Inhibition of Retinoblastoma Protein (Rb) Phosphorylation at Serine Sites and an Increase in Rb-E2F Complex Formation by Silibinin in Androgen-dependent Human Prostate Carcinoma LNCaP Cells: Role in Prostate Cancer Prevention 1 This work was supported in part by United States Army Medical Research and Materiel Command Prostate Cancer Program DAMD17-98-1-8588. In part, this work was done during the tenure of the authors at AMC Cancer Research Center, Denver, CO 80262. 1

Alpana Tyagi, Chapla Agarwal and Rajesh Agarwal

Mol Cancer Ther 2002;1:525-532.

Inhibition of Retinoblastoma Protein (Rb) Phosphorylation at Serine Sites and an Increase in Rb-E2F Complex Formation by Silibinin in Androgen-dependent Human Prostate Carcinoma LNCaP Cells: Role in Prostate Cancer Prevention[1]

Alpana Tyagi, Chapla Agarwal, and Rajesh Agarwal[2]

Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, Denver, Colorado 80262

Abstract

Several studies have identified silibinin as an anticarcinogenic agent. Recently, we showed that silibinin inhibits cell growth via G_1 arrest, leading to differentiation of androgen-dependent human prostate carcinoma LNCaP cells (X. Zi and R. Agarwal, Proc. Natl. Acad. Sci. USA, *96:* 7490–7495, 1999). Here, we extend this study to assess the effect of silibinin on total retinoblastoma protein (Rb) levels and its phosphorylation status, levels of E2F family members, and Rb-E2F binding in LNCaP cells. Compared with controls, silibinin resulted in an increase in total Rb levels that was largely attributable to an increase in unphosphorylated Rb (up to 4.1-fold). This effect of silibinin was mainly attributable to a large decrease (70–97%) in the amount of Rb phosphorylated at specific serine sites. In other studies, silibinin showed a moderate effect on E2F1 but up to 98 and 90% decreases in E2F2 and E2F3 protein levels, respectively. Silibinin treatments also resulted in an increase in the amount of Rb binding to E2F1 (3.8-fold), E2F2 (2.2-fold), and E2F3 (2.2-fold). Cyclin-dependent kinases (CDKs), together with their catalytic subunit cyclins, phosphorylate Rb, which makes transcription factor E2Fs free from Rb-E2F complexes, resulting in cell growth and proliferation. Conversely, CDK inhibitors inhibit this phosphorylation, maintaining E2Fs bound to Rb, which causes growth inhibition. On the basis of our data showing that silibinin induces both unphosphorylated Rb levels and Rb-E2F binding, we also assessed its effect on upstream cell cycle regulators. Silibinin-treated cells showed up to 2.4- and 3.6-fold increases in Cip1/p21 and Kip1/p27 levels, respectively, and a decrease in CDK2 (80%), CDK4 (98%), and cyclin D1 (60%). Consistent with these results, silibinin showed both G_1 arrest and growth inhibition. Together, these findings identify modulation of Rb levels and its phosphorylation status as a molecular mechanism of silibinin-induced neuroendocrine differentiation of human prostate carcinoma LNCaP cells and suggest that this could be a novel approach for prostate cancer prevention by silibinin.

Introduction

PCA[3] is the most commonly diagnosed invasive malignancy and second leading cause of cancer deaths in United States men with 198,100 new cases and 31,500 deaths in 2001 (1). Epidemiological studies suggest that diet plays an important role in an increased risk of PCA as well as its prevention (2–4). Accordingly, extensive efforts are being made to prevent PCA by altering dietary choices or the use of dietary supplements. In this regard, polyphenolic flavonoids are receiving increased attention for the prevention and intervention of human cancers, including PCA. Silibinin, isosilibinin, silycristine, and silydianine are polyphenolic flavonoids found in dried fruits of *Silybum marianum*. Together, these compounds are known as the silymarin group. Silibinin is the major active constituent of silymarin (5), and both silibinin and silymarin are used clinically to prevent or treat several hepatic disorders in Europe and Asia (Ref. 6 and references therein) and are consumed as dietary supplements around the world, including the United States. Several recent studies by us and others have shown both cancer preventive and therapeutic efficacy of silibinin and silymarin using *in vivo* and *in vitro* cancer models (7–11).

In eukaryotes, cell growth and differentiation are governed through cell cycle progression, which is regulated by CDKs and their catalytic subunit cyclins, and CDKIs (12, 13). Cip1/p21 has been shown to inhibit cell cycle progression through its interaction with cyclin-CDK complexes and proliferating cell nuclear antigen (14, 15). Cyclin-CDK is required for cell cycle progression, where CDK phosphorylates Rb in the G_1 phase, although the phosphorylation status of Rb differs throughout the cell cycle (16). Rb-P exerts most of its effects

Received 11/28/01; revised 1/25/02; accepted 3/12/02.
[1] This work was supported in part by United States Army Medical Research and Materiel Command Prostate Cancer Program DAMD17-98-1-8588. In part, this work was done during the tenure of the authors at AMC Cancer Research Center, Denver, CO 80262.
[2] To whom requests for reprints should be addressed, at Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, 4200 East Ninth Street, Box C238, Denver, CO 80262. Phone: (303) 315-1381; Fax: (303) 315-6281; E-mail: Rajesh.Agarwal@UCHSC.edu.

[3] The abbreviations used are: PCA, prostate cancer; Rb, retinoblastoma protein; Rb-P, phosphorylated Rb protein; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; PI, propidium iodide; FACS, fluorescence-activated cell sorter.

in a specific time frame during the first two-thirds of the G_1 phase, where mammalian cells make most of their decisions for proliferation *versus* quiescence. Release of the E2F family of transcription factors from Rb-E2F complexes after Rb phosphorylation induces expression of a number of genes required for S-phase transition (17, 18). Rb plays a critical role in G1-S checkpoint control, where unphosphorylated Rb prevents cellular proliferation by binding with E2Fs and inhibiting cell cycle progression (19, 20). Phosphorylation of Rb by CDK/cyclin complex results in the release of active E2F species to stimulate the transcription of genes involved in DNA synthesis and S-phase progression (21–23).

In a recent study, we showed that silibinin treatment of LNCaP cells results in inhibition of cell growth via G_1 arrest, leading to neuroendocrine differentiation (10). Here we extended this study to assess the effect of silibinin on Rb levels and its phosphorylation status, specific phosphorylation sites of Rb, levels of E2Fs, and Rb-E2F interaction during G_1 arrest of LNCaP cells. Because Rb phosphorylation is mediated via an upstream effector, CDK, the levels of CDKs, cyclins, and CDKIs during G_1 phase were also assessed to establish a cause-and-effect relationship for the observed activity of silibinin on G_1 arrest, growth inhibition, and neuroendocrine differentiation of LNCaP cells.

Materials and Methods

Cell Lines and Reagents. Human prostate carcinoma LNCaP cells were from American Type Culture Collection (Manassas, VA). RPMI 1640 and all other culture materials were from Life Technologies, Inc. (Gaithersburg, MD). Silibinin was from Sigma Chemical Co. (St. Louis, MO), and its purity was checked as 100% (10). Anti-Cip1/p21 antibody was from Calbiochem (San Diego, CA), and anti-Kip1/p27 was from Neomarkers (Fremont, CA). Antibodies for cyclin D1, cyclin E, CDK2, CDK4, Rb (recognizes both Rb-P and unphosphorylated Rb forms as slow- and fast-migrating bands, respectively), E2F1, E2F2, E2F3, rabbit antimouse immunoglobulin, and goat antirabbit immunoglobulin-horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Phosphoplus Rb antibodies (Ser-780, Ser-795, Ser-807/811, Ser-249/Thr-252, and Thr-373) were from New England Biolabs, Inc. (Beverly, MA). The ECL detection system was from Amersham (Arlington Heights, IL).

Cell Culture and Silibinin Treatments. LNCaP cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin under standard culture conditions. To assess the effect of silibinin on different cell cycle regulators, 60% confluent cultures were treated with either DMSO alone or different doses of silibinin (50, 100, and 200 μM) in DMSO for 12, 24, and 48 h. The final concentration of DMSO in culture medium during silibinin treatment did not exceed 0.1% (v/v), and the same concentration of DMSO was present in control dishes. After these treatments, medium was aspirated, cells were washed two times with cold PBS, and total cell lysates were prepared as described recently (10, 11). Nuclear extracts were prepared by the method of Schreiber *et al.* (24).

Immunoprecipitation and Immunoblotting. To study the binding of Rb with E2Fs, 50 μg of nuclear extract protein/sample were precleared with protein A/G plus agarose for 1 h and then incubated overnight with anti-Rb antibody plus protein A/G plus agarose beads, and immunocomplexes were washed three times with lysis buffer. For immunoblotting, immunocomplexes, total cell lysates, or nuclear extracts (30–60 μg protein/sample) were denatured with 2$\times$ sample buffer; samples were subjected to SDS-PAGE on 6, 12, or 16% gel, and separated proteins were transferred onto membrane by Western blotting. The levels of total Rb, Phosphoplus Rb (Ser-780, Ser-795, Ser-807/811, Ser-249/Thr-252, and Thr-373), E2F1, E2F2, E2F3, CDK2, CDK4, cyclin D1, cyclin E, Cip1/p21, and Kip1/p27 were determined using specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibody and ECL detection.

Densitometry Analysis. Autoradiograms of the immunoblots were scanned using Adobe Photoshop (Adobe System, Inc., San Jose, CA). The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed using the Scion Image program (NIH, Bethesda, MD). In each case, the numerical data shown under the blots are arbitrary units, where silibinin treatments at 50, 100, and 200 μM doses are compared with DMSO controls (denoted as 0). Furthermore, for each set of treatment times, *e.g.,* 12, 24, and 48 h, respective DMSO control densitometry value was used as 1, and a comparison was then made for densitometry values obtained after silibinin treatments. The comparative data are presented as "fold change" that is compared to DMSO control for individual treatment time in each case. Both autoradiograms and respective densitometry data shown are representative of two to four independent experiments with reproducible findings.

Cell Cycle Analysis. LNCaP cells at 60% confluency were treated with either DMSO alone or various doses of silibinin. After 24 h of treatments, medium was aspirated, cells were quickly washed two times with cold PBS and trypsinized, and cell pellets were collected. Approximately 0.5×10^6 cells in 0.5 ml of saponin/PI solution (0.3% saponin, 25 μg/ml PI, 0.1 mM EDTA, and 10 μg/ml RNase in PBS) were incubated at 4°C for 24 h in the dark. Cell cycle distribution was then analyzed by flow cytometry using the FACS analysis core services of the University of Colorado Cancer Center (Denver, CO).

Cell Growth Assay. LNCaP cells were plated at 5000 cells/cm^2 density in 35-mm dishes under standard culture conditions. After 24 h, cells were fed with fresh medium and treated with either DMSO alone or different doses of silibinin. After 2, 4, and 6 days of treatments, cells were trypsinized, collected, and counted using a hemocytometer. Trypan blue dye exclusion was used to determine cell viability.

Cell Morphology Assay. LNCaP cells were cultured in 10% FBS as detailed above and treated with either vehicle alone or with 50 or 100 μM silibinin for 2, 4, or 6 days. Pictures were then taken using phase-contrast microscope (Nikon TMS, Tokyo, Japan) and a Polaroid MicroCam Camera (Whealthampstead, United Kingdom) at $\times$200.

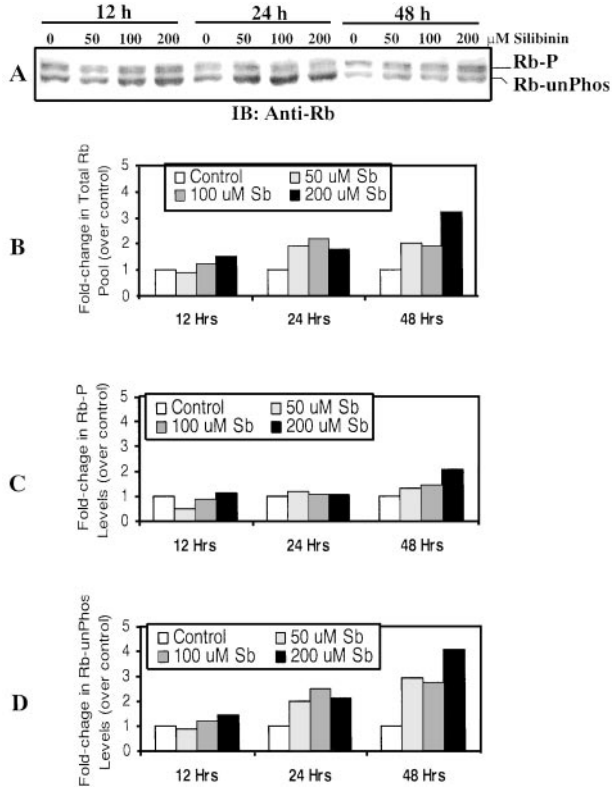

Fig. 1. Silibinin induces unphosphorylated Rb levels in human PCA LNCaP cells. *A,* cell culture and silibinin treatments were done, followed by nuclear extract preparation, SDS-PAGE on 6% gels, and Western blotting as described in "Materials and Methods." Membrane was probed with anti-Rb antibody, followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled. The densitometry data shown in *B, C,* and *D* for total Rb pool, Rb-P, and Rb-unPhos (unphosphorylated Rb), respectively, are from the blot shown in *A*. The details of densitometry analysis are described in "Materials and Methods."

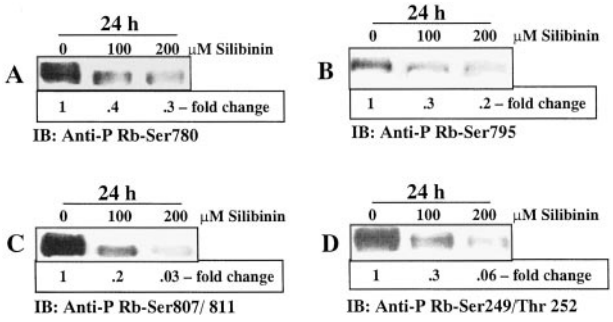

Fig. 2. Silibinin inhibits Rb phosphorylation at serine sites in human PCA LNCaP cells. Experimental details are same as described in Fig. 1, except gels used were 8%. Membranes were probed with Phosphoplus anti-Rb: Ser-780 (*A*), Ser-795 (*B*), Ser-807/811 (*C*), or Ser-249/Thr-252 (*D*) antibody, followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled. The data shown under each blot are from the densitometry analysis of that blot as described in "Materials and Methods."

Results

Silibinin Increases Total Rb Pool Largely by an Increase in Unphosphorylated Rb Levels in LNCaP Cells. To assess changes in total Rb levels and its phosphorylation status by silibinin, LNCaP cells were treated with 50, 100, and 200 μM doses of silibinin for 12, 24, and 48 h, and total Rb pool, Rb-P, and unphosphorylated Rb levels were analyzed in nuclear extracts. As shown in Fig. 1*A,* silibinin treatment of LNCaP cells resulted in an absolute increase in the amount of total Rb pool at all doses and time points studied, except 12-h treatment at the 50 μM dose. The densitometry analysis of the blots (both upper and lower bands together in each case representing total Rb levels in terms of a sum of Rb-P and unphosphorylated Rb, respectively) showed ~2-fold increase in total Rb pool after 24- and 48-h treatments at all doses, except in the case of the 200 μM dose and 48-h exposure, where silibinin showed an ~3-fold increase in total Rb levels as compared with DMSO control (Fig. 1*B*). When this increase in total Rb pool by silibinin was analyzed in terms of Rb-P levels, densitometry analysis of the blots in Fig. 1*A* (*upper band*) revealed that up to 24-h silibinin treatment at all three doses did not result in a noticeable change; only 48-h treatment time showed an ~2-fold increase at the 200 μM dose (Fig. 1*C*). However, there was a clear increase in unphosphorylated Rb levels after silibinin treatment at all doses and time points studied, except 12-h treatment at the 50 μM dose (Fig. 1*A*). In this case, densitometry analysis of the blots shown in Fig. 1*A* (*lower band*) revealed that compared with DMSO-treated controls, 12- and 24-h silibinin treatment at various doses results in a 1.5–2.5-fold increase in unphosphorylated Rb levels (Fig. 1*D*). A much stronger increase, however, was evidenced after 48 h of treatment, where the 50, 100, and 200 μM doses showed a 2.9-, 2.7-, and 4.1-fold increase in unphosphorylated Rb levels, respectively (Fig. 1*D*). When Western immunoblot bands shown in Fig. 1*A* were analyzed for a ratio of unphosphorylated Rb *versus* Rb-P, compared with DMSO control, silibinin treatment at 50, 100, and 200 μM doses for 24 and 48 h showed an almost 2-fold increase in this ratio (data not shown), which further supports other analyses that silibinin increases unphosphorylated Rb levels in LNCaP cells.

Silibinin Decreases the Amount of Rb Phosphorylated at Specific Serine Residues in LNCaP Cells. On the basis of the above results showing up to a 4.1-fold increase in unphosphorylated Rb levels by silibinin in LNCaP cells, we assessed whether this effect of silibinin is attributable to a change in Rb phosphorylation at specific Ser/Thr phosphorylation sites. Using nuclear extracts from silibinin-treated LNCaP cells and phospho-specific Rb antibodies in immunoblotting, we observed that silibinin strongly decreases the amount of Rb phosphorylated at serine sites (Ser-780, Ser-795, Ser-807/811, and Ser-249/Thr-252; Fig. 2); we did not find any change in Rb phosphorylation at the Thr-393 site (data not shown). Densitometry analysis of these blots showed that compared with DMSO control, 24-h silibinin treatment at 100 and 200 μM doses resulted in 60 and 70% decrease in Ser-780 (Fig. 2*A*), 70 and 80% decrease in Ser-795 (Fig. 2*B*), 80 and 97% decrease in Ser-807/811 (Fig. 2*C*), and 70 and 94% decrease in Ser-249/Thr-252 (Fig. 2*D*) phosphorylated Rb levels, respectively.

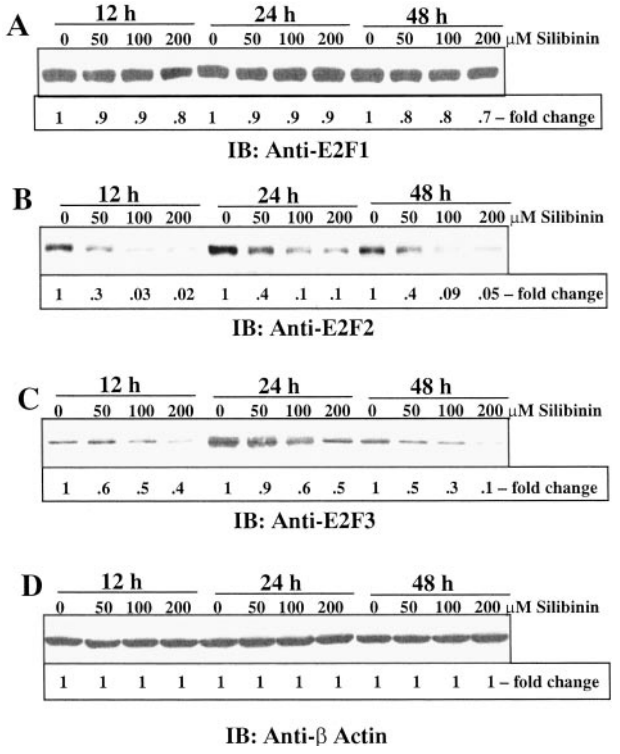

Fig. 3. Silibinin decreases E2F1, E2F2, and E2F3 protein levels in human PCA LNCaP cells. Experimental details are the same as described in Fig. 1, except that total cell lysates were used in the analysis and subjected to SDS-PAGE on 12% gels. Membranes were probed with anti-E2F1 (*A*), E2F2 (*B*), E2F3 (*C*), or β-actin (*D*) antibody, followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Different treatments are as labeled. The data shown under each blot are from the densitometry analysis of that blot as described in "Materials and Methods."

Silibinin Decreases the Levels of E2Fs but Increases their Binding with Rb in LNCaP Cells. Various members of the E2F transcription factor family are known to exist in a bound form with Rb inhibiting their transcriptional activity; however, after a growth signal that phosphorylates Rb, E2Fs are released from Rb and thus allow specific gene transcription (18, 25, 26). On the basis of our findings showing that silibinin increases the levels and amount of Rb and decreases its phosphorylation in LNCaP cells, we next assessed the effect of silibinin on E2F levels and their binding with Rb. As shown in Fig. 3, compared with DMSO controls, silibinin treatment of LNCaP cells resulted in only a moderate change in E2F1 but a significant decrease in E2F2 and E2F3 protein levels. Densitometry analysis of the blots showed that silibinin was not effective in modulating E2F1 protein levels, except 48-h treatment at the 200 μM dose, which showed a 30% decrease compared with its respective DMSO control (Fig. 3*A*). However, in the cases of E2F2 and E2F3, silibinin exerted a strong dose-dependent decrease in protein levels, even after 12 h of treatment. Compared with their DMSO controls, as high as 98 and 90% decreases in E2F2 (Fig. 3*B*) and E2F3 (Fig. 3*C*) protein levels were observed after different silibinin treatments of LNCaP cells, respectively. When the same blots were reprobed with anti-β-actin antibody, they confirmed equal protein loading (Fig. 3*D*).

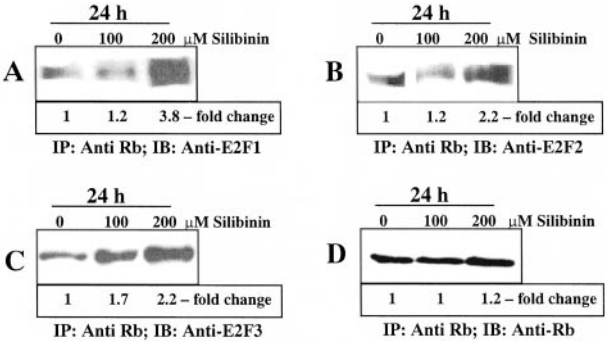

Fig. 4. Silibinin increases the binding of Rb with E2F1, E2F2, and E2F3 in human PCA LNCaP cells. Experimental details are same as described in Fig. 1. Nuclear extracts were subjected to immunoprecipitation with anti-Rb antibody, followed by SDS-PAGE on 12% gels and Western blotting. Membranes were probed with anti-E2F1 (*A*), anti-E2F2 (*B*), and anti-E2F3 (*C*), or anti-Rb (*D*) antibody, followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Different treatments are as labeled. The data shown under each blot are from the densitometry analysis of that blot as described in "Materials and Methods."

To assess the effect of silibinin on the binding of E2Fs with Rb, nuclear extracts were subjected to immunoprecipitation using anti-Rb antibody, and after SDS-PAGE and blotting, membranes were probed with anti-E2F1, anti-E2F2, anti-E2F3, or anti-Rb antibodies. As shown in Fig. 4, compared with DMSO controls, treatment of LNCaP cells with the 200 μM dose of silibinin for 24 h resulted in an increase in the binding of Rb with E2F1 × 3.8-fold (Fig. 4*A*), with E2F2 × 2.2-fold (Fig. 4*B*), and with E2F3 × 2.2-fold (Fig. 4*C*). The observed increase in the binding of Rb with different E2Fs was not attributable to an overall considerable increase in Rb immunoprecipitation after silibinin treatments (Fig. 4*D*).

Silibinin Modulates the Levels of G_1 Cell Cycle Regulators in LNCaP Cells. On the basis of our findings showing an increase in unphosphorylated Rb, inhibition of Rb phosphorylation at serine sites, and an increase in Rb-E2F complex levels by silibinin in LNCaP cells, we next assessed the effect of silibinin on upstream regulators of cell cycle progression, specifically CDKIs, CDKs, and cyclins involved in G_1 phase. Similar silibinin treatment of LNCaP cells showed an increase in CDKI Kip1/p27 and Cip1/p21 protein levels (Fig. 5). In the case of Kip1/p27, compared with its respective DMSO control, maximum induction was evidenced at 48 h of 200 μM silibinin treatment that accounted for 3.6-fold increase over control (Fig. 5*A*). However, maximum increase (2.4-fold over DMSO control) in Cip1/p21 protein expression was evident after 24-h treatment of silibinin at the 200 μM dose (Fig. 5*B*).

In the studies assessing the effect of silibinin on CDKs and cyclins, it showed a dose-dependent decrease in their protein levels in LNCaP cells (Fig. 6). Compared with their respective DMSO controls, densitometry analysis of the blots for CDK2 and CDK4 showed a maximum of 80 and 98% decrease in their protein levels after 48-h treatment at the 200 μM dose of silibinin (Figs. 6, *A* and *B*), respectively.

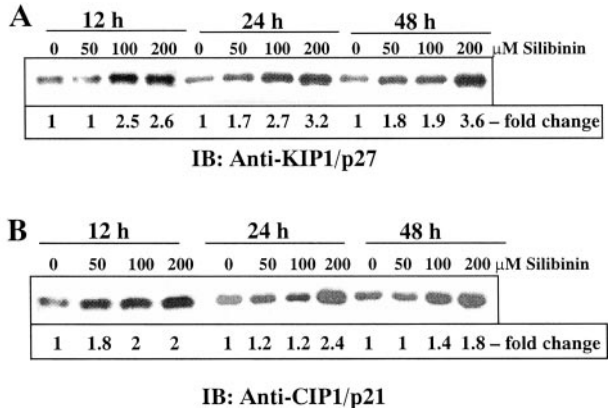

Fig. 5. Silibinin induces CDKI Kip1/p27 and Cip1/p21 protein levels in human PCA LNCaP cells. Experimental details are the same as described in Fig. 1. Total cell lysates were subjected to SDS-PAGE on 12% gels, followed by Western blotting. Membranes were probed with anti-Kip1/p27 (*A*) or anti-Cip1/p21 (*B*) antibody, followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Different treatments are as labeled. The data shown under each blot are from the densitometry analysis of that blot as described in "Materials and Methods."

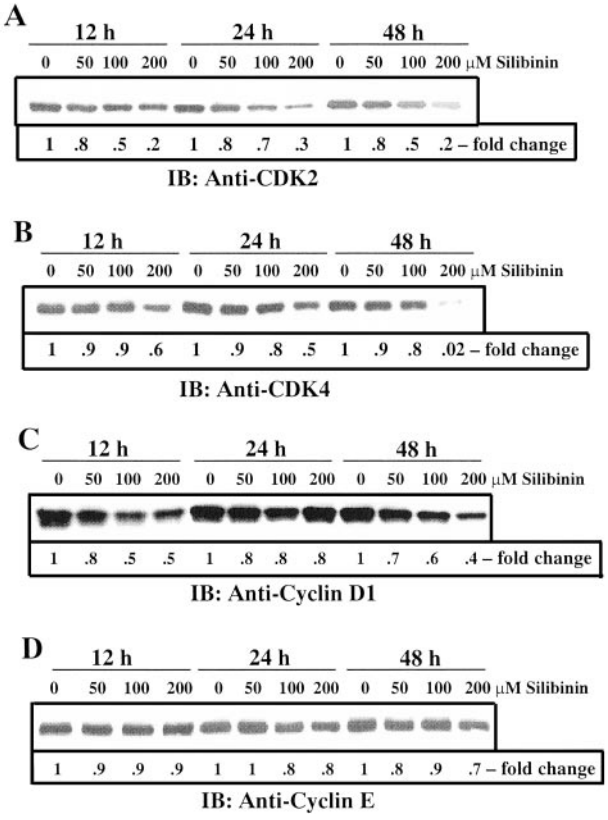

Fig. 6. Silibinin decreases CDK and cyclin protein levels in human PCA LNCaP cells. Experimental details are the same as described in Fig. 1. Total cell lysates were subjected to SDS-PAGE on 12% gels, followed by Western blotting. Membranes were probed with anti-CDK2 (*A*), anti-CDK4 (*B*), anti-cyclin D1 (*C*), or anti-cyclin E (*D*) antibody, followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Different treatments are as labeled. The data shown under each blot are from the densitometry analysis of that blot as described in "Materials and Methods."

Similarly, silibinin also showed a strong decrease (60%) in cyclin D1 protein level after 48-h treatment at the 200 μM dose (Fig. 6*C*). In case of cyclin E, silibinin was overall not effective in modulating its levels at any of the doses and time points studied, except the 48-h treatment at the 200 μM dose, which showed a 30% decrease compared with its DMSO control (Fig. 6*D*).

Silibinin Causes G_1 Arrest, Growth Inhibition, and Neuroendocrine Differentiation of LNCaP Cells. On the basis of results shown in Figs. 1–6, we next examined the biological effect of silibinin on LNCaP cells. As shown in Fig. 7*A,* FACS analysis of DMSO control and silibinin-treated cells showed that silibinin induces G_1 arrest in cell cycle progression. The effect of silibinin was dose dependent when compared with the DMSO-treated control showing 51% cells in the G_1 phase; 50, 100, and 200 μM doses of silibinin treatment for 24 h resulted in 60, 68, and 71% cells in the G_1 phase, respectively (Fig. 7*A*). The observed G_1 arrest by silibinin was largely attributable to a decrease in S-phase cell population, whereas cells in G_2-M remained largely unchanged (Fig. 7*A*). In the studies assessing effect of silibinin on LNCaP cell growth, it showed significant to complete inhibition in both dose- and time-dependent manner (Fig. 7*B*). Two days of silibinin treatment at 50, 100, and 200 μM doses showed 54, 54, and 66% inhibition, respectively (Fig. 7*B*). A much stronger effect of silibinin was evidenced after 4 and 6 days of treatments, where 100 and 200 μM doses showed almost complete cell growth inhibition (Fig. 7*B*).

Treatment of LNCaP cells as described above resulted in morphological changes suggestive of neuroendocrine differentiation (Fig. 8) as reported earlier (10). Compared with vehicle-treated controls, 6 days of silibinin treatment at 50 and 100 μM doses caused significant changes in their morphology; cells became elongated in length with prominent dendrite-like cytoplasmic extensions, and most of them were connected among neighboring cells (Fig. 8). These morphological changes were similar to that of neuroendocrine morphology, suggesting that silibinin induces neuroendocrine differentiation of LNCaP cells (Fig. 8). Similar but less profound neuroendocrine differentiation effects were also observed after 2 and 4 days of silibinin treatment of LNCaP cells at these doses (data not shown).

Discussion

The progression of cells from G_1 to S-phase is accompanied by the phosphorylation of retinoblastoma gene product, a tumor suppressor gene that is active in controlling the G_1 phase (27, 28). Phosphorylation of Rb protein by serine/threonine kinases, known as CDKs, inactivates Rb (29). Distinct phases of the cell cycle are controlled by specific CDKs complexed with their respective catalytic subunit cyclins. CDK4 and CDK6 complexed with cyclin D1 are responsible for cell cycle progression through the G_1 phase (11), and CDK2/cyclin E complex functions in the progression of the cell from late G_1 to early S-phase (30). In recent years, a growing number of proteins classified as

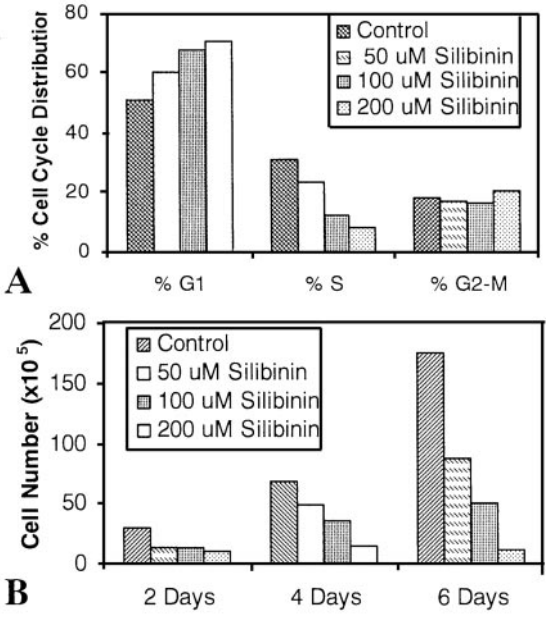

Fig. 7. Silibinin exhibits G_1 arrest and growth inhibition of human PCA LNCaP cells. *A,* cells were cultured as described in "Materials and Methods" and treated with either vehicle alone (*Control*) or various concentrations of silibinin for 24 h. At the end of these treatments, cells were collected, incubated with saponin/PI solution, and subjected to FACS analysis for cell cycle progression as detailed in "Materials and Methods." *B,* cells were plated in 35-mm dishes, treated with vehicle (*Control*) or different concentrations of silibinin, and after 2, 4, or 6 days, cells were counted as detailed in "Materials and Methods."

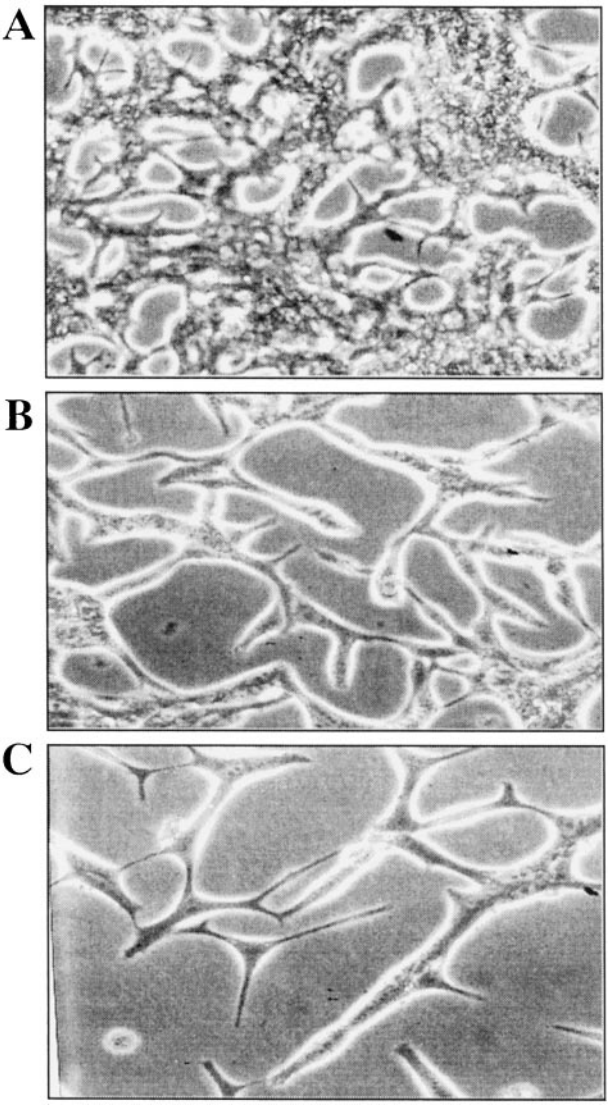

Fig. 8. Silibinin exhibits differentiation of human PCA LNCaP cells. Cells were cultured as described in "Materials and Methods" and treated with either vehicle (*A*) or 50 μM (*B*) or 100 μM (*C*) dose of silibinin, and after 6 days, phase-contrast photography was done at $\times$200, as detailed in "Materials and Methods."

CDKIs have also been characterized as negative regulators of CDKs (14, 31, 32). Previous studies from our group showed that silibinin induces the expression of CDKIs, leading to an increased binding with CDKs and an inactivation of the kinase activity associated with CDKs and cyclin (10). Here we extended this study to assess the effect of silibinin on total Rb pool, Rb-P, and unphosphorylated Rb status, specific phosphorylation sites in Rb, and Rb-E2F interaction. The results obtained were consistent with the role of unphosphorylated Rb levels in silibinin-treated LNCaP cells and an increase in Rb binding with E2Fs during G_1 arrest as well as cellular differentiation (27, 28). In addition, our data provide convincing evidence that an upstream effect of silibinin in inducing CDKI levels is, in part, responsible for inactivating CDK-associated kinase activity, as reported by us recently (10), which possibly induces unphosphorylated Rb levels in LNCaP cells leading to an increase in Rb-E2F binding. This effect of silibinin could be responsible for G_1 arrest, growth inhibition, and neuroendocrine differentiation of LNCaP cells as observed in this study and reported earlier (10). We also observed that in addition to the above-described cause and effect cascade for the effect of silibinin on Rb phosphorylation status, other mechanisms of action of silibinin are also operational in LNCaP cells, causing a significant decrease in the protein levels of CDKs, cyclins, and E2Fs. Whether these are direct effects of silibinin or are part of the same cascade remains to be studied.

Rb contains 16 Ser/Thr-Pro motifs, which are potential CDK phosphorylation sites (18, 33, 34). The kinases responsible for Rb phosphorylation *in vivo* are known to include members of the CDK family including CDK2 in association with cyclins E and A as well as CDK4 and CDK6 in association with D-type cyclin (35). Evidence is emerging that various CDKs differentially phosphorylate Rb at distinctive residues *in vitro* (36, 37). Phosphorylation at particular Rb residues may only affect the binding of Rb to particular subsets of its interacting partners (38). This suggests that different sites of Rb phosphorylation by distinct CDKs may result in differential regulation of downstream effector pathway(s). The results obtained in the present study suggest that silibinin induces unphosphorylated Rb levels, possibly

by inhibiting Rb phosphorylation at different serine sites. Whether this effect of silibinin is associated with specific CDKs remains to be established.

In summary, the major findings in the present study are that silibinin increases the total intracellular Rb pool and the levels and amount of unphosphorylated Rb by a large decrease in the amount of Rb phosphorylated at specific serine sites in human PCA LNCaP cells. It is an increase in the absolute amount of unphosphorylated Rb that might be leading to a subsequent increase in Rb-E2F interaction as well as cellular growth inhibition and induction of differentiation. Together, these findings lend more molecular understanding to our previous results showing that silibinin inhibits cell growth via G_1 arrest, leading to neuroendocrine differentiation of LNCaP cells (10). In this regard, it is important to emphasize here that, in recent years, new agents are being studied to induce neuron-like morphological changes and an associated decrease in malignancy of human PCA LNCaP cells (39). Accordingly, our recent finding (10) and the results of the present study suggest that the efficacy of silibinin in inducing neuroendocrine differentiation of human PCA cells should be studied in more detail to develop this nontoxic phytochemical for the prevention of human PCA growth.

References

1. Greenlee, R. T., Hill-Harman, M. B., Murry, T., and Thun, M. Cancer Statistics, 2001. CA Cancer J. Clin., *51:* 15–36, 2001.

2. Anderson, K. E., Rosner, W., Khan, M. S., New, M. I., Pang, S. Y., Wissel, P. S., and Kappas, A. Diet-hormone interactions: protein/carbohydrate ratio alters reciprocally the plasma levels of testosterone and cortisol and their respective binding globulins in man. Life Sci., *40:* 1761–1768, 1987.

3. Adlercreutz, H., Fotsis, T., Bannwart, C., Wahala, K., Makela, T., Brunow, G., and Hase, T. Determination of urinary lignans and phytoestrogen metabolites, potential antiestrogens and anticarcinogens, in urine of women on various habitual diets. J. Steroid Biochem., *25:* 791–797, 1986.

4. Morton, M. S., Chan, P. S., Cheng, C., Blaclock, N., Matos-Ferreira, A., Abranches-Morteiro, L., Correia, R., Lloyd, S., and Griffiths, K. Lignans and isoflavonoids in plasma and prostatic fluid in men: samples from Portugal, Hong Kong, and the United Kingdom. Prostate, *32:* 122–128, 1997.

5. Wagner, H., Diesel, P., and Seitz, M. The chemistry and analysis of silymarin from *Silybum marianum*. Gaertn. Arzneimittelforschung, *24:* 466–471, 1974.

6. Flora, K., Hahn, M., Rosen, H., and Benner, K. Milk thistle (*Silybum marianum*) for the therapy of liver disease. Am. J. Gastroenterol., *93:* 139–143, 1998.

7. Agarwal, R. Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents. Biochem. Pharmacol., *60:* 1051–1059, 2000.

8. Katiyar, S. K., Korman, N. J., Mukhtar, H., and Agarwal, R. Protective effects of silymarin against photocarcinogenesis in mouse skin model. J. Natl. Cancer Inst., *89:* 556–566, 1997.

9. Lahiri-Chatterjee, M., Katiyar, S. K., Mohan, R. R., and Agarwal, R. A flavonoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in SENCAR mouse skin tumorigenesis model. Cancer Res., *59:* 622–632, 1999.

10. Zi, X., and Agarwal, R. Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: Implications for prostate cancer intervention. Proc. Natl. Acad. Sci. USA, *96:* 7490–7495, 1999.

11. Zi, X., Grasso, A. W., Kung, H. J., and Agarwal, R. A flavonoid antioxidant silymarin inhibits activation of erbB1 signaling, and induces cyclin-dependent kinase inhibitors: G_1 arrest and anti-carcinogenic effects in human prostate carcinoma DU145 cells. Cancer Res., *58:* 1920–1929, 1998.

12. Grana, X., and Reddy, E. P. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CDKIs). Oncogene, *11:* 211–219, 1995.

13. Lees, E. Cyclin dependent kinase regulation. Curr. Opin. Cell Biol., *7:* 773–780, 1995.

14. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 Cdk- interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell, *75:* 805–816, 1993.

15. Waga, S., Hannon, G. J., Beach, D., and Stillman, B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature (Lond.), *369:* 574–578, 1994.

16. Howard, C. M., Claudio, P. P., De Luca, A., Stiegler, P., Jori, F. P., Safdar, N. M., Caputi, M., Khalili, K., and Giordano, A. Inducible pRb2/p130 expression and growth-suppressive mechanism: evidence of a pRb2/p130, p27kip1, and cyclin E negative feedback regulatory loop. Cancer Res., *60:* 2737–2744, 2000.

17. Sherr, C. J., and Roberts, J. M. Inhibitors of mammalian G1 cyclin dependent kinases. Genes Dev., *9:* 1149–1163, 1995.

18. Weinberg, R. A. The retinoblastoma protein and cell cycle control. Cell, *81:* 323–330, 1995.

19. Paggi, M. G., Baldi, A., Bonetto, F., and Giordano, A. Retinoblastoma protein family in cell cycle and cancer: a review. J. Cell. Biochem., *62:* 418–430, 1996.

20. Wang, J. Y., Knudsen, E. S., and Welch, P. J. The retinoblastoma tumor suppressor protein. Adv. Cancer Res., *64:* 25–85, 1994.

21. Qin, X. Q., Chittenden, T., Livingston, D. M., and Kaelin, W. G., Jr. Identification of a growth suppression domain within the retinoblastoma gene product. Genes Dev., *6:* 953–964, 1992.

22. Qian, Y., Luckey, C., Horton, L., Esser, M., and Templeton, D. J. Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. Mol. Cell. Biol., *12:* 5363–5372, 1992.

23. Hiebert, S. W. Regions of the retinoblastoma gene product required for its interaction with the E2F transcription factor are necessary for E2 promoter repression and pRb-mediated growth suppression. Mol. Cell. Biol., *13:* 3384–3391, 1993.

24. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. Rapid detection of octamer binding proteins with mini-extracts, prepared from a small number of cells. Nucleic Acids Res., *17:* 6419, 1989.

25. Taya, Y. RB Kinase and RB-binding proteins: new points of view. Trends Biochem. Sci., *22:* 14–17, 1997.

26. Shirodkar, S., Ewen, M., DeCaprio, J. A., Morgan, J., Livingston, D. M., and Chittenden, T. The transcription factor E2F interacts with the retinoblastoma product and a pp107-cyclin A complex in a cell cycle-regulated manner. Cell, *68:* 157–166, 1992.

27. Hatakeyama, M., and Weinberg, R. A. The role of RB in cell cycle control. Prog. Cell Cycle Res., *1:* 9–19, 1995.

28. Morgan, D. O., Fisher, R. P., Espinoza, F. H., Farrell, A., Nourse, J., Chamberlin, H., and Jin, P. Control of eukaryotic cell cycle progression by phosphorylation of cyclin-dependent kinases. Cancer J. Sci. Am., *4* (Suppl. 1)*:* S77–S83, 1998.

29. Morgan, D. O. Cyclin-dependent kinases: engines, clocks, and microprocessor. Annu. Rev. Cell Dev. Biol., *13:* 261–291, 1997.

30. Sherr, C. J. Cancer cell cycles. Science (Wash. DC), *274:* 1672–1677, 1996.

31. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature (Lond.), *366:* 704–707, 1993.

32. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. Cell, *75:* 817–825, 1993.

33. Lees, J. A., Buchkovich, K. J., Marshak, D. R., Anderson, C. W., and Harlow, E. The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J., *10:* 4279–4290, 1991.

34. Lin, B. T., Gruenwald, S., Morla, A. O., Lee, W. H., and Wang, J. Y. Retinoblastoma cancer suppressor gene product is a substrate of the cell cycle regulator cdc2 kinase. EMBO J., *10:* 857–864, 1991.

35. Vesely, J., Havlicek, L., Strand, M., Blow, J. J., Donella-Deana, A., Pinna, L., Letham, D. S., Kato, J., Detivaud, L., Leclerc, S., *et al.* Inhibition of cyclin-dependent kinases by purine analogues. Eur. J. Biochem., *224:* 771–786, 1994.

36. Zarkowska, T., and Mittnacht, S. Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J. Biol. Chem., *272:* 12738–12746, 1997.

37. Kitagawa, M., Higashi, H., Jung, H. K., Sujuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J., Segawa, K., Yoshida, E., Nishimura, S., and Taya, Y. The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. EMBO J., *15:* 7060–7069, 1996.

38. Knudsen, E. S., and Wang, J. Y. Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. J. Biol. Chem., *271:* 8313–8320, 1996.

39. Shimizu, T., Ohta, Y., Ozawa, H., Matsushima, H., and Takeda, K. Papaverine combined with prostaglandin E2 synergistically induces neuron-like morphological changes and decreases malignancy in human prostatic cancer LNCaP cells. Anticancer Res., *20:* 761–767, 2000.