μM dose of silibinin in DU145 cells. In additional studies identifying the mechanism of observed effect, we found that silibinin strongly synergizes doxorubicin-caused G_2-M arrest in cell cycle progression. G_2-M transition provides an effective checkpoint in cell cycle progression that is regulated by the sequential activation and deactivation of cdc-family proteins (tyrosine kinases and phosphatases) and cyclin complexes (25). cdc25C functions as a mitotic activator by dephosphorylating cdc2/p34 that forms a complex with cyclin B1 and drives the cell from G_2 to M phase. The activation of cdc25C is controlled via its inhibitory phosphorylation by an upstream kinase Chk1/2, in response to DNA damage, and thus inhibits mitosis (26). Therefore, the regulation of G_2-M transition could be an effective target to control the growth and proliferation of cancer cells and facilitate their apoptotic death. Consistent with this notion, a combination of silibinin with doxorubicin strongly inhibited cdc2/p34 kinase activity accompanied by down-regulation of cdc25C and cyclin B1. Apoptotic death of cancer cells is considered to be a potential anticancer mechanism, which could control their proliferation (27–29). There are several reports that G_2-M arrest sensitizes cell death through the apoptotic pathway (24, 30–32). Consistent with these reports, the data of our present study show clearly that silibinin and doxorubicin combination induces strong apoptotic cell death that was ~3-fold higher than each agent alone.

Silibinin, a flavonoid antioxidant, is an active constituent in milk thistle extract, which is one of the most commonly consumed dietary supplements, and used clinically as an antihepatotoxic agent worldwide including the United States (19, 33). In recent studies, we have demonstrated preventive and therapeutic efficacy of silibinin/silymarin in several epithelial cancer models including PCA, both *in vitro* as well as *in vivo* (11–18). Our completed studies have also shown (18, 34) and found[4] that depending on the doses, mode, duration of administration, and preparation of silibinin used in animal tumor studies, its achievable plasma levels were in the range of 25–100 μM. These data suggest that the strong synergistic efficacy of silibinin at 50 and 100 μM doses in combination with doxorubicin, observed in the present study, is at physiologically achievable concentrations at least in animals. However, the relevance of these silibinin concentrations in humans remained to be studied and established.

Recent epidemiological studies show that many PCA patients use alternative medicine, mostly of herbal origin. On the basis of these reports, the present finding indicates that the synergistic effect of silibinin-doxorubicin combination for PCA therapy could be achieved at relatively lower doses of doxorubicin with lesser side effects. Collectively, these studies suggest that a preclinical study is warranted to establish the synergistic efficacy of silibinin on doxorubicin chemotherapy of human prostate cancer in an *in vivo* model, the results of which could find their implication in Phase I trial in human PCA patients.

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