

p21 and p27 induction by silibinin is essential for its cell cycle arrest effect in prostate carcinoma cells

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Abstract

Recent studies have shown that silibinin induces p21/Cip1 and p27/Kip1 and G₁ arrest in different prostate cancer cells irrespective of p53 status; however, biological significance and mechanism of such induction have not been studied. Here, using two different prostate cancer cell lines DU145 and 22Rv1, representing androgen-independent and androgen-dependent stages of malignancy, first we investigated the importance of p21 and p27 induction in silibinin-mediated G₁ arrest. Silencing p21 and p27 individually by RNA interference showed marked reversal in G₁ arrest; however, their simultaneous ablation showed additional reversal of G₁ arrest in 22Rv1 but not DU145 cells. These results suggest that whereas relative importance of these molecules might be cell line specific, their induction by silibinin is essential for its G₁ arrest effect. Next, studies were done to examine mechanisms of their induction where cycloheximide-chase experiments showed that silibinin increases p21 and p27 protein half-life. This effect was accompanied by strong reduction in Skp2 level and its binding with p21 and p27 together with strong decrease in phosphorylated Thr¹⁸⁷ p27 without considerable change in proteasomal activity, suggesting a posttranslational mechanism. Skp2 role was further elucidated using Skp2-small interfering RNA-transfected cells, where decreased G₁ arrest and attenuated Cip/Kip induction were observed with silibinin treatment. Further, silibinin caused a marked increase in p21 and p27 mRNA levels together with an increase in their promoter activity, also indicating a transcriptional mechanism. Together, our results for the

first time identify a central role of p21 and p27 induction and their regulatory mechanism in silibinin-mediated cell cycle arrest. [Mol Cancer Ther 2007;6(10):2696–707]

Introduction

Silibinin, the active constituent of silymarin isolated from the dried fruits of milk thistle (*Silybum marianum*) plant, is long being used as a dietary supplement for its strong antihepatotoxic efficacy in Europe and Asia. However, in the last 15+ years, significant research efforts have focused on investigating its anticancer efficacy and thus far, several studies by us and others have shown that silibinin has promising anticancer and chemopreventive efficacy in both cell culture and animal models of a variety of epithelial carcinomas (1–5). Moreover, silibinin has been found to be nontoxic in both chronic and acute toxicity studies, and no LD₅₀ has been reported for this flavolignan, which further underscores its promise in cancer prevention.

Prostate cancer is the second leading cause of cancer deaths in the U.S. male population. The conventional treatment regimen starts with androgen ablation that causes regression of primary lesions (6). However, this effect is transient and once the disease progresses to the androgen-refractory stage, it becomes resistant to chemotherapy and no suitable cure is available thus far for this advanced stage of malignancy (7). Silibinin has shown promising anticancer potency against both androgen-dependent and androgen-independent models of prostate cancer (4, 8). In xenograft models using androgen-independent DU145 cells, administration of silibinin dramatically reduced tumor volume and tumor weight (3). In prostate cancer cell culture studies, silibinin has been shown to impair mitogenic signaling and modulate cell cycle-related molecules, and thus induces growth arrest mainly via G₁ arrest in cell cycle progression (9). In a recently completed phase I dose-escalation clinical trial in patients with prostate cancer, biologically relevant levels of free silibinin were detected typically between 50 and 150 μmol/L range (10). Based on this study outcome, a phase II study has been planned to be conducted that will analyze biomarkers in prostate and serum from the patients receiving silibinin or placebo before prostatectomy.

Cell cycle progression in a eukaryotic cell is intricately regulated by the activity of the cyclin-dependent kinase (Cdk)-cyclin complexes. Upon mitogenic stimulation, the cyclin D–Cdk4/6 as well as cyclin E–Cdk2 complexes mediate phosphorylation of retinoblastoma (Rb), which thereafter releases transcriptionally active E2F thereby ensuring G₁-S transit (11). However, in the event of tumorigenesis, several genetic and epigenetic changes occur that culminate in the deregulation of cell cycle events (12). Because the threshold kinase activity of Cdks is a

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crucial determinant of cell cycle progression, they represent attractive targets for the intervention of sustained proliferation of carcinoma cells (13). The Cip/Kip and the INK4 are the two most important families of Cdk inhibitors (CDKI). The Cip/Kip interacts with the cyclin subunit and through a conserved NH₂-terminal domain, bind with Cdk and block its ATP loading region (14). Numerous studies have shown that up-regulation of p21/Cip1 and/or p27/Kip1 causes growth inhibition in various cancer models (15–17). Moreover, despite being central players in cell cycle regulation, and unlike p53 and p16/INK4, the frequency of somatic mutations in the Cip/Kip genes in cancers is very rare (18, 19), which underlines the importance of these molecules as promising therapeutic targets. Both p21 and p27 are up-regulated, by a variety of regulatory pathways, at the transcriptional as well as posttranscriptional levels (20–22). p21 was initially identified to be transcriptionally up-regulated by p53 in response to DNA damage (23); however, recent studies have shown that p21 can also be induced by various transcription factors and subsequently mediates growth arrest, senescence, and apoptosis in a p53-independent manner (24–26). Moreover, p21 mRNA stability can be posttranscriptionally regulated by HuR, a RNA-binding protein, in response to stress (27, 28); p27 translation can be regulated by an internal ribosome entry site element in its 5' untranslated region (29). At the posttranslational level, these CDKIs are primarily regulated by S-phase kinase-associated protein (Skp2), an E3 ubiquitin ligase, which targets them for ubiquitylation and proteasomal degradation (30).

Our previous studies have shown that silibinin-mediated G₁ arrest is associated with an increase in p21 and p27 levels in a p53-independent manner, a reduction in Cdk2 level, and hypophosphorylation of retinoblastoma-like proteins in DU145 cells (31). However, the precise molecular events orchestrating silibinin-induced growth arrest in prostate cancer models have not been clearly elucidated. Here, we report for the first time that Cip/Kip family of proteins play a central role in silibinin-induced G₁ arrest. We also observed that the induction of p21 and p27 by silibinin occurs at the posttranslational level involving down-regulation of Skp2, as well as at the transcriptional level via promoter activation and mRNA induction.

Materials and Methods

Reagents

Silibinin, bromodeoxyuridine (BrdUrd), anti- β -actin antibody, and propidium iodide were from Sigma. Antibodies to Cdk2, Cdk4, Skp2, Rb-GST fusion protein, normal rabbit IgG, A/G plus agarose beads, and Skp2–small interfering RNA (siRNA) were from Santa Cruz Biotechnology. Antibodies against p27 and p21, and p27-siRNA (sequences were not revealed by the manufacturer), were from Upstate. p21-siRNA (sequences were not revealed by the manufacturer) was from Cell Signaling Technology; control-siRNA was from Dharmacon; and Trans-IT TKO transfection reagent was from Mirus. Anti-p27^{Thr-187}

antibody was from Zymed, and [γ -³²P] ATP and enhanced chemiluminescence detection system were from Amersham Biosciences. Histone H1 was from Boehringer Mannheim Corp. SV total RNA isolation system kit, Dual Luciferase Reporter assay kit, and phRL-TK vector were from Promega.

Cell Growth and Cell Cycle Analysis

DU145 and 22Rv1 cells were from American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/mL penicillin/G-100 μ g/mL streptomycin, in a 5% CO₂ humidified atmosphere at 37°C. Cells were plated at a density of 5,000/cm² in 60-mm dishes and were treated the next day with different doses of silibinin in DMSO. After the desired treatment time, cells were harvested and analyzed for total cell number and dead cell population by trypan blue exclusion method, or cell cycle distribution by flow cytometry after overnight staining with saponin/propidium iodide solution as described earlier (4).

Western Blotting, and Immunoprecipitation and Kinase Assays

After desired treatments, cells were harvested and lysates were prepared in nondenaturing lysis buffer as reported earlier (4). For immunoblotting, 50 to 80 μ g of protein per sample were denatured in 2 \times SDS-PAGE sample buffer and subjected to SDS-PAGE using Tris-glycine gels; the separated proteins were then transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween 20) for 1 h, and then incubated with the desired primary antibody overnight at 4°C and finally incubated with peroxidase-conjugated appropriate secondary antibody and visualized by ECL detection system.

For immunoprecipitation and kinase activity studies, lysates (500 μ g protein) were precleared with A/G-plus agarose beads for 2 h, and then Cdk4, Cdk2, and Skp2 were immunoprecipitated from the cleared lysates by overnight incubation at 4°C with specific antibody and A/G-plus agarose beads. In control immunoprecipitation, normal rabbit IgG was used with A/G-plus agarose beads, instead of an antibody for Cdk2 or Skp2. The beads with immunoprecipitates were washed thrice with lysis buffer, boiled in 2 \times sample buffer, centrifuged, and supernatants were subsequently processed as in Western blotting. Cdk2 and Cdk4 kinase activity assays were done as described earlier (32).

siRNA Transfection

Cells were plated to a confluency of 50% to 60% and were transfected with 50 nmol/L of p21- and/or p27-siRNAs, Skp2-siRNA, or control-siRNA for 24 h using Trans-IT TKO transfection reagent as per manufacturer's protocol. The cells were subsequently treated with DMSO or 100 μ mol/L silibinin for 24 h in fresh medium. The extent of knockdown of corresponding protein level was assessed by Western blotting.

BrdUrd Incorporation Assay

After 24 h of treatment with DMSO or 100 μ mol/L silibinin, cells were labeled with 50 μ mol/L BrdUrd solution for 1 h and then harvested by trypsinization. Cells

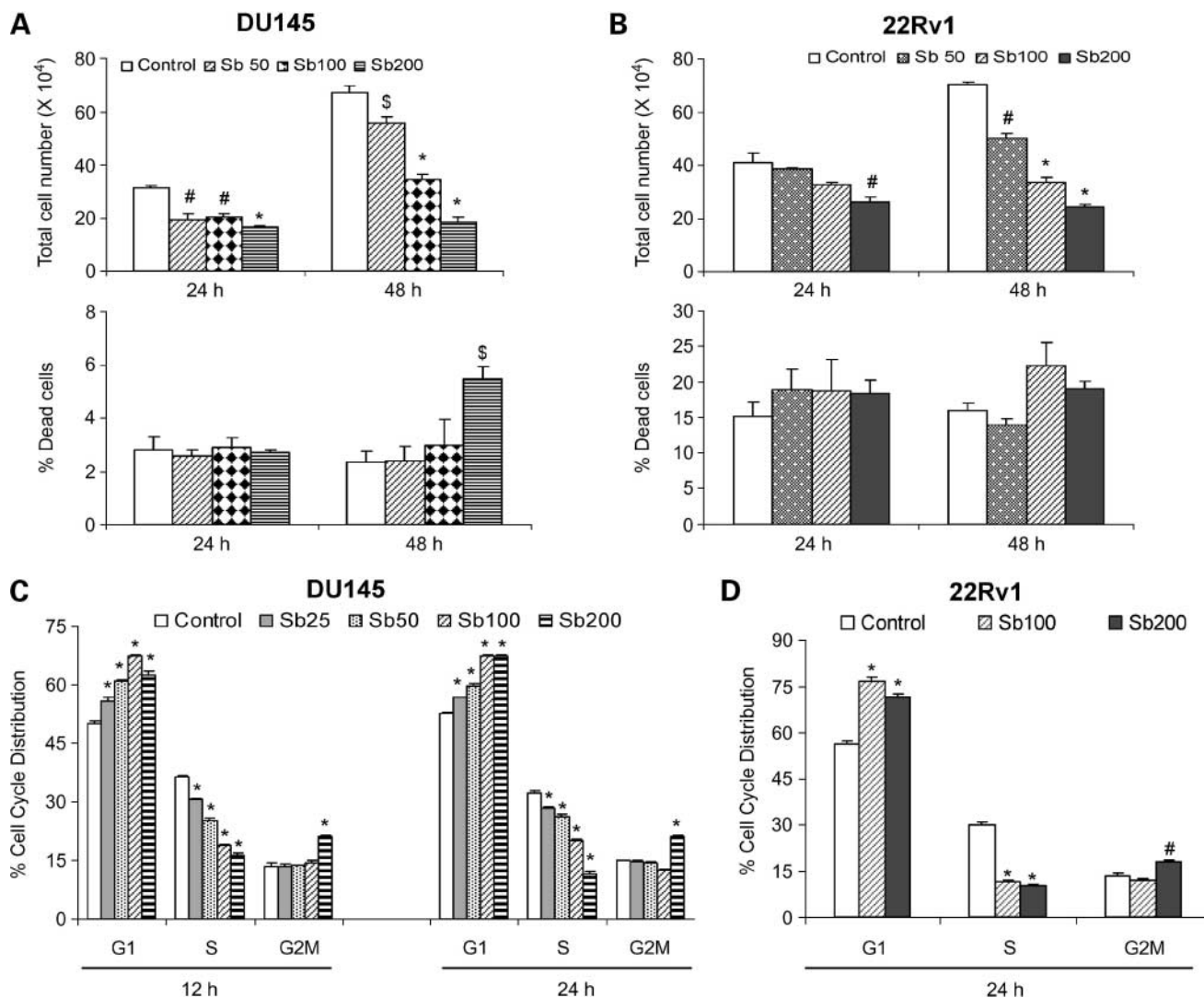


Figure 1. Silibinin inhibits cell growth and induces prominent G₁ arrest in human prostate cancer cells. **A** and **B**, DU145 and 22Rv1 cells were treated with either DMSO or increasing doses of silibinin (50–200 μmol/L) for 24 and 48 h and total number of cells and percentage of dead cells were determined by trypan blue exclusion method. **C**, DU145 cells were treated with 25 to 200 μmol/L doses of silibinin for 12 to 24 h. **D**, 22Rv1 cells were treated with 100 and 200 μmol/L doses of silibinin for 24 h. The percentage of cells in the different phases of the cell cycle was determined by flow cytometry. Columns, mean of three independent treatment samples and were reproducible in additional one to two independent experiments; bars, SE. \$, $P < 0.05$; #, $P < 0.01$; *, $P < 0.001$ versus DMSO control. Sb, silibinin.

were fixed with ice-cold ethanol for 2 h and collected by centrifugation at 4,000 rpm. Cells were next incubated in 2 N HCl at room temperature for 30 min and then neutralized with 0.1 mol/L sodium tetraborate (pH 8.5). The cells were centrifuged and resuspended in PBS to a final concentration of 1×10^6 /mL and incubated with 20 μL of anti-BrdUrd-FITC antibody for 45 min. Cells were washed with PBS-Tween 20 (0.5%), and then resuspended in 25 μg/mL propidium iodide solution and the percentage of BrdUrd incorporation was analyzed by fluorescence-activated cell sorting.

Reporter Assay

The p21-Firefly luciferase reporter construct was prepared as described by Xiao et al. (33). The p27-LUC reporter

plasmid, which contains -3,568 to -12 bp of the p27 promoter 5' to LUC was a kind gift from T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). To assess the transcriptional activity of p21 and p27 promoters, the cells were plated at a density of 1.5×10^5 per plate in 35-mm dishes, and next day were transfected with 1.5 μg of p21- or p27-Firefly luciferase reporter construct along with 0.15 μg phRL-TK Renilla luciferase as an internal control. The next day, cells were treated with DMSO or 100 μmol/L silibinin for 12 to 36 h, and Firefly and Renilla luciferase activities were analyzed by using Dual-Luciferase reporter assay system (Promega). The Firefly luciferase activity was normalized with the Renilla luciferase activity and protein concentration.

Real-time Reverse Transcription-PCR

Total RNA was isolated using SV total RNA isolation system. The mRNA levels for p21 and p27 were measured by real-time quantitative reverse transcription-PCR using ABI PRISM 7700 at the Molecular Biology Core Facility of the University of Colorado Cancer Center. The primers used were 5'-TGGAGACTCTCAGGGTCGAAA-3' and 5'-CGGCGTTTGGAGTGGTAGAA-3' for p21 and 5'-CCGGTGGACCACGAAGAGT-3' and 5'-GCTCGCCTCTTCCATGTCTC-3' for p27. Briefly, reactions were done in a 50 μ L reaction volume containing 8% glycerol; 1 \times TaqMan buffer A [500 mmol/L KCl, 100 mmol/L Tris-HCl, 0.1 mol/L EDTA, and 600 nmol/L passive reference dye ROX (pH 8.3)]; 300 μ mol/L each of dATP, dGTP, and dCTP; 600 μ mol/L dUTP; 5.5 mmol/L MgCl₂; 900 nmol/L each of forward and reverse primers; 200 nmol/L probe; 1.25 units AmpliTaq Gold DNA polymerase (Perkin-Elmer); 12.5 units Moloney murine leukemia virus reverse transcriptase (Invitrogen Corporation); 20 units RNasin RNase inhibitor (Promega); and template RNA (2 μ g). First, reverse transcription was done at 48°C for 30 min followed by activation of TaqGold at 95°C for 10 min and subsequent 40 cycles of amplification at 95°C for 15 s and 60°C for

1 min. A standard curve was generated using fluorescent data from 10-fold serial dilutions of control RNA, which was then used to calculate relative amounts of p21 and p27 in test samples. Quantities of p21 and p27 mRNA in each sample were normalized to the corresponding 18S rRNA levels.

Proteasomal Activity in Whole-Cell Extract

Whole-cell extracts (20 μ g) from DU145 cells were incubated at 37°C for 45 min with 40 μ mol/L of the fluorogenic peptide substrate Suc-Leu-LeuVal-Tyr-AMC, for chymotrypsin-like proteasomal activity, in 200 μ L of proteasome assay buffer [50 mmol/L Tris (pH 7.4), 5 mmol/L MgCl₂, DTT, 5 mmol/L ATP]. The fluorescent intensity of the 7-amido-4-methyl coumarin products thus formed was measured using a multiwell plate reader SpectraMAX (Molecular Devices) at excitation 380 nm and emission 460 nm.

Statistical Analysis

Statistical analysis was done using SigmaStat 2.03 software (Jandel Scientific). Data were analyzed using one-way ANOVA and a statistically significant difference was considered to be present at $P < 0.05$. The immunoblots/autoradiograms were scanned by Adobe Photoshop 6.0 (Adobe Systems, Inc.), and the mean density of each

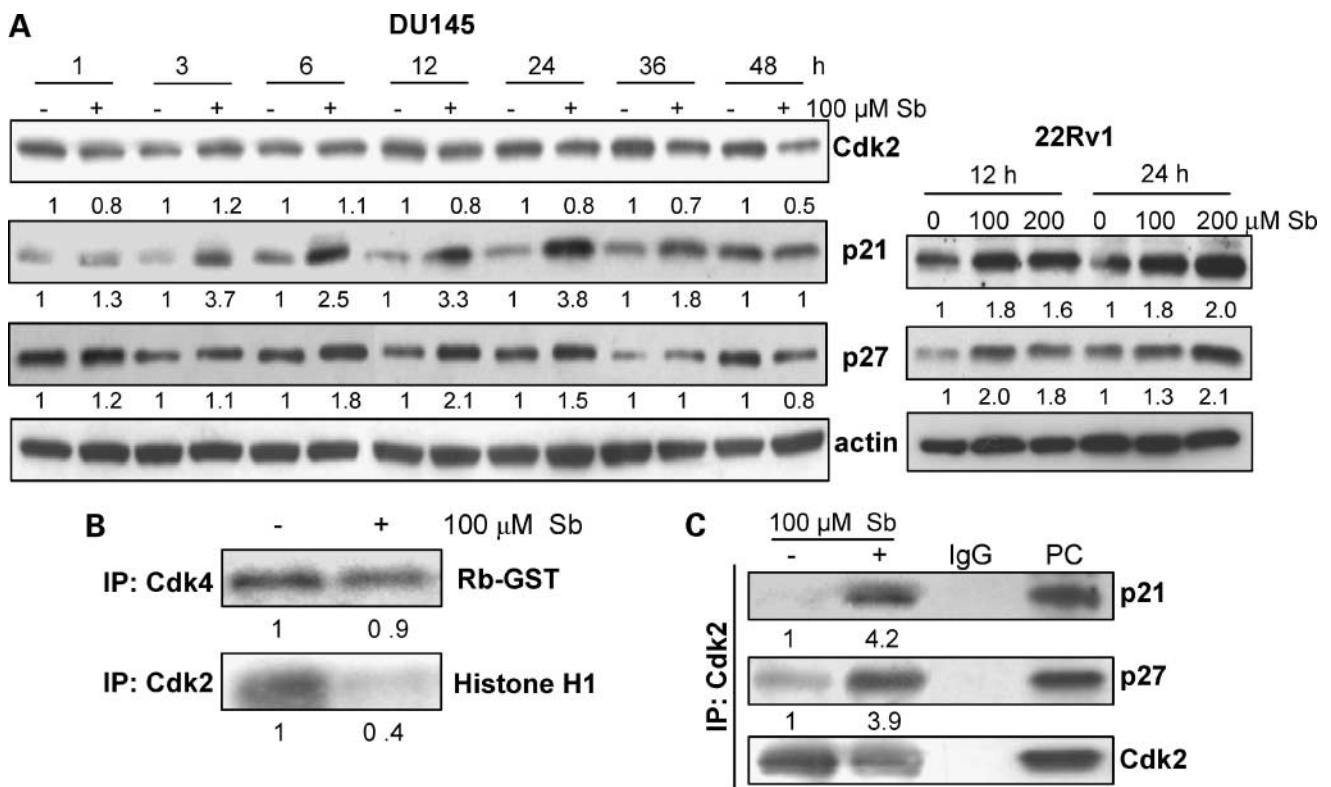


Figure 2. Silibinin modulates the protein expression of G₁-associated cell cycle molecules in DU145 cells. **A**, DU145 cells were treated with DMSO or 100 μ mol/L silibinin for 1 to 48 h, and 22Rv1 cells were treated with DMSO, 100 or 200 μ mol/L silibinin for 12 and 24 h. Western immunoblotting was done for Cdk2, p21, p27, and the membranes were reprobed for actin to confirm equal protein loading. **B**, DU145 cells were treated with DMSO or 100 μ mol/L silibinin for 24 h and Cdk4 and Cdk2 kinase activity in the cell extracts were analyzed *in vitro* using Rb-GST and histone H1 as their respective substrates. **C**, cell extracts were immunoprecipitated with anti-Cdk2 antibody or normal rabbit IgG (negative control) and immunoblotted for p21, p27, and Cdk2. Positive control (PC) represents cell lysate that was not subjected to immunoprecipitation. Data in each panel are representative of at least two independent experiments.

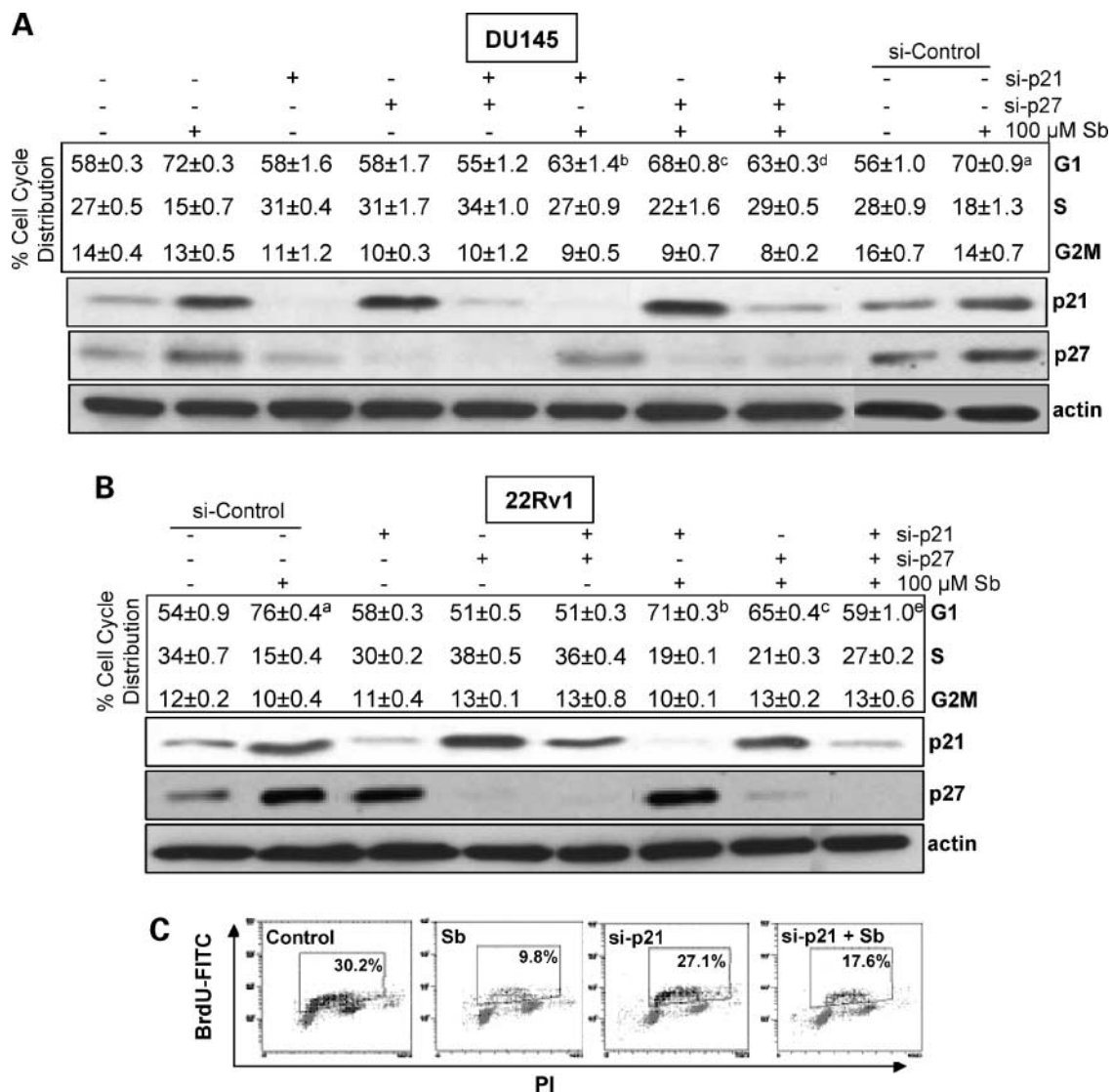


Figure 3. Silibinin fails to induce prominent G₁ arrest in cells transiently transfected with p21 and/or p27 siRNAs. **A**, DU145 cells were either untransfected or transiently transfected with control-, p21-, and/or p27-siRNAs and then treated with DMSO or 100 μ mol/L silibinin for 24 h. Cell cycle distribution was quantitated by fluorescence-activated cell sorting analysis (*top*). Western blotting for p21 and p27 was done to show the efficiency of transfection in p21 and/or p27 knockdown in DU145 cells (*bottom*). **B**, 22Rv1 cells were transfected with control, p21-, and/or p27-siRNAs; after identical treatment conditions as in DU145 cells, cell cycle distribution (*top*) and Western blotting (*bottom*) were done. **C**, fluorescence-activated cell sorting analysis of BrdUrd-FITC positive cells was done in untransfected and p21-siRNA-transfected cells treated with or without silibinin for 24 h. The quantitative data in each panel are mean \pm SE of three independent treatment samples, and were reproducible in an additional independent experiment. The immunoblot results in each panel are representative of at least two independent experiments. *a*, $P < 0.001$ versus si-Control; *b*, $P < 0.001$ versus si-p21; *c*, $P < 0.001$ versus si-p27; *d*, $P < 0.001$ versus si-p21 + p27; *e*, $P < 0.05$ versus si-p21 + p27. si-p21, p21-siRNA; si-p27, p27-siRNA; si-control, control-siRNA.

band was analyzed by Scion Image program (NIH). Unless specified otherwise, the densitometry data presented below in each band represent fold change compared with the corresponding control.

Results

Silibinin Inhibits Cell Growth and Induces G₁ Arrest in DU145 and 22Rv1 Cells

We first examined the effect of silibinin on cell growth and cell death in prostate cancer cells by trypan blue

exclusion assay. Silibinin treatment (50–200 μ mol/L) for 24 and 48 h resulted in a dose-dependent manner in both DU145 and 22Rv1 cells. In DU145 cells, silibinin caused 36% to 47% ($P < 0.01$ – 0.001) and 17% to 72% ($P < 0.05$ – 0.001) inhibition in cell growth in terms of a reduction in cell number after 24 and 48 h treatments, respectively (Fig. 1A). In 22Rv1 cells, under similar treatment conditions, the growth inhibition was 6% to 36% ($P < 0.001$) and 28% to 65% ($P < 0.01$ – 0.001) in 24 and 48 h treatments, respectively (Fig. 1B). No significant

induction in cell death was observed in either cell line by these silibinin treatments, except at 48 h with 200 $\mu\text{mol/L}$ dose in DU145 cells showing 5.5% cell death compared with 2.3% in vehicle control.

To identify the underlying mechanism of silibinin-mediated growth arrest, we next treated asynchronously growing DU145 cells with 25 to 200 $\mu\text{mol/L}$ doses of silibinin, and then stained with saponin/propidium iodide and cell cycle distribution was analyzed. Silibinin treatments induced strong G_1 arrest ($P < 0.001$) in DU145 cells in a dose-dependent manner (Fig. 1C), which was consistent with earlier observations (34). Analyses of the results showed that DU145 cells started accumulating in G_1 phase between 12 and 24 h at the expense of a dose-dependent decrease ($P < 0.001$) in S-phase population. The maximum effect (31% increase in G_1 population by silibinin over vehicle control, $P < 0.001$) was observed in 24 h treatment with 100 $\mu\text{mol/L}$ dose with no additional increase in G_1 population at higher dose (200 $\mu\text{mol/L}$), although the cells also showed a moderate G_2 -M arrest ($P < 0.001$) at this dose (Fig. 1C). Comparable observations were also evidenced in 22Rv1 cells where maximum effect (41% increase in G_1 population by silibinin over vehicle control, $P < 0.001$) was observed with 100 $\mu\text{mol/L}$ silibinin treatment for 24 h, and that higher dose once again also showed a moderate G_2 -M arrest ($P < 0.01$) (Fig. 1D). The observed cell cycle arrest effects of silibinin in 22Rv1 cells were at the expense of a decrease ($P < 0.001$) in S-phase population. Based on all these cell cycle arrest effects of silibinin in both DU145 and 22Rv1 cells, a 100 $\mu\text{mol/L}$ dose of silibinin was selected for further mechanistic studies.

Silibinin Induces p21 and p27 Levels, but Decreases Protein Level and Kinase Activity of Cdk2

A time kinetics study was done for the cell cycle regulatory proteins Cdk2, p21, and p27; the selection of these molecules was based on our earlier studies where silibinin treatment showed the most prominent changes in their protein levels in DU145 cells (34). In the present study, protein level of Cdk2 started decreasing at 12 h of silibinin treatment and by 48 h, there was a 50% reduction in Cdk2 levels (Fig. 2A). On the other hand, silibinin treatment of cells for 1 to 48 h showed a strong increase (3.7-fold) in p21 protein level within 3 h that remained comparably sustained up to 24 h (Fig. 2A). In case of p27, its protein level started to increase within 6 h of silibinin treatment and remained sustained until 24 h before declining to control levels at later time points (Fig. 2A). In 22Rv1 cells, silibinin induced p21 and p27 levels by up to 2.1- and 2.0-fold, respectively (Fig. 2A). The induction in protein levels of these CDKIs is probably p53 independent because DU145 cells harbor mutant p53 and earlier studies have shown that silibinin does not affect wild-type p53, although induced both p21 and p27 levels, in LNCaP cells (35).

Because transit from G_1 phase of cell cycle is primarily determined by the sequential activation of Cdk4 and Cdk2 in early and mid/late G_1 phase (9), respectively, the kinase activity of these two serine-threonine kinases in DU145 cells was next assessed after silibinin treatment. Whereas

Cdk4 kinase activity was minimally altered by silibinin treatment in present experimental conditions, Cdk2 kinase activity was reduced by $\sim 63\%$ after 24 h of silibinin treatment (Fig. 2B), suggesting that silibinin possibly mediates late- G_1 arrest in DU145 cells. Because physical association of Cdks with their corresponding CDKIs (p21 and p27 for Cdk2) plays a primary role in inhibiting their kinase activity (14), we next examined the binding of Cdk2 with p21 and p27. We observed that silibinin treatment increases the association of Cdk2 with both p21 and p27 by 4.2- and 3.9-fold, respectively, without any nonspecific pull downs in immunoprecipitation controls (Fig. 2C), which further suggested a possible role of p21 and p27 in silibinin-caused G_1 arrest.

p21 and p27 Play Central Roles in Silibinin-Caused G_1 Arrest

To dissect the roles of p21 and p27 in silibinin-caused G_1 arrest, expression of these two molecules was reduced using RNA interference. p21- and/or p27-depleted cells were then treated with 100 $\mu\text{mol/L}$ silibinin for 24 h. Western blot analyses of cell lysates showed that siRNAs targeted against p21 and p27 reduced their protein levels by 91% and 84%, respectively (Fig. 3A) in DU145 cells, and by 51% and 93%, respectively, in 22Rv1 cells (Fig. 3B). However, knocking down the expression of p21 and p27 proteins reversed silibinin-induced G_1 arrest to different degrees in these cell lines. In DU145 cells, silibinin induced comparable increase in G_1 population in untransfected and control-siRNA transfected cells (31% and 27% increases over their respective controls), but caused only 8.5% and 17% increase in G_1 population in p21- and p27-siRNA-transfected cells, respectively (Fig. 3A, top), indicating a significant ($P < 0.001$) reversal in G_1 arrest response of silibinin when these CDKIs are individually knocked down. Because knocking down either p21 or p27 significantly reversed, but did not completely abolish, silibinin-caused G_1 arrest, there was a possibility for an overlapping effect of p21 and p27 for each other specifically when one of them was knocked down. To address this issue, we next knocked down both of them and then assessed silibinin effect on G_1 arrest. As shown in Fig. 3A, we did not observe any additional reversal in silibinin-induced G_1 arrest when both these molecules were knocked down compared with the results obtained for p21 alone knockdown experiment. Interestingly, silencing p27 expression markedly induced p21 level, whereas p21 protein knockdown did not have any effect on p27 expression. This might explain why knocking down p21 shows a more prominent reversal of G_1 arrest compared with p27 knockdown in DU145 cells. However, why induction of p21 in p27-siRNA-transfected cells, in the absence of silibinin treatment, did not affect the cell cycle distribution is not clear at this stage and needs further investigations.

In 22Rv1 cells, silibinin caused 41%, 20%, and 26% increase in G_1 arrest over respective control-, p21- and p27-siRNA-transfected cells (Fig. 3B, top). However, in this case, knocking down both p21 and p27 together showed an additional reversal in silibinin-caused G_1 arrest where it

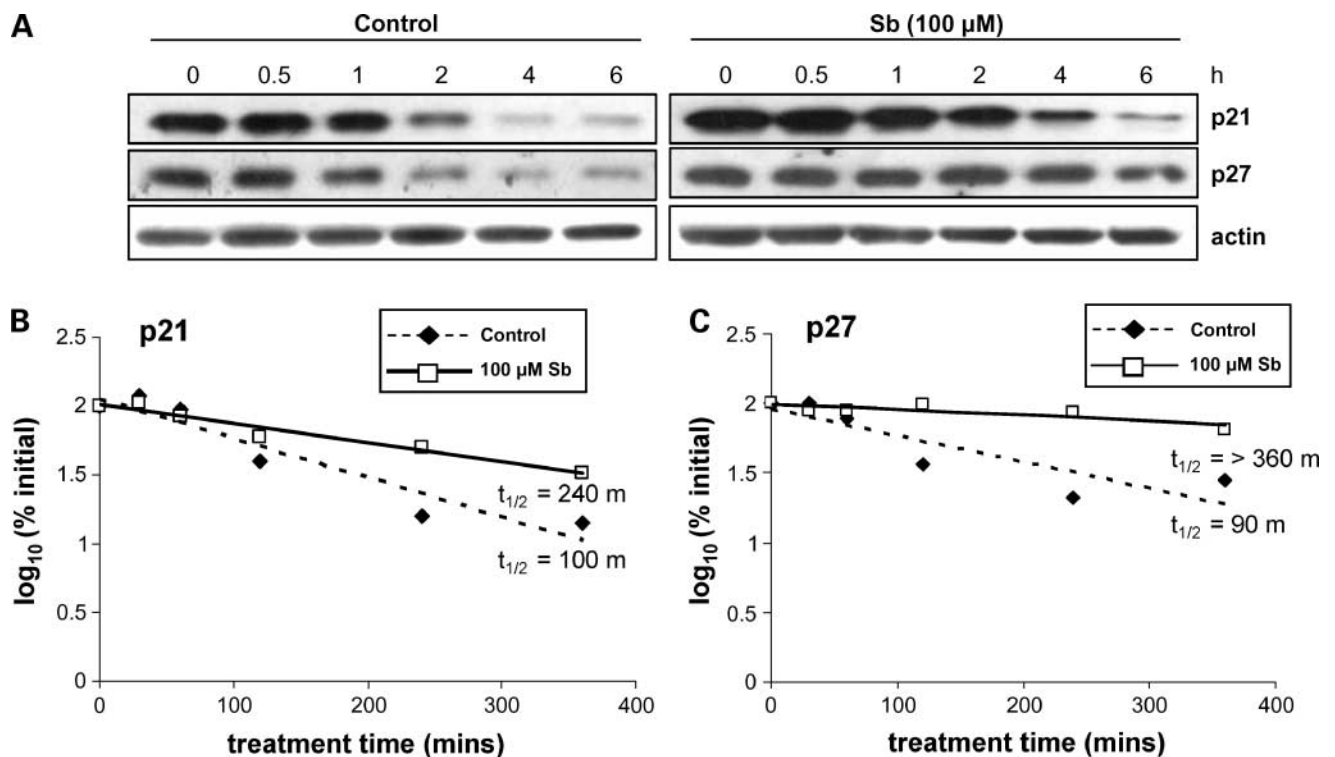


Figure 4. Silibinin treatment induces p21 and p27 protein stabilization. **A**, DU145 cells were treated with 100 $\mu\text{mol/L}$ dose of silibinin for 12 h and then exposed to 5 $\mu\text{g/mL}$ cycloheximide for 0 to 6 h, and the cell lysates were immunoblotted for p21 (*top*) and p27 (*middle*). The membranes were reprobed for actin to correct for protein loading (*bottom*). **B** and **C**, the protein levels of p21 and p27 at the different time points, in both treated and untreated cells, were quantified by densitometric analysis and calculated as percentage of the initial protein level (time 0 h). \log_{10} of the percentage was plotted against time and the $t_{1/2}$ values shown in each case were calculated as the time corresponding to the \log_{10} of 50%.

showed only 15% increase in G_1 population over the control samples (Fig. 3B, *top*). Western blot analyses of various samples showed that knocking down p21 induces p27 level and vice versa, indicating a complementary role of these CDKIs in 22Rv1 cells, which is in accord with the results where silencing them together showed an additional reversal of silibinin-caused G_1 arrest. Taken together, whereas these studies underline the importance of Cip/Kip proteins in silibinin-induced G_1 arrest, they also show that the relative contribution of these inhibitors might be cell line specific.

Because the reversal of silibinin-induced G_1 arrest by p21 or p27 siRNA was associated with a corresponding increase in the S-phase population, BrdUrd incorporation assay was next done to consolidate the fact that the cells coming out of G_1 phase are actually participating in DNA replication. Silibinin treatment of DU145 cells reduced BrdUrd-positive S-phase cells by $\sim 66\%$; however, after p21-siRNA transfection and then silibinin treatment, the reduction in BrdUrd population was only 35% (Fig. 3C), suggesting that an absence of p21 protein level indeed facilitates silibinin-treated cells to enter S phase and participate in DNA synthesis.

Silibinin Increases Protein Half-Lives of Both p21 and p27

Cellular levels of CDKIs are intricately regulated at the levels of protein synthesis and degradation. To determine

the mechanism of p21 and p27 up-regulation by silibinin, we first focused on its effect on p21 and p27 protein stability by performing cycloheximide chase experiments. DU145 cells were pretreated with DMSO or 100 $\mu\text{mol/L}$ silibinin for 12 h, and then protein synthesis was blocked by the addition of 5 $\mu\text{g/mL}$ cycloheximide and relative protein levels in both treatments were subsequently analyzed over a time period by immunoblotting (Fig. 4A). Quantitative densitometric analysis of the immunoblots showed an altered pattern of p21 and p27 degradation in silibinin-treated cells compared with their corresponding controls. The half-life ($t_{1/2}$) of p21 protein was increased from 100 min in controls to 240 min in silibinin-treated cells (Fig. 4A and B), and this effect was even stronger in case of p27 where silibinin increased the $t_{1/2}$ to >360 min compared with $t_{1/2} \sim 90$ min in controls (Fig. 4A and C).

Silibinin Regulates p21 and p27 Protein Levels in a Skp2-Dependent Manner

The results from cycloheximide chase experiments suggested protein stabilization of p21 and p27 by silibinin. Treatment of DU145 cells with 100 $\mu\text{mol/L}$ silibinin for 3 to 24 h did not show any significant alteration in the chymotrypsin-like (Fig. 5A) or trypsin-like (data not shown) proteasomal activity compared with their corresponding controls, whereas MG132 (used as a positive control) showed prominent effect ($\sim 70\%$ inhibition in 12 h; Fig. 5A).

Furthermore, silibinin treatment of cells for various times up to 24 h resulted in marginal decrease in phosphorylated Thr¹⁸⁷ p27 levels (Fig. 5B); however, because total p27 concomitantly increases with silibinin treatment, phosphorylated Thr¹⁸⁷ p27 levels were normalized with total p27 levels, which showed a remarkable decrease in the effective phosphorylated Thr¹⁸⁷ p27 levels (~60% reduction by 24 h; Fig. 5B). In other studies, silibinin also significantly decreased protein level of Skp2 starting as early as 3 h of its treatment and by 24 h, a 60% reduction in Skp2 level was observed (Fig. 5B). The functional significance of this observation was next studied using immunoprecipitation assay that showed 20% and 36% (after normalization to their

corresponding Skp2 levels) decreased association of both p21 and p27 with Skp2 after silibinin treatment, respectively, without any nonspecific pull downs in immunoprecipitation controls (Fig. 5C).

To further delineate the importance of Skp2 decrease by silibinin, we knocked down Skp2 protein level by RNA interference. Immunoblotting showed that 50% reduction in basal level of Skp2 in Skp2-siRNA-transfected cells (Fig. 5D, bottom). This was accompanied by a decreased S-phase population (30% S-phase population in Skp2-siRNA-transfected cells compared with 37% in control-siRNA-transfected cells) and an increased G₁ population, which underlined the importance of Skp2 in driving G₁-S

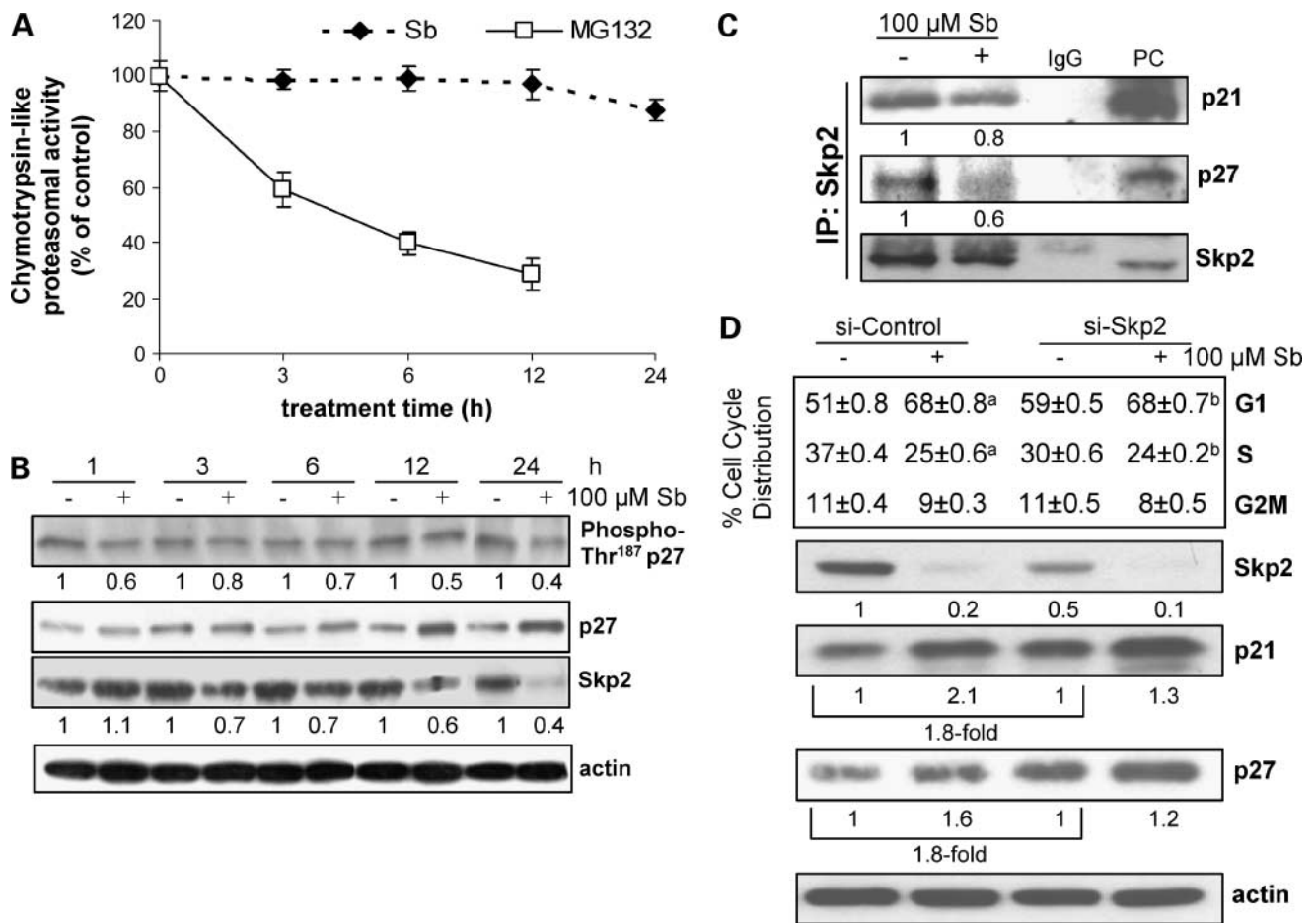


Figure 5. Silibinin treatment strongly reduces Skp2 protein levels and its interaction with p21 and p27. **A**, DU145 cells were treated with 100 μmol/L silibinin or 10 μmol/L MG 132 for 1 to 24 h and then processed for proteasome activity. The proteasomal activity for silibinin- and MG132-treated cells at various times was expressed as percentage of their corresponding controls. The proteasomal activity for MG132-treated cells at 24 h is not shown as there were no viable cells at that time. Points, mean of three independent observations; bars, SE. **B**, DU145 cells were treated with either DMSO or 100 μmol/L silibinin for 1 to 24 h. Western immunoblotting was done for phosphorylated Thr¹⁸⁷ p27, p27, and Skp2, and the membranes were reprobed for actin. The relative changes in phosphorylated p27 levels were normalized to total p27 protein levels. **C**, cellular lysates from DU145 cells, treated with 100 μmol/L silibinin for 12 h, were immunoprecipitated with anti-Skp2 antibody or normal rabbit IgG, and immunoblotted for p21, p27, and Skp2. **D**, DU145 cells were transfected with control- or Skp2-siRNA for 24 h and then treated with DMSO or 100 μmol/L silibinin for 24 h. Cell cycle distribution was quantitated by fluorescence-activated cell sorting analysis (top) and Western blotting was done for Skp2, p21, and p27 (bottom). The densitometric analyses data are either between DMSO and silibinin for both control or Skp2-siRNA-transfected cells, or between DMSO-treated samples to show an increase in p21 and p27 by Skp2-siRNA transfection alone. Positive control represents cell lysate that was not subjected to immunoprecipitation. Data are representative of at least two independent experiments. *a*, $P < 0.001$ versus si-Control; *b*, $P < 0.001$ versus si-Skp2.

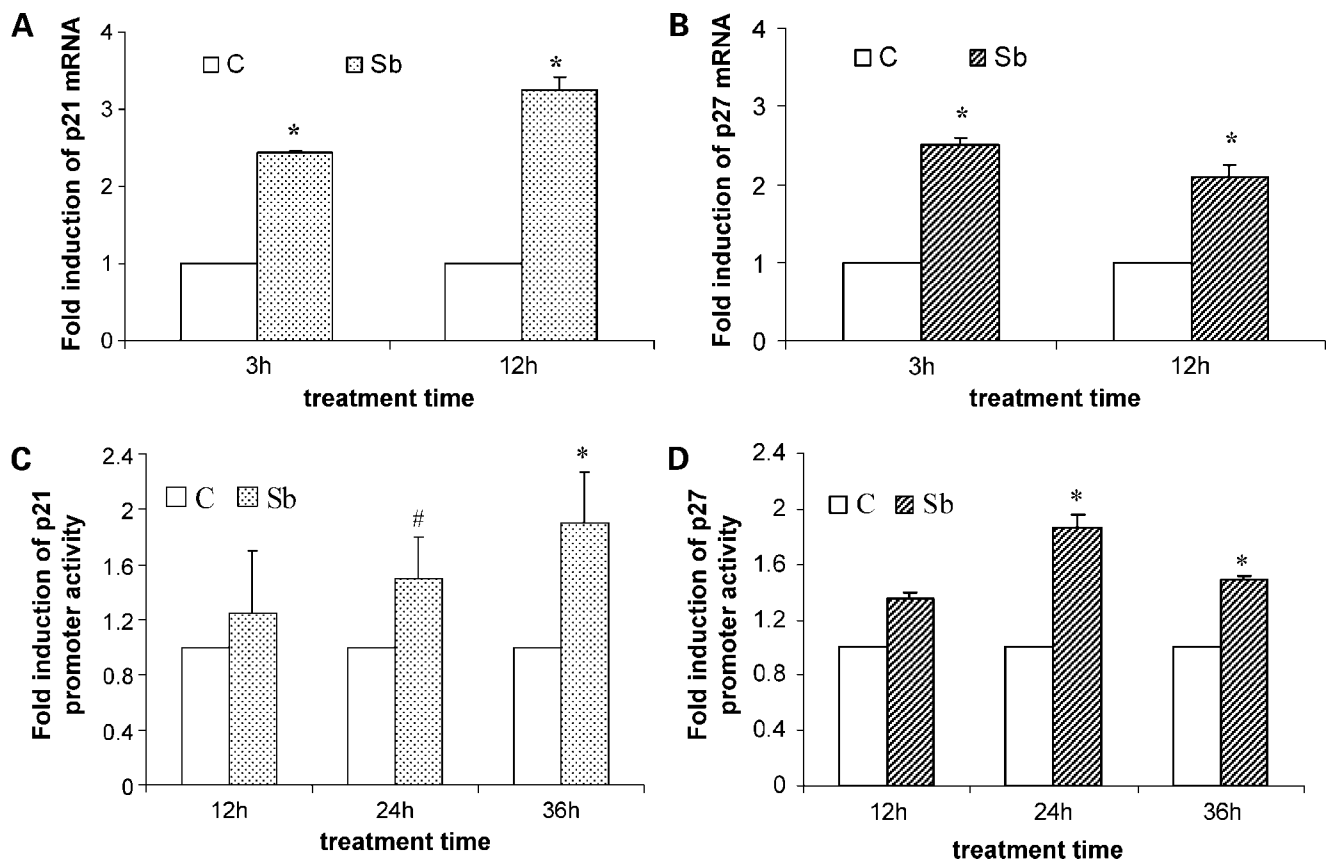


Figure 6. Silibinin treatment increases mRNA levels and stimulates promoter activity of both p21 and p27. **A** and **B**, DU145 cells were treated with 100 $\mu\text{mol/L}$ silibinin for 3 and 12 h and the mRNA levels of p21 and p27 were estimated by real-time RT-PCR and normalized with the rRNA. **C** and **D**, DU145 cells were transfected with full-length firefly luciferase-p21 or firefly luciferase p27 promoter constructs as well as pHRL-TK reporter construct, treated with silibinin for 12 to 36 h, and assayed for luciferase activity. The graphs represent luciferase activity in each sample, normalized for efficiency of transfection and protein expression. Columns, mean of three independent observations; bars, SE. #, $P < 0.05$; *, $P < 0.001$.

transition (Fig. 5D, top). Interestingly, silibinin-mediated G_1 -S block was significantly attenuated in Skp2-knockdown cells [33% increase in G_1 cell population ($P < 0.001$) by silibinin in control-siRNA-transfected cells compared with only 15% increase in G_1 cell population ($P < 0.001$) in Skp2-siRNA-transfected cells], suggesting a key role of Skp2 in silibinin-induced G_1 arrest (Fig. 5D, top). To further validate our results that silibinin-mediated Cip/Kip regulation is via Skp2 modulation, we immunoblotted for p21 and p27 protein levels under these treatment conditions. We observed that knocking down Skp2 levels by itself resulted in an increased expression of Cip/Kip proteins (1.8-fold increase compared with control-siRNA-transfected cells), which correlated with an increased G_1 population in these cells. However, silibinin treatment failed to increase p21 and p27 protein expression in Skp2-siRNA-transfected cells (only 1.3- and 1.2-fold increase, respectively, over vehicle control) to the same extent as in control-siRNA-transfected cells (2.1-fold increase in p21 and 1.6-fold increase in p27 levels in control-siRNA cells; Fig. 5D, bottom). These results emphasize that Skp2 is an important target of silibinin for the stabilization of Cip/Kip proteins as well as for cell cycle arrest in DU145 cells.

Silibinin Also Increases mRNA Levels and Promoter Activity of Both p21 and p27

Several studies have shown that p21 and p27 levels can be regulated at transcriptional, translational, and posttranslational levels (29, 30, 36, 37). In our studies, we found that p27 protein half-life was increased significantly, whereas p21 protein half-life increased by up to 4 h only. This extent of increase in p21 half-life might be insufficient to explain sustained elevated p21 protein levels as observed with silibinin treatment. In previous studies by Agarwal et al. (1), silibinin was found to increase p21 and p27 mRNA levels in HT-29 colon cancer cells. Accordingly, using real-time RT-PCR, we next investigated whether silibinin also induces these molecules in DU145 cells at the transcriptional level. Both p21 and p27 mRNA levels were induced as early as 3 h, with maximum induction of 3.5-fold (Fig. 6A) and 2.3-fold (Fig. 6B) after 12 h of silibinin treatment, respectively. Longer silibinin treatment did not show any additional increase in either p21 or p27 mRNA levels (data not shown). To further assess whether the accumulation of the mRNA levels is due to increased transcription, we studied the effect of silibinin treatment on

luciferase reporter constructs of p21 and p27 promoters. A time kinetics study showed that silibinin treatment results in the stimulation of p21 promoter activity, in a time-dependent manner (Fig. 6C), and also of p27 promoter activity with maximum effect at 24 h (Fig. 6D). A very early (3 h) induction of mRNA levels, together with stimulation of promoter activity at relatively later time points (12–36 h), implicates that posttranscriptional mechanism(s) might be playing a predominant role than transcriptional mechanisms in overall up-regulation of p21 and p27 levels by silibinin.

Discussion

Silibinin has shown promising efficacy against several cancer models, especially prostate cancer where following the completion of phase I dose-escalation study recently (10), it is currently in a phase II pilot clinical trial in prostate cancer patients; however, the molecular targets that play a central role in silibinin efficacy are not well-established. For the first time in the present study, our findings convincingly show that an up-regulation of p21 and p27 is the most important and central event in silibinin-induced G₁ arrest in both androgen-dependent and androgen-independent human prostate carcinoma cells. It is important to highlight here that silibinin showed all its significant biological effects at 100 μmol/L concentration, which are directly relevant to our recently completed phase I clinical trial showing that peak plasma levels of free silibinin achievable in prostate cancer patients are in excess of 100 μmol/L (10). Taken together, the results of the present study and the completed phase I clinical trial convincingly represent the practical translation of the findings of our *in vitro* studies with silibinin into the clinical settings. Furthermore, based on the findings of the present study showing that p21 and p27 up-regulation plays a central role in silibinin efficacy in human prostate cancer cells irrespective of their androgen dependence, in our on-going phase II clinical trial in prostate cancer patients, our major focus is to analyze the levels of p21 and p27 as molecular markers of silibinin efficacy in human prostate cancer tissue collected after prostatectomy from the patients who are on silibinin treatment. The other biomarkers to be studied in this phase II clinical trial are Rb phosphorylation status and proliferating cell nuclear antigen staining. These selections are based on the fact that after their up-regulation; p21 and p27 negatively regulate the kinase activity of the CDKs that alters the phosphorylation of Rb protein, keeping it in hypophosphorylated form and bound to transcription factor E2Fs (38). The Rb-bound E2Fs are inactive and therefore fail to activate their downstream target genes involved in cell cycle progression and DNA replication, specifically proliferating cell nuclear antigen (38). Accordingly, both Rb phosphorylation status and proliferating cell nuclear antigen staining are the logical downstream targets of p21 and/or p27 up-regulation, and therefore are part of our marker studies in phase II clinical trial.

As mentioned earlier, studies done by us have revealed that silibinin induces marked G₁ arrest in both androgen-dependent and androgen-independent prostate cancer cells, accompanied by changes in the expression and activity of several cell cycle molecules associated with G₁-S transition (34). Hence, further studies were needed to identify the primary target(s) of silibinin, which is instrumental in mediating the cell cycle arrest at G₁ phase. We selected two prostate cancer cell lines (DU145 and 22Rv1), which not only are representative of different stages of the malignancy but also differ in their status of p53 and Rb, the two very important molecular players controlling the proliferative potential of a given cell. Loss of Rb results in unrestrained proliferation and p53-mediated apoptosis via activation of E2F1 and its responsive genes (38, 39). Regarding prostate cancer, loss of Rb function due to loss of heterozygosity of *Rb* locus (13q14) has been observed in 60% clinical cases (40); thus, it became pertinent to conduct our mechanistic studies in cell lines differing in the status of Rb as well as p53. DU145 cells are androgen insensitive and harbor both p53 and Rb mutations. 22Rv1 cells are androgen-sensitive and have wild-type p53 and Rb. Irrespective of these molecular differences, silibinin induced comparable growth inhibition and cell cycle arrest in both the cell lines, suggesting that its effects are mediated through certain specific molecular targets that remain functional in both early and advanced stages of prostate cancer. Of all the G₁-associated cell cycle regulators affected by silibinin, in our study, p21 and p27 showed the earliest as well as the most prominent response to silibinin treatment and their time of induction preceded the onset of cell cycle arrest at the G₁ phase (~12 h). Together, these findings provided a strong rationale to further investigate the role and mechanism of p21 and p27 up-regulation in silibinin-induced G₁ arrest.

In our studies, the pivotal role of p21 and p27 induction in mediating silibinin-induced G₁ arrest was supported by siRNA experiments where knocking down p21 and/or p27 levels caused significant reversal of silibinin-induced G₁ arrest, although ablation of p21 compared with p27 had a greater effect on this reversal, indicating that p21 might be the prime/preferential target of silibinin. Whereas the exact mechanism for any preference of silibinin toward p21 as its main executor of G₁ arrest remains unclear, one possibility might be that in addition to CDKs, p21 regulates the activity of several other targets, for example, signal transducers and activators of transcription 3, proliferating cell nuclear antigen, etc. (41, 42), making its presence more crucial for observed silibinin effects. Once we identified that an up-regulation of p21 and p27 plays a central role in silibinin caused G₁ arrest, our next aim was to identify at what level these CDKs are up-regulated. As such, Cip/Kip proteins are extremely short-lived and their metabolic stability and timely degradation are crucial determinants of cell cycle progression. The p27 proteolytic pathway is well characterized and has two rate-limiting steps, namely phosphorylation of p27 at Thr¹⁸⁷ residue by cyclinE/A-Cdk2 complex in S phase and subsequent recognition of the

phosphorylated protein by Skp2 for ubiquitylation and proteasomal degradation (43, 44). Skp2 is considered as an oncoprotein that is elevated in transformed cells, and when overexpressed is found to be capable of driving G₁-S progression. Our studies showed a strong reduction in Skp2 levels in silibinin-treated DU145 cells. Moreover, knocking down Skp2 levels showed a marked attenuation in silibinin-mediated G₁ arrest and Cip/Kip induction, which implicates the role of Skp2 in CDKI regulation in this system. The role of Skp2 in p27 regulation is well defined. An inverse correlation of p27 and Skp2 expression has been observed in a variety of cancer tissues, including prostate cancer (44). Recent studies have also suggested a Thr¹⁸⁷ phosphorylation-independent proteasomal degradation of p27, which occurs at early G₁ phase in response to mitogenic signaling (45). Our findings showed that p27 protein half-life is significantly increased (>6 h) in silibinin-treated DU145 cells concomitant with a reduction in phosphorylated Thr¹⁸⁷ p27 levels and reduced binding of Skp2 to p27. Regarding p21, multiple proteolytic pathways have been proposed for its degradation depending on the experimental milieu, including its ubiquitylation by SCF-Skp2 complex (46, 47). Recent studies showed that p21 half-life is significantly greater in Skp2^{-/-} mouse embryonic fibroblasts compared with wild-type cells (48). However, the same study also showed that phosphorylation of p21 is not obligatory for its ubiquitylation. Moreover, p21 can also undergo proteasome-dependent but ubiquitin-independent degradation (46). Taking these reports together in the context of our findings, whereas silibinin treatment decreased Skp2 protein levels by up to 60% together with its decreased binding to p21, the half-life of p21 increased by only 4 h, suggesting that its turnover might still be maintained in an ubiquitin-independent manner in silibinin-treated cells.

Contrary to p27, which is mostly regulated through protein stabilization, p21 is widely regulated at both the transcriptional and posttranscriptional levels (27, 28, 37). To identify any additional mechanisms by which silibinin increases Cip/Kip protein levels, we also did quantitative RT-PCR studies and found that silibinin treatment increases mRNA levels of both p21 and p27. Further studies revealed that silibinin also stimulates promoter activity of both these CDKIs. However, the time of induction of mRNA by silibinin precedes the time of its effect on the promoter activity of both the molecules, indicating that transcriptional stimulation might be having a minor contribution to their mRNA accumulation, whereas additional posttranscriptional mechanism(s) such as message stabilization by RNA-binding proteins might also be involved. Furthermore, because part of the 5' end of the p21 and p27 mRNAs is in the reporter constructs of p21 and p27 promoters used in our promoter activity studies (33), the observed increase in promoter activity might in part be due to some translational control. Additional studies, however, are required in the future to determine the overall contribution of these mechanisms in silibinin-induced up-regulation of these CDKIs.

In summary, silibinin up-regulates the levels of p21 and p27 at both protein and mRNA levels and their up-regulation is indispensable in G₁ arrest effect of silibinin in human prostate cancer cells at the concentration that is physiologically achievable in prostate cancer patients. This highlights the significance of present findings in terms of using p21 and p27 as molecular markers and their downstream targets namely Rb phosphorylation status and proliferating cell nuclear antigen staining as additional markers in our on-going pilot phase II clinical trial with silibinin in prostate cancer patients.

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