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Silibinin decreases prostate-specific antigen with cell growth inhibition via G_1 arrest, leading to differentiation of prostate carcinoma cells: Implications for prostate cancer intervention

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ABSTRACT **Reduction in serum prostate-specific antigen (PSA) levels has been proposed as an endpoint biomarker for hormone-refractory human prostate cancer intervention. We examined whether a flavonoid antioxidant silibinin (an active constituent of milk thistle) decreases PSA levels in hormone-refractory human prostate carcinoma LNCaP cells and whether this effect has biological relevance. Silibinin treatment of cells grown in serum resulted in a significant decrease in both intracellular and secreted forms of PSA concomitant with a highly significant to complete inhibition of cell growth via a G_1 arrest in cell cycle progression. Treatment of cells grown in charcoal-stripped serum and 5α-dihydrotestosterone showed that the observed effects of silibinin are those involving androgen-stimulated PSA expression and cell growth. Silibinin-induced G_1 arrest was associated with a marked decrease in the kinase activity of cyclin-dependent kinases (CDKs) and associated cyclins because of a highly significant decrease in cyclin D1, CDK4, and CDK6 levels and an induction of Cip1/p21 and Kip1/p27 followed by their increased binding with CDK2. Silibinin treatment of cells did not result in apoptosis and changes in p53 and bcl2, suggesting that the observed increase in Cip1/p21 is a p53-independent effect that does not lead to an apoptotic cell death pathway. Conversely, silibinin treatment resulted in a significant neuroendocrine differentiation of LNCaP cells as an alternative pathway after Cip1/p21 induction and G_1 arrest. Together, these results suggest that silibinin could be a useful agent for the intervention of hormone-refractory human prostate cancer.**

Prostate cancer (PCA) is the most common invasive malignancy and second leading cause of cancer deaths in United States males (1). Clinical PCA incidence is low in Asians and highest in African-Americans and Scandinavians (2, 3). However, once moved to the United States, incidence and mortality because of PCA increase in Asians, approximating those of Americans (3). Epidemiological studies suggest that dietary and environmental factors are major causes for an increase in PCA (2, 3). Low-fat and high-fiber diets significantly affect sex hormone metabolism in men (4). In Japan and other Asian countries, despite the same incidence of latent small or noninfiltrating PCA, mortality rate is low (3). This could be explained, at least partly, by a diet-related lowering of biologically active androgen (4). The importance of androgen in PCA also is suggested by the observations that PCA rarely occurs in eunuchs or men with deficiency in 5α-reductase, the enzyme that converts testosterone to its active metabolite 5α-dihydrotestosterone (DHT) (5). In addition, at least 75% of PCAs with metastatic potential are androgen-dependent at initial diagnosis (6).

Androgen receptors (ARs) are required for development of both normal prostate and PCA (7). A high proportion of mutations are shown in the ligand-binding domain of AR in hormone-refractory and metastatic PCA (7), and mutant ARs could be activated by estrogen and progesterone (7). Changes in specificity of AR may provide a selective advantage in metastatic androgen-independent PCA because they remain active after androgen ablation (7). A notable gene regulated by androgen in normal prostate and PCA cells is prostate-specific antigen (PSA) (8). PSA is demonstrated to be a sensitive and specific tumor marker for PCA screening and assessment (9) and is used as an indicator of disease and response to PCA therapy (10). Several trials also have shown a direct relationship between decline in PSA and shrinkage of PCA (11). Whereas stimulation of mutant AR in human PCA LNCaP cells by androgen does not differ from stimulation of wild-type AR, estrogenic substance and some antiandrogens bind to AR in LNCaP cells with higher affinity, efficiently stimulate its transactivation function, and increase PSA (7).

Traditional Asian diets are low in animal proteins and fat, high in starch and fiber, and rich in "weak plant estrogens," which are released in large amounts in urine and serum (12, 13). Some of these phytoestrogens possess weak estrogenic, antiestrogenic, and antioxidant activity, and, therefore, possess the potential for exerting an influence on hormone-dependent cancers including PCA (12, 13). Two groups of phytoestrogens, polyphenolic flavonoid antioxidants and lignans, are receiving attention for the prevention and intervention of human cancers including PCA (12–14). Silymarin, a polyphenolic flavonoid isolated from the seeds of milk thistle (*Silybum marianum*), is composed mainly of silibinin (or silybin; Fig. 1*A*), with small amounts of other stereoisomers isosilybin, dihydrosilybin, silydianin, and silychristin (15). Silymarin and silibinin have human acceptance, being used clinically in Europe and Asia for the treatment of liver diseases (reviewed in refs. 16–19). Human populations in Europe have been using silymarin or silibinin in a whole range of liver conditions (16, 17). As therapeutic agents, both silymarin and silibinin are well tolerated and largely free of adverse effects (15–19). Silymarin is sold in the United States and Europe as a dietary supplement, and silibinin is used clinically as silipide, a lipophilic silibinin–phosphatidylcholine complex (16).

Recently, we showed that silymarin affords high to complete protection against tumorigenesis in mouse skin models (18, 19). Likewise, in a mammary gland culture initiation–promotion protocol, silymarin inhibits tumor promotion (19). More recent studies by us found that both silibinin and silymarin possess comparable inhibitory effects on human carcinoma cell growth

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Abbreviations: AR, androgen receptor; CDK, cyclin-dependent kinase; CDKIs, CDK inhibitors; DHT, 5α-dihydrotestosterone; EC, electrochemical; cFBS, charcoal-stripped FBS; K8 & K18, cytokeratins 8 and 18; PCA, prostate cancer; PSA, prostate-specific antigen; RB, retinoblastoma.

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and DNA synthesis and are equally strong antioxidants (R.A. and colleagues, unpublished observations). Based on (*i*) structural similarity of silibinin with phytoestrogens for a polyphenolic flavonoid skeleton, (*ii*) strong antioxidant and anticarcinogenic effects of silibinin, (*iii*) the fact that silibinin is used clinically and marketed as dietary supplement, and (*iv*) the bioavailability of silibinin in prostate after its oral administration to mice (R.A. and colleagues, unpublished observations), we reasoned that silibinin also could be a useful agent for the intervention of human PCA. Here, we show that silibinin decreases intracellular and secreted levels of PSA in human PCA LNCaP cells under both serum- and androgen-stimulated conditions concomitant with inhibition of cell growth via a G_1 arrest in cell cycle progression. The G_1 arrest by silibinin does not lead to apoptosis but causes neuroendocrine differentiation of the cells.

MATERIALS AND METHODS

Cells and Cultures. Human prostate carcinoma LNCaP cells and NIH 3T3 cells were obtained from American Type Culture Collection. Normal human epithelial prostate cells were from Clonetics (San Diego). LNCaP and NIH 3T3 cells were cultured in RPMI 1640 medium and DMEM, respectively, with 10% FBS and 1% penicillin-streptomycin (P-S). LNCaP cells also were cultured in 10% charcoal-stripped FBS (cFBS) and 1% P-S with or without 1 nM DHT. Normal prostate cells were cultured in defined medium as suggested by the vendor.

Silibinin and Its Purity. Silibinin (Fig. 1*A*), International Union of Pure and Applied Chemistry name: 3,5,7-trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxan-6-yl]-4-chromanon, was from U. Mengs (MADAUS AG, Cologne, Germany) and Sigma. Purity of silibinin from both sources was checked by HPLC equipped with UV followed by electrochemical detectors (EC). The HPLC system consisted of two ESA 580 pumps, an ESA RP-C18 column (3 mm, 4.6 × 250 mm), a UV detector (at λ 270 nm), an EC detector (at 500 mV potential), and an ESA 5600 control and analysis software. HPLC mobile phase contained solvent A [7.5% methanol in 100 mM of acetate buffer with 50 mM of triethylamine (TEA)/1 mM of 1-octanesulfonic acid (OSA), pH 4.8] and solvent B (80% methanol in 100 mM of acetate buffer with 50 mM TEA/1 mM OSA, pH 4.8). The linear gradient, at 0.6 ml/min, was 0–5 min, 75% A and 25% B; 5–15 min, 50% of both A and B; 15–20 min, 30% A and 70% B; 20–25 min, isocratic 30% A and 70% B; and 25 min, stop of run. Column eluate was monitored at 270 nm followed by EC detection. As shown in Fig. 1*B*, using these HPLC conditions, silibinin showed a single peak in both 270 nm UV and EC detections, with a retention time of 13.5 min. These HPLC profiles also show the purity of silibinin to be 100%.

Silibinin Treatments. Silibinin was dissolved in ethanol. Final volume of ethanol in culture during silibinin treatment and controls did not exceed 0.5%. LNCaP cells were grown in 10% FBS to 80% confluency and treated with ethanol or varying doses of silibinin for 20 hr or 75 μg/ml of silibinin for varying times. Cells also were treated with paclitaxel (1 μM final concentration) for 20 hr. Cells then were lysed in 0.5 ml lysis buffer as detailed recently (20). In another study, cells grown in 10% FBS were treated with ethanol or 25 and 75 μg/ml of silibinin for 24, 48, and 72 hr, and medium was collected. Cells also were grown in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 24 hr, were treated with ethanol or 50 μg/ml of silibinin. Cell lysates then were prepared (20).

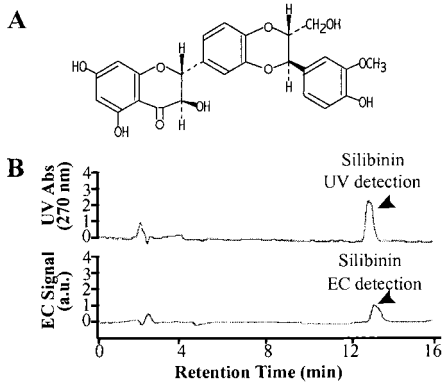

FIG. 1. Chemical structure of silibinin (*A*) and HPLC profiles of silibinin by UV and EC detection (*B*).

Western Blotting and Kinase Assays. Levels of PSA, cell cycle and apoptosis regulatory molecules, cytokeratins 8 and 18 (K8 & K18), and chromogranin A were determined by Western blotting. Equal amounts of protein (10–80 μg) from cell lysate or 20 μl of medium sample was denatured in sample buffer and subjected to SDS/PAGE on a 12% gel, and proteins were transferred onto membrane. The blots were probed with specific primary followed by secondary antibody and visualized by enhanced chemiluminescence. The binding of cyclin-dependent kinase inhibitors (CDKIs) with CDKs, CDK2- and cyclin E-H1 histone kinase activity, and CDK4-, CDK6-, and cyclin D1-retinoblastoma (RB) kinase activity were determined as detailed recently (20).

Cell Growth Assay. LNCaP cells were plated at 1×10^4 cells per 60-mm plate in RPMI 1640 medium containing 10% FBS. To assess the effect of silibinin on normal cell growth, NIH 3T3 cells were plated at the same density, and normal human prostate cells were plated at 2,500 cells/cm^2. On day 2, cells were fed with fresh medium and treated with ethanol or varying doses of silibinin (5, 25, 50, and 75 μg/ml). The cultures were fed with fresh medium with the same treatments on alternate days. After 1–6 days of treatments, cells were trypsinized and counted (20). In other studies, LNCaP cells were cultured in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 24 hr, were treated with ethanol or 50 μg/ml of silibinin. Cells then were collected and counted (20). To assess cytotoxicity of silibinin, cell viability was determined by Trypan blue assay.

FACS Analysis. LNCaP cells were cultured in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 24 hr, were treated with ethanol or 50 μg/ml of silibinin. Cells then were trypsinized, and cell cycle distribution was analyzed as detailed recently (20).

DNA Ladder Assay. LNCaP cells at 70–80% confluency were treated with different doses of silibinin for 24 and 48 hr, and, thereafter, trypsinized cells (together with any floating cells) were collected. The DNA ladder analysis then was done as detailed recently (21).

Morphological Analysis. LNCaP cells were cultured in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 48 hr, were treated with ethanol or 50 μg/ml of silibinin. Pictures then were taken by using a phase-contrast microscope at ×200 magnification.

RESULTS

Silibinin Decreases Serum- and DHT-Stimulated PSA Expression in LNCaP Cells. PSA has its acceptance and approval from FDA as a screening tool for human PCA. Therefore, to evaluate the usefulness of silibinin for PCA intervention, we assessed its effect on PSA levels in LNCaP cells. Consistent with an earlier study (8), LNCaP cells showed high levels of intracellular PSA as evidenced by a 33- to 34-kDa band (Fig. 2*A*). However, treatment of cells grown in 10% FBS with silibinin resulted in a highly significant decrease in intracellular PSA levels in a dose- and time-dependent manner (Fig. 2*A*). In a quantitative analysis, 50, 75, and 100 μg/ml of silibinin showed 54, 66, and 79% reduction in intracellular PSA levels, respectively. Similarly, cells grown in 10% FBS with 25 and 75 μg/ml of silibinin for 24 and 48 hr also showed a significant decrease in secreted PSA (Fig. 2*B*). Silibinin treatment for 24 hr at 25- and 75-μg/ml doses led to a 45 and 59% reduction in PSA secretion in medium, respectively. Because promoter of PSA gene contains functional androgen-responsive element (8) and DHT increases PSA production in LNCaP cells

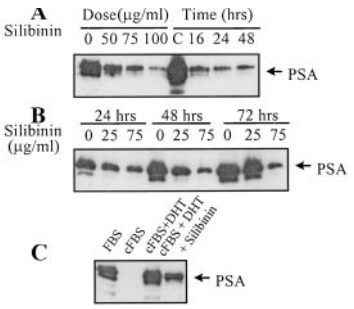

FIG. 2. Silibinin decreases serum- and DHT-stimulated PSA expression in LNCaP cells. (*A*) Effect of silibinin on intracellular PSA in cells grown in 10% FBS. Cells were treated with silibinin for 20 hr or for indicated times at 75 μg/ml; C, control cells treated with ethanol for 48 hr. (*B*) Effect of silibinin on secreted (medium) PSA in cells grown in 10% FBS. Cells were treated with silibinin for the indicated doses and time, and medium was collected. (*C*) Effect of silibinin on intracellular PSA in cells grown in 10% cFBS + 1 nM DHT. Cells were grown in: 1, 10% serum; 2, 10% cFBS; 3, 10% cFBS + 1 nM DHT; or 4, 10% cFBS supplemented with 1 nM DHT + 50 μg/ml of silibinin, and cell lysates were prepared. The data in *C* are at 5 days of cultures; silibinin was added at day 4. PSA protein levels were determined in cell lysates and medium as detailed in *Materials and Methods*. The Western blot data shown are representative of three independent experiments with similar findings.

(7), we next examined whether inhibitory effects of silibinin on PSA levels are mediated via AR. Compared with cells grown in 10% FBS showing strong PSA levels, cells grown in 10% cFBS showed no reactivity for PSA protein (Fig. 2*C*). However, cells grown in 10% cFBS + 1 nM DHT showed levels of PSA comparable to that for 10% FBS (Fig. 2*C*). Treatment of cells grown in 10% cFBS and 1 nM DHT with 50 μg/ml of silibinin resulted in a 56% reduction in DHT-stimulated intracellular PSA levels (Fig. 2*C*).

Silibinin Inhibits Serum- and DHT-Stimulated Growth of LNCaP Cells with No Effects on Normal Cells. To assess whether an observed decrease in PSA by silibinin is a biological response, we examined its effect on LNCaP cell growth. Treatment of cells grown in 10% FBS with silibinin resulted in a highly significant to complete inhibition of their growth in both a dose- and time-dependent manner (Fig. 3*A*). An inhibitory effect of silibinin was evident at 2 days, but a more profound effect was observed during 4–6 days of treatment. The 5- and 25-μg/ml doses of silibinin showed 42 and 61% inhibition in cell growth, respectively (Fig. 3*A*). Cells treated with 50 and 75 μg/ml of silibinin showed 93% and complete growth inhibition, respectively (Fig. 3*A*). At these doses of silibinin, cells stopped growing as early as 1 and 2 days, with a small reduction in initial cell number at 75 μg/ml (Fig. 3*A*). In studies assessing the effect of silibinin on androgen-stimulated growth of LNCaP cells, compared with cells grown in 10% FBS, cells grown in 10% cFBS showed a 68% reduction in growth (Fig. 3*B*). This was an expected finding because cFBS is devoid of hormones and other growth agents. Cells grown in 10% cFBS + 1 nM DHT showed much higher growth, but it was only 77% of that observed in 10% FBS (Fig. 3*B*). Silibinin treatment, however, showed 38% inhibition of DHT-stimulated cell growth (Fig. 3*B*). Together, the inhibitory effects of silibinin on FBS- and DHT-stimulated LNCaP cell growth were consistent with a decrease in PSA levels. Silibinin, however, did not show a considerable inhibition of NIH 3T3 and normal human prostate cell growth (data not shown). In cell viability, silibinin did not show cytotoxicity at present doses (data not shown).

Silibinin Induces G_1 Arrest and Decreases CDK and Cyclin Kinase Activity in LNCaP Cells. We next assessed whether cell growth-inhibitory effects of silibinin are via perturbation in cell cycle progression. Fluorescence-activated cell sorter (FACS) analysis of control and silibinin-treated cells grown in 10% FBS clearly indicated a G_1 arrest by silibinin (Fig. 4). The increase in

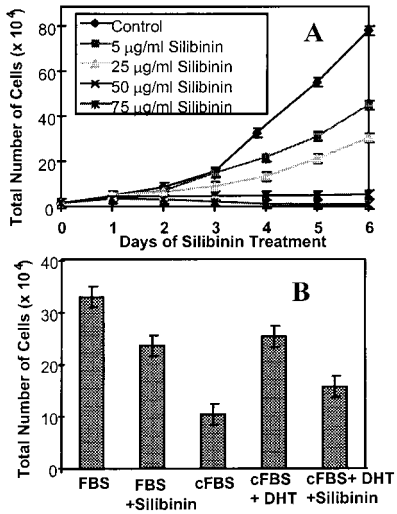

FIG. 3. Silibinin inhibits serum- and DHT-stimulated growth of LNCaP cells. (*A*) Dose- and time-dependent inhibitory effect of silibinin on serum-stimulated cell growth. Cells were treated with ethanol (control) or indicated doses of silibinin. (*B*) Inhibitory effect of silibinin on DHT-stimulated cell growth. Cells were grown in FBS, 10% serum; FBS + silibinin, 10% serum + 50 μg/ml of silibinin; cFBS, 10% cFBS; cFBS + DHT, 10% cFBS + 1 nM DHT; or cFBS + DHT + silibinin, 10% cFBS + 1 nM DHT + 50 μg/ml of silibinin. The data in *B* are at 5 days of cultures; silibinin was added at day 4. After desired treatments, cells were trypsinized and counted as described in *Materials and Methods*. Each data point represents mean ± SE of four independent plates; each sample was counted in duplicate.

G_1 population by silibinin (82.8 vs. 63% in control) was accompanied by a large decrease of cells in both S and G_2/M phases (Fig. 4 *B* vs. *A*). G_1 arrest by silibinin also was found at other time points (data not shown). Similar to silibinin, when cells were

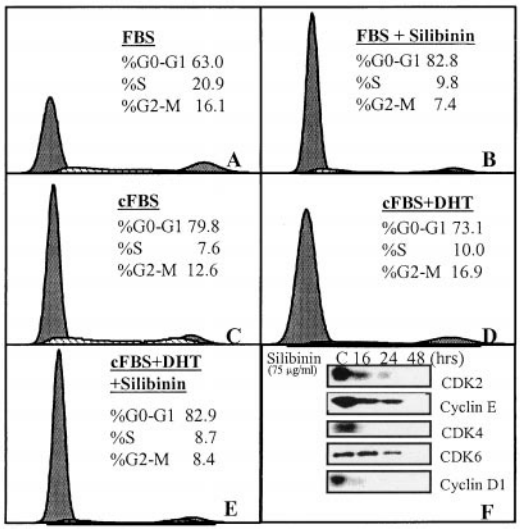

FIG. 4. Silibinin induces G_1 arrest and decreases CDK and cyclin-associated kinase activity in LNCaP cells. Cell cycle phase distribution of LNCaP cells grown in 10% serum (*A*); 10% serum + 50 μg/ml of silibinin (*B*); 10% cFBS (*C*); 10% cFBS + 1 nM DHT (*D*); and 10% cFBS + 1 nM DHT + 50 μg/ml of silibinin (*E*). The data are at 5 days of cultures; silibinin was added at day 4. After desired treatments, cells were trypsinized and FACS analysis was done as described in *Materials and Methods*. (*F*) Inhibitory effect of silibinin on CDK and cyclin kinase activity. Cells were treated with 75 μg/ml of silibinin for the indicated time, and CDK and cyclin kinase activity was determined as described in *Materials and Methods*; C, control cells treated with ethanol for 48 hr. The cell cycle phase distribution and kinase activity data shown are representative of three independent experiments with similar findings.

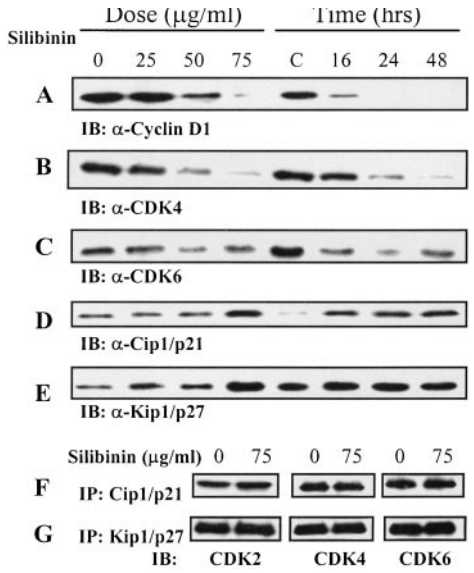

FIG. 5. Silibinin modulates protein levels of cyclin D1, CDKs, and CDKIs and increases binding of CDKIs to CDK2 in LNCaP cells. Dose- and time-dependent effect of silibinin on levels of cyclin D1 (*A*); CDK4 (*B*); CDK6 (*C*); Cip1/p21 (*D*); and Kip1/p27 (*E*). Cells were treated with silibinin for 20 hr or for the indicated time at 75 μg/ml; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared and subjected to SDS/PAGE, Western blotting, and enhanced chemiluminescence detection as described in *Materials and Methods*. Shown also is the effect of silibinin on binding of CDKs with Cip1/p21 (*F*) and Kip1/p27 (*G*). Cells were treated with vehicle or 75 μg/ml of silibinin for 16 hr, and cell lysates were prepared. CDKIs binding with CDKs was determined as described in *Materials and Methods*. The data shown are representative of three independent experiments with similar findings.

grown in 10% cFBS, a G_1 arrest also was observed (Fig. 4 *C* vs. *A*). This finding suggests a possibility that observed G_1 arrest by silibinin may be due to its inhibitory effect on growth-stimulating factors that are not present in cFBS. Additional studies also were performed to answer two questions: first, whether absence of androgen in cFBS was a major factor for observed G_1 arrest in 10% cFBS grown cells and, second, whether silibinin inhibits DHT-stimulated cell cycle progression. Compared with 10% cFBS, cells grown in 10% cFBS + 1 nM DHT showed a release from G_1 arrest (Fig. 4 *D* vs. *C*). However, when FACS data for 10% cFBS + 1 nM DHT were compared with 10% FBS, DHT-stimulated release from G_1 arrest in 10% cFBS cells was not complete (Fig. 4 *D* vs. *A*). DHT-stimulated release of cells from G_1 arrest, however, was blocked completely by silibinin (Fig. 4 *D* vs. *E*). Together, these data suggest that, in addition to androgen, there are other growth factors in serum responsible for growth and cell cycle progression of LNCaP cells and that silibinin results in a G_1 arrest in cell cycle progression of cells that are stimulated for growth by serum or only androgen.

Cell cycle progression is regulated via irreversible transitions propelled by CDKs and cyclins (22, 23). Whereas CDK4 (or CDK6)/cyclin D1 are involved in early G_1 phase, transition from G_1 to S is regulated by CDK2/cyclin E (23). Therefore, we reasoned that observed G_1 arrest by silibinin could be due to a decrease in kinase activity of CDKs and cyclins. Indeed, 75 μg/ml of silibinin showed a time-dependent decrease in CDK2 and cyclin E kinase activity (Fig. 4*F*); at 48 hr, kinase activity was not detectable in both cases. Similarly, silibinin also resulted in a highly significant decrease in CDK4, CDK6, and cyclin D1 kinase activity (Fig. 4*F*). Together, these data suggest that G_1 arrest induced by silibinin is due to a significant decrease in kinase activity of both CDKs and cyclins associated with early G_1 phase and late G_1- to S-phase transition.

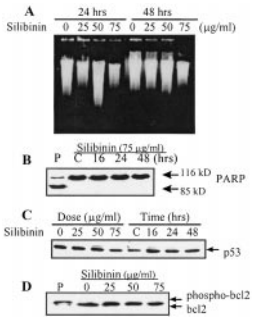

FIG. 6. Silibinin does not induce apoptosis and modulation of p53 and Bcl2 in LNCaP cells. (*A*) Agarose gel electrophoresis of cellular DNA showing a lack of DNA ladder by silibinin treatment. Cells, at 80% confluency, were treated with silibinin for the indicated doses and time. Cells were collected and cellular DNA was isolated, followed by agarose gel electrophoresis as described in *Materials and Methods*. (*B*) A lack of silibinin's effect on PARP cleavage. Cells were treated with paclitaxel (P) for 20 hr at 1 μM or for the indicated time at 75 μg/ml of silibinin; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared, and PARP protein level and cleavage were detected as described in *Materials and Methods*. (*C*) Dose- and time-dependent effect of silibinin on p53 expression. Cells were treated with silibinin for 20 hr or for the indicated time at 75 μg/ml of silibinin; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared, and p53 levels were detected as described in *Materials and Methods*. (*D*) Dose-dependent effect of silibinin on bcl2 expression. Cells were treated with paclitaxel (P) for 20 hr at 1 μM or silibinin for 20 hr, cell lysates were prepared, and bcl2 levels were detected as described in *Materials and Methods*. The data shown are representative of three independent experiments with similar findings.

Silibinin-Induced Decrease in Kinase Activity of CDKs and Cyclins Is Mediated via a Decrease in Cyclin D1, CDK4, and CDK6 Levels and an Induction of Cip1/p21 and Kip1/p27 and Their Increased Binding with CDK2 in LNCaP Cells. CDK activity is regulated positively by cyclins and negatively by CDKIs (22, 23). Based on silibinin's effect on kinase activity, we assessed its effect on (*i*) CDK and cyclin levels and (*ii*) CDKI Cip1/p21 and Kip1/p27 levels and their binding with CDKs. Silibinin resulted in a significant to complete reduction in cyclin D1 protein (Fig. 5*A*) and showed a strong decrease in CDK4 and CDK6 (Fig. 5 *B* and *C*). No effect of silibinin, however, was evident on CDK2 and cyclin E (data not shown). In other studies, silibinin resulted in both dose- and time-dependent induction of CDKIs Cip1/p21 (Fig. 5*D*) and dose-dependent induction of Kip1/p27 (Fig. 5*E*); maximum increase was evident at 24 and 16 hr, respectively. Because an induction in CDKI normally leads to an increase in its binding to and subsequent inactivation of CDK-cyclin complex (22, 23), we also investigated whether an observed decrease in CDK and cyclin kinase activity also is due to an increased CDK binding with up-regulated Cip1/p21 and Kip1/p27 by silibinin. As shown in Fig. 5 *F* and *G*, silibinin resulted in an increase only in CDK2 binding to Cip1/p21 and Kip1/p27; quantification of bands showed 1.4- and 2.6-fold increases, respectively. No effect of silibinin, however, was observed on CDK4 and CDK6 binding to either Cip1/p21 or Kip1/p27 (Fig. 5 *F* and *G*). Together, these results clearly indicate that whereas the resultant effect of silibinin was a G_1 arrest, its causes were different in terms of molecular mechanisms at early G_1 and late G_1- to S-phase transition.

Silibinin Does Not Induce Apoptosis and Modulation of p53 and bcl2 Protein Levels in LNCaP Cells. Based on observed effects of silibinin, we next assessed whether silibinin causes apoptotic death of LNCaP cells. The 25-, 50-, and 75-μg/ml doses of silibinin for 24 and 48 hr did not result in apoptosis as evidenced by a lack of DNA fragmentation (Fig. 6*A*) and a lack of poly (ADP ribose) polymerase (PARP) cleavage that otherwise was clearly evident in a paclitaxel-treated sample used as a positive control (Fig. 6*B*). Because p53 and bcl2 are considered to be crucial in apoptosis (24), we also assessed their levels after

silibinin treatment. As shown in Fig. 6 *C* and *D*, silibinin also did not result in any change in p53 and bcl2 expression; however, paclitaxel (a positive control) showed a clear phosphorylation of bcl2 (Fig. 6*D*), a process associated with inactivation of bcl2 that causes apoptosis in LNCaP cells (25). Paclitaxel also showed clear morphological changes suggestive of apoptosis (data not shown), but no such effect was evident with silibinin, and, in fact, cells started showing differentiation (Fig. 7). These results suggest that silibinin-induced G_1 arrest in LNCaP cells does not lead to an apoptotic cell death.

Silibinin Induces Neuroendocrine Differentiation and Expression of K8 & K18 and Chromogranin A in LNCaP Cells. LNCaP cells treated with silibinin manifested unique morphologic changes. Compared with cells growing in 10% FBS as piled up layers attached loosely to the surface, cells treated with silibinin primarily were monolayer and attached firmly to the surface with better anchoring (Fig. 7 *A* vs. *B*). Significant changes in morphology also were observed with silibinin as cells became elongated with prominent dendrite-like cytoplasmic extensions where some of the dendrite-like extensions were connected to each other among neighboring cells (Fig. 7*B*). These morphological changes were similar to that of neuroendocrine morphology, suggesting that silibinin induces neuroendocrine differentiation of LNCaP cells (Fig. 7*B*). LNCaP cells grown in 10% cFBS also showed similar morphological changes (Fig. 7*C*), which were reversed to normal growth morphology by 1 nM DHT (Fig. 7*D*); the addition of silibinin reversed DHT-stimulated growth effect and induced similar neuroendocrine morphology in LNCaP cells (Fig. 7*E*). Silibinin treatment of cells grown in 10% FBS (or cells grown in 10% cFBS + 1 nM DHT; data not shown) also resulted in a significant induction of K8 & K18 and chromogranin A expression under identical conditions that showed neuroendocrine differentiation (Fig. 7*F*). The observed increases in K8 & K18 and chromogranin A by silibinin were optimum at both 24 and 48 hr

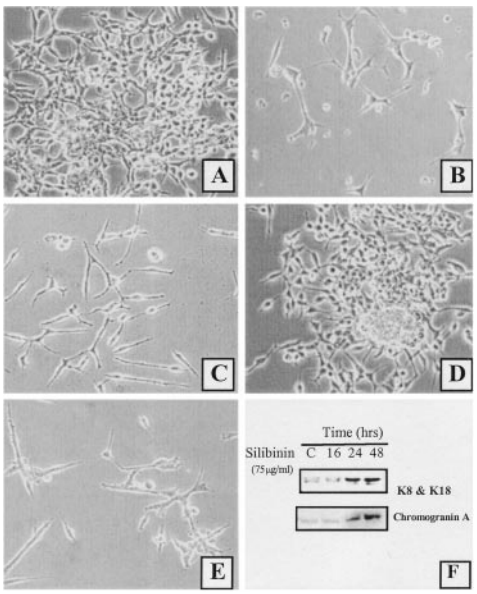

FIG. 7. Silibinin induces neuroendocrine differentiation and expression of K8 & K18 and chromogranin A in LNCaP cells. Morphology of LNCaP cells grown in 10% serum (*A*); 10% serum + 50 μg/ml silibinin (*B*); 10% cFBS (*C*); 10% cFBS + 1 nM DHT (*D*); and 10% cFBS + 1 nM DHT + 50 μg/ml of silibinin (*E*). The data are at 5 days of cultures; silibinin was added at day 3. The phase-contrast photography was done at ×200 magnification as described in *Materials and Methods*. (*F*) Stimulatory effect of silibinin on K8 & K18 and chromogranin A levels. Cells were treated with 75 μg/ml of silibinin for the indicated time; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared, and levels of K8 & K18 (*Upper*) and chromogranin A (*Lower*) were determined as described in *Materials and Methods*. The data shown are representative of three independent experiments with similar findings.

(Fig. 7*F*). K8 & K18 have been shown to be markers of prostate tissue differentiation, and both K8 & K18 and chromogranin A are induced during differentiation of LNCaP cells with similar neuroendocrine-morphological changes (26, 27). These data suggest that silibinin induces neuroendocrine differentiation of LNCaP cells after G_1 arrest in cell cycle progression coupled with inhibition of growth-stimulatory pathways mediated by both serum as well as androgen.

DISCUSSION

LNCaP cells are one of the best *in vitro* models for human PCA studies because they possess an aneuploid male karyotype, produce PSA, and express a high-affinity mutant AR (28). These cells are responsive to androgenic stimulation and form tumors in nude mice (29). Because reduction in serum PSA levels has been proposed as an endpoint biomarker for hormone-refractory human PCA intervention (9–11), our results showing that silibinin significantly decreases both intracellular and secreted levels of PSA in androgen-dependent human PCA LNCaP cells have useful implications for human PCA intervention.

PSA is an abundant serine protease produced by prostate epithelial cells (30) and can cleave predominant seminal vesicle protein (31). PSA secretion by tumor cells into prostate stroma might augment cleavage of IGFBP3-IGF-1 and the activation of transforming growth factor β or other growth factors in extracellular matrix and then endow cancerous cells with a growth advantage leading to tumor progression (8). This hypothesis explains why PCA cells tend to diffusely infiltrate prostatic stroma rather than forming a localized tumor (8). Therefore, inhibition of PSA secretion may be an important strategy to prevent PCA progression. Here, we showed that a percentage decrease by silibinin in secreted PSA levels was comparable to intracellular PSA, suggesting that a decrease in PSA secretion by silibinin may be due to its inhibitory effect on PSA protein expression in LNCaP cells. Because silibinin also inhibited DHT-induced PSA and cell growth, we suggest that silibinin may have a direct effect on AR-mediated PSA expression.

Mammalian cell growth and proliferation are mediated via cell cycle progression (22, 23). However, defects in cell cycle are one of the most common features of cancer cells, because they divide under conditions in which their normal counterparts do not (22, 23). Androgen is shown to regulate genes controlling cell cycle, and that abnormally activated AR activity (e.g., gain-of-function by mutations in AR) may malignantly stimulate cell growth (32). Therefore, agents that inhibit cell cycle progression of cancer cells could lead to a cell growth arrest. We provide convincing evidence that silibinin inhibits both serum- and androgen-stimulated LNCaP cell growth by inducing G_1 arrest. The results from molecular mechanism studies showed that G_1 arrest by silibinin involves a significant decrease in cyclin D1, CDK4, and CDK6, resulting in a marked decrease in their kinase activity, and a significant increase in Cip1/p21 and Kip1/p27 that leads to their increased binding with CDK2, resulting in a marked decrease in CDK2 and cyclin E kinase activity.

Cyclin D1 is involved in cell cycle during early G_1 phase (23). In controlled cell growth, association of cyclin D1 with CDK4 or CDK6 leads to phosphorylation of RB; hyperphosphorylated RB leads to its release from E_2F (33). The free E_2F then activates c-*myc*, resulting in cell proliferation by progression via G_1 (34). However, overexpression of cyclin D1 is associated with various cancers and tumor-derived cell lines, explaining their uncontrolled growth (35). One of the aspects of cyclin D1 overexpression in cells is a shorten G_1 phase, resulting in a more rapid entry into S phase and increased proliferation (35). Based on these and other studies (34–36), a significant decrease in protein levels of cyclin D1, CDK4, and CDK6 by silibinin suggests that silibinin should be a useful agent for the intervention of malignancies overexpressing cyclin D1, CDK4, and/or CDK6. The observed inhibitory effects of silibinin on cyclin D1, CDK4, and CDK6 in

LNCaP cells are of particular significance for the intervention of hormone-refractory PCA because cyclin D1 is strongly associated with androgen-stimulated growth of LNCaP cells (37). Cyclin D1 is also constitutively expressed in androgen-independent human PCA PC3 and DU145 cells, but it is significantly lower in LNCaP cells grown without serum (38). In a recent study, overexpression of cyclin D1 in LNCaP cells was shown to increase cell growth and tumorigenicity in nude mice (39). Consistently, we found that LNCaP cells grown in cFBS arrest mostly in G_1 phase, which is reversed by DHT. This finding suggests the involvement of androgen-mediated growth after the release of cells from G_1 arrest because of a significant decrease in cyclin D1 in the absence of androgen. Similarly, silibinin treatment of LNCaP cells grown in serum or cFBS + DHT also showed a G_1 arrest together with a decrease in serum- and androgen-stimulated PSA levels and cell growth inhibition. These results suggest that observed effects of silibinin are those mediated via AR in terms of PSA levels, cell growth, cell cycle progression, as well as modulation of cyclin D1 and associated CDKs. In support of this suggestion, we recently have shown that treatment of human PCA DU145 cells with silymarin does not involve alterations in cyclin D1 for G_1 arrest (40). More detailed studies are in progress to support the involvement of AR in the inhibitory effects of silibinin.

p53 is an important tumor-suppressor gene, and mutations in p53 are the most commonly observed genetic lesions in human tumors (41). In response to genotoxic stress, p53 induces Cip/p21, resulting in a G_1 arrest (42). However, activation of Cip1/p21 also occurs independent of p53 as observed by transforming growth factor β stimulation during differentiation or upon cellular senescence (43). In each case, up-regulation of Cip1/p21 correlated with an arrest in cell growth, suggesting that it plays a fundamental role in the decision fork between cell proliferation, differentiation, and death. For example, inhibition of Cip1/p21 expression through transfection of Cip1/p21 antisense oligonucleotides was shown to block growth factor-induced differentiation of SH-SY5Y neuroblastoma cells and resulted in their death (44). Cip1/p21 induction also is shown in a variety of cell differentiation, including myogenic, keratinocytic, promyelocytic (HL-60), and human melanoma cells (45–47); Kip1/p27 also has been reported to be involved in cell differentiation (48). Consistently, we observed that silibinin-caused induction of Cip1/p21 was p53-independent and that, together with resultant G_1 arrest, did not induce apoptosis in LNCaP cells. Because treatment of LNCaP cells with silibinin showed neuroendocrine differentiation like morphologic changes and increased K8 & K18 and chromogranin A levels, induction of both Cip1/p21 and Kip1/p27 is likely to be involved with cell cycle exit that is associated with differentiation.

Together, the central finding in the present study is that silibinin, an active constituent of milk thistle, inhibits both serum- and androgen-stimulated PSA protein levels in LNCaP cells concomitant with cell growth inhibition via a G_1 arrest in cell cycle progression. The silibinin-treated LNCaP cells that are unable to grow follow a differentiation pathway as evidenced by neuroendocrine-like morphology, elevated prostate tissue-differentiation markers K8 & K18 and chromogranin A, and altered cell cycle-regulatory molecules. More detailed mechanistic studies are in progress to identify and define the effect of silibinin on the growth-stimulatory signals in hormone-refractory prostate carcinoma cells at molecular levels and to assess the inhibitory effect of silibinin on human PCA tumor xenograft growth in nude mice. In summary, however, based on the present findings, we conclude that silibinin has strong potential to be developed as an antiproliferative differentiating agent for the intervention of hormone-refractory human prostate cancer.

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