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Silibinin causes cell cycle arrest and apoptosis in human bladder transitional cell carcinoma cells by regulating CDKI–CDK–cyclin cascade, and caspase 3 and PARP cleavages

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Bladder cancer is the fourth and eighth most common cancer in men and women in the USA, respectively. Flavonoid phytochemicals are being studied for both prevention and therapy of various human malignancies including bladder cancer. One such naturally occurring flavonoid is silibinin isolated from milk thistle. Here, we assessed the effect of silibinin on human bladder transitional cell carcinoma (TCC) cell growth, cell cycle modulation and apoptosis induction, and associated molecular alterations, employing two different cell lines representing high-grade invasive tumor (TCC-SUP) and high-grade TCC (T-24) human bladder cancer. Silibinin treatment of these cells resulted in a significant dose- and time-dependent growth inhibition together with a G_1 arrest only at lower doses in TCC-SUP cells but at both lower and higher doses in T-24 cells; higher silibinin dose showed a G_2/M arrest in TCC-SUP cells. In other studies, silibinin treatment strongly induced the expression of Cip1/p21 and Kip1/p27, but resulted in a decrease in cyclin-dependent kinases (CDKs) and cyclins involved in G_1 progression. Silibinin treatment also showed an increased interaction between cyclin-dependent kinase inhibitors (CDKIs)–CDKs and a decreased CDK kinase activity. Further, the G_2/M arrest by silibinin in TCC-SUP cells was associated with a decrease in pCdc25c (Ser216), Cdc25c, pCdc2 (Tyr15), Cdc2 and cyclin B1 protein levels. In additional studies, silibinin showed a dose- and a time-dependent apoptotic death only in TCC-SUP cells that was associated with cleaved forms of caspase 3 and poly(ADP-ribose) polymerase. Together, these results suggest that silibinin modulates CDKI–CDK–cyclin cascade and activates caspase 3 causing growth inhibition and apoptotic death of human TCC cells, providing a strong rationale for future studies evaluating preventive and/or intervention strategies for silibinin in bladder cancer pre-clinical models.

Introduction

Bladder cancer is the fourth most common cancer among men and the eighth most common among women in the USA (1). In the USA alone, there are 57 000 new bladder cancer cases each year with 12 500 associated deaths (1). Overall, bladder cancer occurs about twice as often in males than in females, and Caucasians develop this malignancy slightly more often than African-Americans (1). Half of the bladder cancer cases are related to tobacco smoking, where smokers have a 4-fold higher incidence of bladder cancer compared with non-smokers (2–4). There is a measurable increase in DNA damage in urinary bladder cells from smokers compared with non-smokers even in the absence of the neoplasm (5). Thus, smoking is probably a major contributing factor for the development of bladder cancer. Another 25% of cases of bladder cancer, mostly in men, appear to be caused by industrial exposure to aromatic polycyclic hydrocarbons or polychlorinated biphenyls such as 2-naphthylamine, 4-aminobiphenyl and benzidine (2). One strategy to control bladder cancer is chemoprevention and/or chemo-intervention.

Chemoprevention and chemo-intervention refer to the administration of one or more chemical entities, either as individual agents in their pure chemical defined forms or as naturally occurring constituents of the diet such as fruits, vegetables, common beverages and several herbs and plants (6). Among several classes of the chemical agents, naturally occurring flavonoids and isoflavones have received increased attention in the last few years (7–9). One such naturally occurring flavonoid is silibinin isolated from milk thistle. Silibinin and its crude form, silymarin, are used clinically as anti-hepatoxic agents, and are consumed as dietary supplements around the world (10). Both silibinin and silymarin are well tolerated and largely free of adverse effects in both animal and human studies (10). Several recent studies by others and us have shown the cancer preventive and therapeutic efficacy of silibinin in different animal tumor models and cell culture systems including prostate, breast, colon and skin cancers (11–14).

The majority of bladder cancers (90%) are transitional cell carcinoma (TCC), where variable morphology, natural history and prognosis demonstrate that it is not a single disease, but occurs in three distinct forms, each possessing characteristic features such as carcinoma *in situ*; low-grade papillary, non-invasive; and high grade, invasive malignancy (15). Recent studies have begun to elucidate the underlying genetic determinants of the morphologic and biologic characteristics of these different forms of bladder cancer (16). The molecular and genetic alterations that precede morphologic changes and are responsible for tumorigenesis and progression of bladder cancer also include alterations in cell cycle regulators causing uncontrolled cancer growth.

In general, the progression of cell cycle in eukaryotes is a complex process involving resting G_0 phase, and cell growth involving G_1, S and G_2/M phases in a step-wise manner (17). These cell cycle phases receive different growth controlling signals that are integrated and processed for the sequential activation of different members of the cyclin-dependent

Abbreviations: CDKs, cyclin-dependent kinases; CDKIs, cyclin-dependent kinase inhibitors; FACS, fluorescence-activated cell sorting; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; Rb, retinoblastoma; TCC, transitional cell carcinoma.

kinases (CDKs), which are serine/threonine kinases (17–20). Different CDKs govern different phases of the cell cycle such as G_1 by CDK4/CDK6, late G_1 to early S by CDK2, and G_2/M by p34Cdc2 (CDK1) kinase (17–20). The kinase activity of CDKs is governed by their regulatory subunits known as cyclins, which form a complex with their catalytic subunit CDKs and are activated at a specific phase of the cell cycle (21–23). The other important components that control CDK kinase activity are cyclin-dependent kinase inhibitors (CDKIs) Cip1/p21 and Kip1/p27 (21–23). CDKI is shown to inhibit the kinase activity of CDK–cyclin complexes and thus modulates retinoblastoma (Rb) phosphorylation events, which are essential for various cell cycle transitions (21,24,25).

Taken together, these observations suggest new approaches that could alter uncontrolled human bladder TCC growth by modulating cell cycle regulators causing cell cycle arrest and could be useful in human bladder cancer prevention and/or intervention. In the present study, employing two different human bladder TCC cell lines, TCC-SUP and T-24, that represent high-grade invasive tumor (TCC-SUP) and high-grade TCC (T-24) bladder cancer, we assessed the efficacy of silibinin on cell growth, cell cycle progression and apoptotic death, and associated alterations in cell cycle regulators. The results obtained clearly demonstrate that silibinin inhibits human bladder TCC cell growth, causes cell cycle arrest and apoptosis, albeit at different levels, in two cell lines, and that a strong increase in CDKI levels together with a decrease in both CDKs and cyclin levels as well as a decrease in CDK kinase activity are responsible for the observed effects of silibinin in cell cycle arrest. Furthermore, silibinin-caused caspase 3 and poly (ADP-ribose) polymerase (PARP) cleavages contribute to its apoptotic response in TCC-SUP cells.

Materials and methods

Cell lines and reagents

Human bladder TCC cell lines TCC-SUP and T-24 were purchased from the American Type Culture Collection (Manassas, VA), and cultured in MEM and DMEM, respectively, with 10% fetal bovine serum under standard culture conditions (37°C, 95% humidified air and 5% CO_2). Silibinin used in the present study was from Sigma-Aldrich Chemical Company (St Louis, MO) and was analyzed by HPLC as a pure agent as reported earlier (11). The primary antibody for anti-Cip1/p21 was from Calbiochem (Cambridge, MA), and for anti-Kip1/p27 was from Neomarkers (Fremont, CA). Antibodies to CDK2, 4, 6, Cdc2; cyclin D1, D3, E, B1; Cdc25c and Wee1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cleaved PARP, cleaved caspase 3, Phospho-Cdc2 (Tyr15), Phospho-Cdc25c (Ser216), Rb-fusion protein, phospho-specific Rb antibodies, and secondary antibodies were purchased from Cell Signaling (Beverly, MA). The antibody for β-actin was from Sigma. Histone H1 was from Boehringer Mannheim (Indianapolis, IN). [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol) and ECL detection system were from Amersham (Arlington Heights, IL). Annexin V-Vybrant apoptosis kit was from Molecular Probes (Eugene, OR).

Cell growth assay

TCC-SUP or T-24 cells were plated at 5000 cells/cm^2 in 60-mm plates under the standard culture condition. After 24 h, cells were fed with fresh medium and treated with DMSO alone or a different concentration of silibinin (50, 100 and 200 μM). After 24, 48 and 72 h of treatments, cells were trypsinized, collected and counted using a hemocytometer. Trypan blue dye exclusion was used to determine cell viability. The highest silibinin concentration used in the present study, 200 μM, has recently been reported by us to be in the physiological/pharmacological range at least in the animal studies (13).

Flow cytometry analysis for cell cycle analysis

TCC-SUP or T-24 cells at 60% confluency were treated with either DMSO alone or various doses of silibinin. After 24, 48 and 72 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/propidium iodide (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 fluorescence-activated cell sorting (FACS) analysis core services of the University of Colorado Cancer Center (Denver, CO).

Cell culture treatments for molecular analyses

TCC-SUP and T-24 cells were cultured in MEM or DMEM medium containing 10% fetal bovine serum and 1% penicillin–streptomycin under standard culture conditions. At 60% confluency, cultures were treated with desired doses of silibinin (50, 100 and 200 μM) in DMSO or DMSO alone for different time points (24–48 h). Following silibinin treatments, cell lysates were prepared in non-denaturing lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, 1 mM EGTA, 0.3 mM phenyl methyl sulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% NP-40, 5 U/ml aprotinin). Briefly, medium was aspirated and cells were washed with ice cold PBS twice followed by incubation in lysis buffer for 10 min on ice. Then cells were scraped and kept on ice for 30 min, and finally cell lysates were cleared by centrifugation at 4°C for 30 min at 14 000 r.p.m. Protein concentrations in lysates were determined using Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA).

Immunoprecipitation and immunoblotting

Cell lysates (200 μg protein/sample) were diluted to 1 ml with lysis buffer and pre-cleared with protein A/G plus agarose for 1 h, then incubated overnight with primary antibody against CDK2 or CDK4 plus protein A/G plus agarose beads, and immunocomplexes were collected and washed three times with lysis buffer. For immunoblotting, immunocomplexes or total cell lysates were denatured with 2× sample buffer. Samples were subjected to SDS–PAGE on 12 or 16% gel and separated proteins were transferred onto membrane by western blotting. Membranes were blocked with blocking buffer for 1 h at room temperature, and, as desired, probed with primary antibody against, Cip1/p21, Kip1/p27, CDK2, CDK4, CDK6, cyclin E, cyclin D1, cyclin D3, cleaved caspase 3, cleaved PARP, pCdc2 (Tyr15), pCdc25c (Ser216), Cdc25c, Cdc2, cyclin B1, Wee1 and β-actin overnight at 4°C followed by peroxidase-conjugated appropriate secondary antibody and ECL detection. For the binding experiments, the secondary antibody used was HRP-conjugated Mouse IgG TrueBlot (eBioscience, San Diego, CA).

Kinase assays

To assess CDK4 kinase activity, 200 μg of protein lysates from each sample was pre-cleared with protein A/G-plus agarose beads and CDK4 protein was immunoprecipitated using anti-CDK4 antibody and protein A/G plus agarose beads. After overnight incubation at 4°C, beads conjugated with antibody and protein were washed three times with Rb-lysis buffer (50 mM HEPES–KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 80 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 0.1% Tween 20, 10% glycerol, 1 mM PMSF and 10 μg/ml aprotinin and leupeptin) and twice with Rb-kinase assay buffer (50 mM HEPES–KOH, pH 7.5, 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 10 mM $MgCl_2$, 1 mM NaF, 0.1 mM sodium orthovanadate). Phosphorylation of Rb was measured by incubating the beads with 30 μl of Rb-kinase solution (1 μg of Rb-fusion protein and 0.1 mM ATP in Rb-kinase buffer) for 30 min at 37°C. The reaction was stopped by boiling the samples in 5× SDS sample buffer for 5 min. Samples were analyzed by SDS–PAGE and western blotting followed by detection of Rb-fusion protein phosphorylation employing phospho-specific Rb antibodies.

Similarly, to determine the CDK2 associated H1 histone kinase activity, CDK2 protein was immunoprecipitated using anti-CDK2 antibody and protein A/G-plus agarose beads. Beads were washed twice with lysis buffer and finally once with kinase assay buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 30 μl of hot kinase solution [2.5 μg of histone H1, 0.5 ml (5 mCi) of [γ-^{32}P]ATP, 0.5 ml of 0.1 μM ATP and 28.75 μl of kinase buffer] for 30 min at 37°C. Boiling the samples in SDS sample buffer for 5 min stopped the reaction. The samples were analyzed by 12% SDS–PAGE, and the gel was dried and subjected to autoradiography.

Quantitative apoptotic cell death assay

To quantify silibinin-induced apoptotic death of TCC-SUP or T-24 cells, annexin V and PI staining was performed followed by flow cytometry. After 24, 48 and 72 h of silibinin (50, 100 and 200 μM) treatment, cells were collected and subjected to annexin V and PI staining using Vybrant Apoptosis Assay Kit2 and following the step-by-step protocol provided by the manufacturer. The kit contains recombinant annexin V conjugated to fluorophores and the Alexa fluro 488 dyes, providing maximum sensitivity. The apoptotic cells stained with annexin V showing green fluorescence, dead cells stained with both annexin V and PI showing red and green fluorescence, and live cells show little or no fluorescence.

Densitometry and statistical analysis

Autoradiograms of the immunoblots were scanned using Adobe Photoshop, Adobe System Incorporated (San Jose, CA). Density for each band was analyzed using the Scion Image program, National Institutes of Health, (Bethesda, MD). The numerical data shown under each blot are arbitrary units where silibinin treatments are compared with DMSO control. In each case, the control densitometry value is employed as '1' and a comparison is then made for densitometry values obtained following silibinin treatments. The comparative data are presented as 'fold change' as compared with respective control for silibinin treatments in each case. Statistical significance of differences between control and treated samples were calculated by Student's *t*-test (SigmaStat 2.0, Jandel Scientific). P values of <0.05 were considered significant. Unless otherwise mentioned, all the data shown in the study for cell growth inhibition, cell cycle phase distribution, immunoblotting, binding studies, kinase activities and quantitative apoptosis are representative of two or three independent studies.

Results

Silibinin inhibits growth of human bladder TCC cells

Our first aim was to investigate whether silibinin treatment imparts an anti-proliferative effect against bladder TCC cells, as this is the first study assessing the effect of silibinin in human bladder TCC cells (TCC-SUP and T-24). As shown in Figure 1, silibinin treatment inhibited the growth of TCC-SUP and T-24 cells in a dose- and a time-dependent manner. Silibinin treatment at 50, 100 and 200 μM doses resulted in 11–41% ($P < 0.001$) and 13–83% ($P < 0.001$) inhibition in the growth of TCC-SUP cells (Figure 1A) and 9–56% ($P < 0.001$) and 37–83% ($P < 0.001$) growth inhibition in T-24 cells (Figure 1B) after 48 and 72 h of treatment, respectively. Much stronger cell growth inhibition was observed at the 200 μM dose of silibinin following 48 and 72 h treatment in both the cell lines; however, cell growth was minimally affected by the 50 μM dose of silibinin following 24 h treatment in both the cells lines (Figure 1A and B).

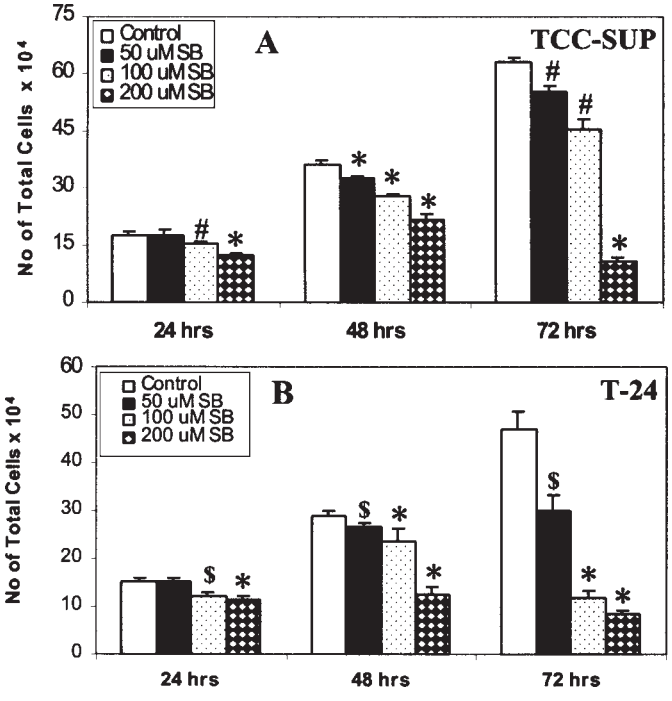

Fig. 1. Silibinin exhibits growth inhibition in human bladder transitional cell carcinoma TCC-SUP (**A**) and T-24 (**B**) cells. Cells (1.05×10^5) were plated in 60 mm dishes, treated with DMSO (control) or different concentrations of silibinin, and after 24, 48 or 72 h cells were counted as detailed in the Materials and methods. The data shown are mean ± SE of three independent plates, which were reproducible in two additional independent experiments. $, $P < 0.05$; #, $P < 0.01$; and *, $P < 0.001$; for differences with control group. SB, silibinin.

Silibinin induces cell-cycle arrest in human bladder TCC cells

To gain an insight into the mechanism of anti-proliferative activity of silibinin, its effect on cell cycle distribution was determined. Silibinin induced G_1 arrest in the cell cycle progression of both TCC cell lines. In the case of TCC-SUP cells, compared with the DMSO-treated control showing a 52% cell population in G_1 phase, silibinin treatment showed an accumulation of 55, 68 and 51% cells in G_1 phase at 50, 100 and 200 μM doses following 24 h treatment (Figure 2A). Overall, the 100 μM dose of silibinin in TCC-SUP cells for 24 h showed maximum G_1 arrest that was accompanied by a decrease in the S phase cell population (Figure 2B). In a time–response study, the G_1 arrest observed after 24 h of silibinin treatment at the 100 μM dose, remained statistically significant ($P < 0.05$ and <0.001) after both 48 and 72 h of treatment, but to a lesser degree than that observed at 24 h (Figure 2A).

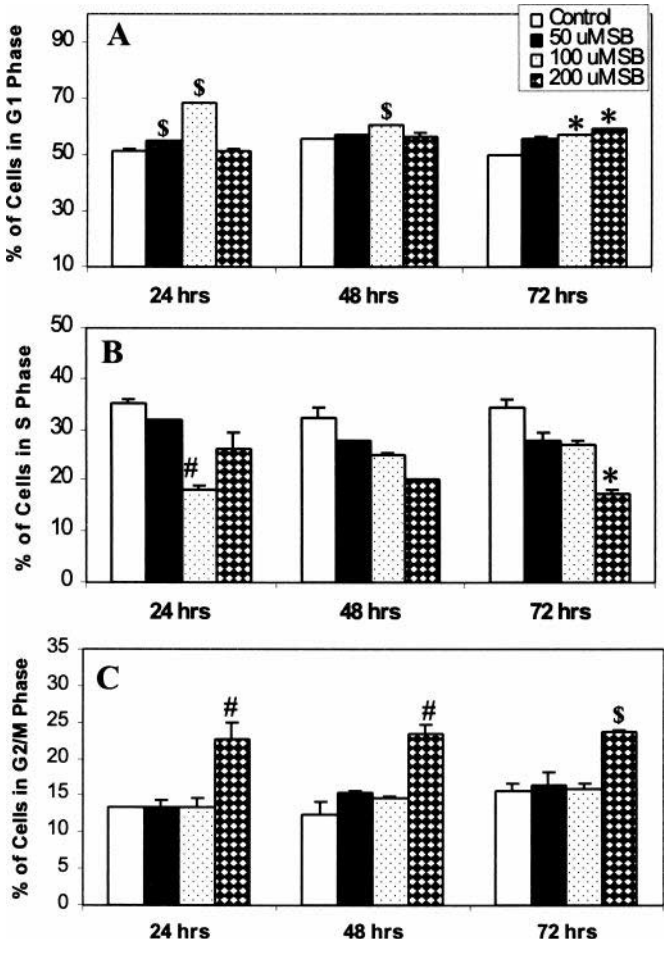

Fig. 2. Silibinin induces G_1 and G_2/M arrests in the cell cycle progression of human bladder transitional cell carcinoma TCC-SUP cells. Cells were cultured as described in the Materials and methods and treated with either DMSO alone (control) or varying concentrations of silibinin for 24, 48 and 72 h. At the end of these treatments, cells were collected and incubated with saponin/PI solution at 4°C for 24 h in dark and subjected to FACS analysis as detailed in the Materials and methods. The data shown are mean ± SE of three independent plates, which were reproducible in two additional independent experiments. $, $P < 0.05$; #, $P < 0.01$; and *, $P < 0.001$; for differences with control group. SB, silibinin.

Interestingly, the higher dose of silibinin (200 μM) treatment of TCC-SUP cells for all time points studied (24, 48 and 72 h), showed a strong G_2/M arrest accounting for 23% cell population in this cell phase ($P < 0.01$) compared with respective controls with 12–14% cells in G_2/M (Figure 2C). In T-24 cells, silibinin treatment for 48 h showed a maximum effect in causing 66, 72 and 65% cells in G_1 phase at 50, 100 and 200 μM doses as compared with 53% cells in control (Figure 3A). Similar to TCC-SUP cells, the effect of silibinin on G_1 arrest in T-24 cells was also largely accompanied by a decrease in S phase cells, whereas the population of cells in G_2/M phase did not change significantly as compared with corresponding DMSO-treated control T-24 cells (Figure 3B and C).

Silibinin induces CDKIs Cip1/p21 and Kip1/p27 in human TCC cells

Based on the above findings where silibinin treatment showed G_1 and G_2/M phase arrest in TCC-SUP cells and a G_1 phase arrest in T-24 cells, we next assessed the effect of silibinin on cell cycle regulatory molecules involved in G_1 phase of the cell cycle progression. Our results revealed that the Cip1/p21 protein was induced 24 h after silibinin treatment and persisted for 48 h in TCC-SUP cells (Figure 4A). Similarly in T-24 cells, Cip1/p21 protein expression was induced at 24 h and remained high up to 48 h of silibinin treatment (Figure 4C). Treatment of cells with silibinin also showed an up-regulation of Kip1/p27 protein level in both cell lines (Figure 4B and D). Overall, silibinin treatment (50, 100 and 200 μM) of TCC-SUP and T-24 cells for 24 and 48 h induced the protein expression of both Cip1/p21 and Kip1/p27 in a dose-dependent manner. The densitometric analysis of the blots for Cip1/p21 showed maximum induction (~3–4 fold) after 24 and 48 h of silibinin treatments at 100 and 200 μM doses in TCC-SUP cells (Figure 4A), but 1–3-fold increase in Cip1/p21 in T-24 cells under identical silibinin treatments (Figure 4C), respectively. In the case of Kip1/p27, comparable induction (~6–6.5-fold) was evident after 24 and 48 h of 100 and 200 μM doses of silibinin treatments in TCC-SUP cells (Figure 4B), but in the case of T-24 cells, maximum induction (4.5-fold) was observed after 24 h of silibinin treatment at 200 μM dose (Figure 4D). The observed strong induction in Cip1/p21 and Kip1/p27 protein levels by silibinin was not due to a change in protein loading as confirmed by probing the same membrane with β-actin antibody (Figure 4E).

Silibinin decreases protein levels of G_1 regulatory CDKs and cyclins in human TCC cells

Using immunoblot analysis, we also observed the effect of silibinin treatment on the protein levels of the CDKs and

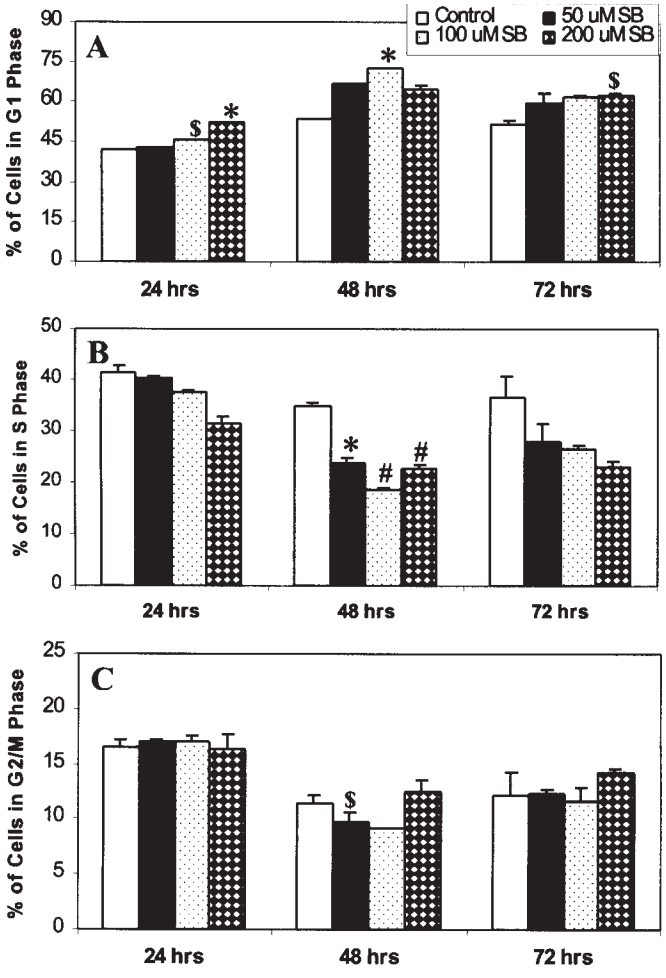

Fig. 3. Silibinin induces G_1 arrest in the cell cycle progression of human bladder transitional cell carcinoma T-24 cells. Cells were cultured as described in the Materials and methods and treated with either DMSO alone (control) or varying concentrations of silibinin for 24, 48 and 72 h. At the end of the treatment, cells were collected and incubated with saponin/PI solution at 4°C for 24 h in dark and subjected to FACS analysis as detailed in the Materials and methods. The data shown are mean ± SE of three independent plates, which were reproducible in two additional independent experiments. $, $P < 0.05$; #, $P < 0.01$; and *, $P < 0.001$; for differences with control group. SB, silibinin.

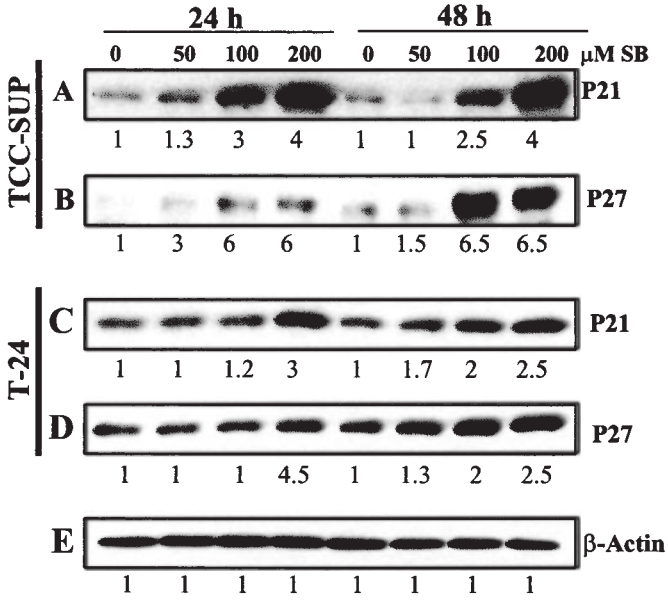

Fig. 4. Silibinin induces Cip1/p21 and Kip1/p27 protein expression in human bladder transitional cell carcinoma TCC-SUP and T-24 cells. Cells were cultured as described in the Materials and methods, and treated with either DMSO alone (control) or varying concentrations of silibinin. Different treatments are as labeled in the figure; lanes labeled as '0' denote DMSO treatment alone. At the end of the treatments, total cell lysates were prepared and subjected to SDS–PAGE followed by western immunoblotting. Membranes were probed with anti-Cip1/p21, Kip1/p27 and β-actin antibodies followed by peroxidase-conjugated appropriate secondary antibodies, and visualized by ECL detection system. The densitometric data (arbitrary) shown under the immunoblots in (**A–E**) are from representative blots. SB, silibinin.

cyclins, which are negatively regulated by CDKIs (Cip1/p21 and Kip1/p27) during G_1 cell cycle progression. Silibinin strongly decreased the expression of CDK2, CDK4 and CDK6 levels in both the cell lines (Figure 5) in a dose-dependent manner. In TCC-SUP cells, silibinin treatment at a 200 μM dose for 48 h resulted in a down regulation in CDK2 (0.02-fold, Figure 5A), CDK4 (0.2-fold, Figure 5B) and CDK6 (0.2-fold, Figure 5C) protein levels. Identical silibinin treatment in T-24 cells also caused a down regulation of CDK2 (0.5-fold, Figure 5A') and CDK4 (0.4-fold, Figure 5B'), whereas no changes were observed in CDK6 protein levels (Figure 5C'). In the studies assessing silibinin effect on cyclin levels, it decreased the protein levels of cyclin D1 (0.5-fold, Figure 5D), D3 (0.3-fold, Figure 5E) and cyclin E (0.5-fold, Figure 5F) in TCC-SUP cells at 200 μM dose following 48 h of treatment. We also observed a decrease in cyclin D3 (0.6-fold, Figure 5E') and cyclin E (0.5-fold, Figure 5F') protein expression, but no change in cyclin D1 levels (Figure 5D') in T-24 cells following similar silibinin treatment. In all these experiments, the lowest dose of silibinin (50 μM) did not show a noticeable effect.

Silibinin increases CDK–CDKI binding in human bladder TCC cells

Based on our findings showing that silibinin strongly induces CDKI expression in both the human bladder TCC cell lines, and since such an induction in CDKIs has been shown to result in an increased interaction with CDKs leading to a decrease in their kinase activity (17), we next assessed whether silibinin causes an increased interaction between induced CDKIs and CDKs. To assess the effect of silibinin on this binding, cell extracts were subjected to immunoprecipitation using CDK2 or CDK4 antibody, and after SDS–PAGE and blotting, membranes were probed with anti-Cip1/p21 or Kip1/p27, as well as CDK2 or CDK4 (to confirm the specificity of immunoprecipitated CDKs). As shown in Figure 6, compared with DMSO-treated controls, silibinin treatment showed an increased binding of Cip1/p21 and Kip1/p27 with CDK2 and CDK4 in both (TCC-SUP and T-24) cell lines. These results suggest that an increased interaction between induced levels of CDKIs (by silibinin) with CDKs plays an important regulatory role in possibly inhibiting CDK kinase activity leading to a G_1 arrest by silibinin in the cell cycle progression of human bladder TCC cell lines.

Silibinin inhibits CDK kinase activity in human bladder TCC cells

To further explore the interaction between CDKIs and CDK by silibinin treatment of TCC cells, which resulted in an inhibition in the kinase activity of CDKs, cell lysates prepared from control and silibinin-treated cells were subjected to immunoprecipitation with CDK2 or CDK4 antibodies followed by

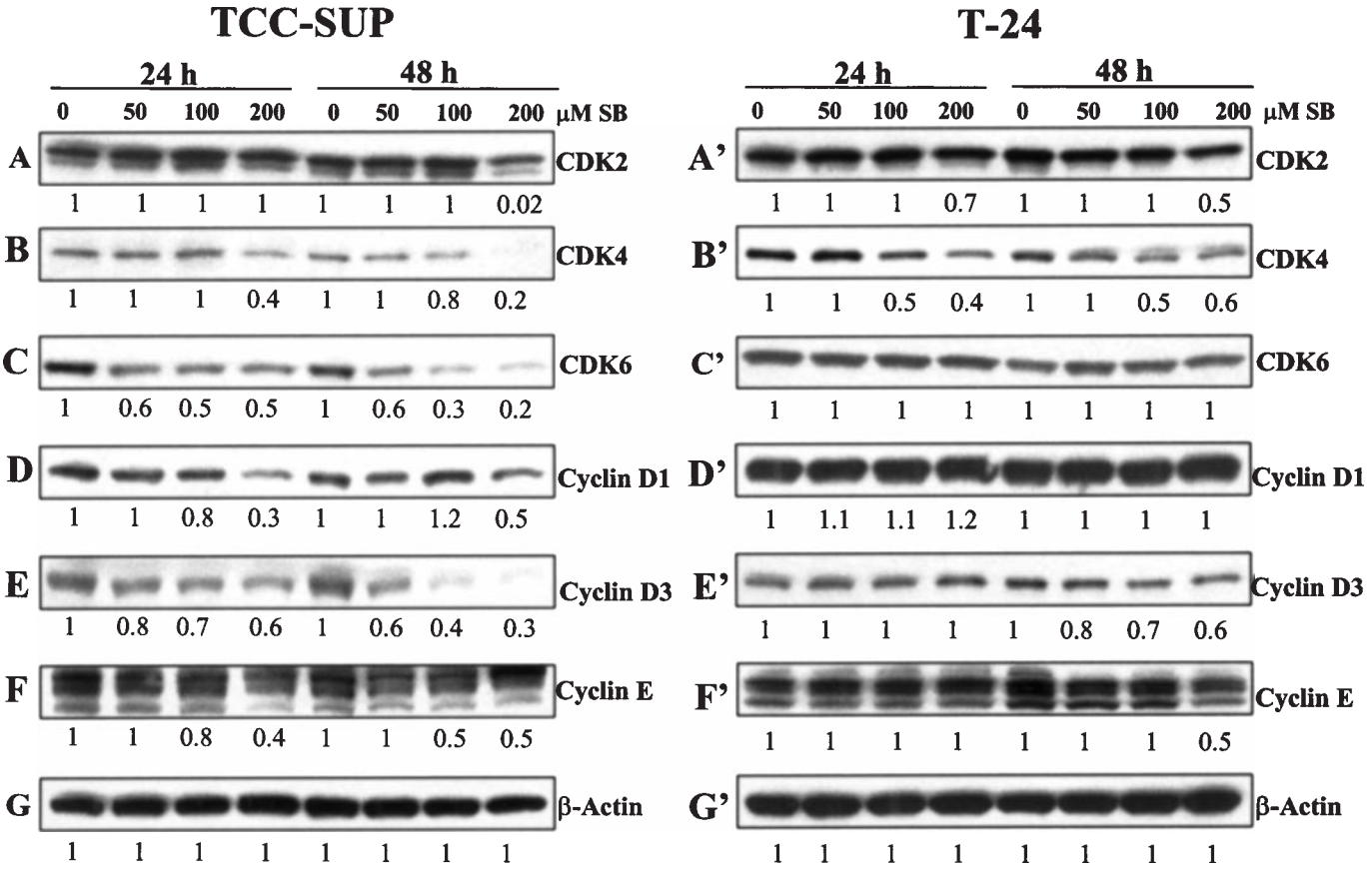

Fig. 5. Silibinin decreases the protein expression of G_1 cell cycle regulatory CDKs and cyclins in TCC-SUP and T-24 cells. Cells were cultured as described in the Materials and methods, and treated with either DMSO alone (control) or varying concentrations of silibinin. Different treatments are as labeled in the figure; lanes labeled as '0' denote DMSO treatment alone. At the end of the treatments, total cell lysates were prepared and subjected to SDS–PAGE followed by western immunoblotting. Membranes were probed with anti-CDK2, CDK4, CDK6, cyclin D1, cyclin D3, cyclin E and β-actin antibodies followed by peroxidase-conjugated appropriate secondary antibodies, and visualized by ECL detection system. The densitometric data (arbitrary) shown under the immunoblots in (**A–G**) (for TCC-SUP cells) and (**A'–G'**) (for T-24 cells) are from representative blots. SB, silibinin.

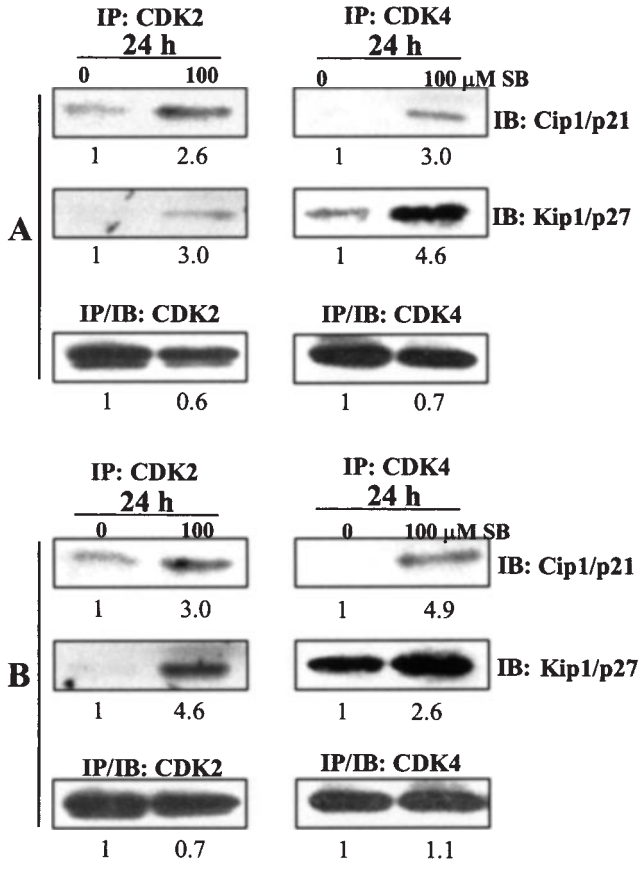

Fig. 6. Silibinin induces CDKs–CDKIs binding in human bladder transitional cell carcinoma TCC-SUP and T-24 cells. Cells were cultured and treated with either DMSO or 100 μM silibinin for 24 h as described in the Materials and methods. At the end of the treatments, total cell lysates were prepared. For binding study, CDK2 and CDK4 were immunoprecipitated from total cell lysates by using specific antibody followed by SDS–PAGE and western immunoblotting as detailed in the Materials and methods. Membranes were probed with anti-Cip1/p21, Kip1/p27 and CDK2/CDK4 antibodies followed by peroxidase-conjugated Mouse IgG TrueBlot secondary antibody, and visualized by ECL detection system as described in the Materials and methods. (**A** and **B**) Data for TCC-SUP and T-24 cells, respectively. The densitometric data (arbitrary) shown under the immunoblots in (A and B) are from representative blots. SB, silibinin.

kinase assays. As shown in Figure 7A and B, compared with DMSO-treated controls, silibinin treatment of both TCC-SUP and T-24 cells resulted in a strong decrease in CDK2 kinase activity as evidenced by a reduction in phosphorylation of histone H1 used as substrate. Similarly, silibinin also showed inhibition of CDK4 kinase activity in both the cell lines as observed by a reduction in ser780 and ser807/811 phosphorylation of Rb-fusion protein used as substrate (Figure 7C–F).

Molecular mechanism of silibinin-induced G_2/M arrest in TCC-SUP cells

Since the cyclin B/Cdc2 complex performs an important function in controlling the G_2/M phase (26), and since treatment of TCC-SUP cells with silibinin at 200 μM dose resulted in a strong increase in G_2/M phase cell population, we next conducted a detailed analysis of the molecules involved in G_2/M phase of the cell cycle. These analyses were done only in TCC-SUP cells, as we observed G_2/M arrest only in these cells by silibinin. Cdc25c phosphatase and Wee1 kinases are responsible for the dephosphorylation and phosphorylation of Cdc2, respectively, where the dephosphorylated form of Cdc2 is active and regulates the entry of all cells into M phase of the cell cycle (17,26). As shown in Figure 8, silibinin treatment of cells also resulted in a decrease in pCdc25c (Ser216), Cdc25c, pCdc2 (Tyr15) and Cdc2 protein levels (Figure 8A–D), without any noticeable change in Wee1 levels (Figure 8F). Similar silibinin treatment also resulted in a strong decrease in cyclin B1 protein levels in TCC-SUP cells (Figure 8E). Taken together, these results suggest that changes in the expression of G_2/M regulatory proteins in TCC-SUP cells by silibinin to its overall efficacy in inducing G_2/M arrest in these cells.

Effect of silibinin on apoptotic cell death in human bladder TCC cells

Apoptosis is a controlled form of cell death and plays an important role in maintaining normal tissue homeostasis; its deregulation leads to the development of various diseases including cancer (27,28). In order to assess whether silibinin also causes the apoptotic death of human bladder TCC cells, we treated both TCC-SUP and T-24 human bladder TCC cells with silibinin under similar condition as in other studies, and

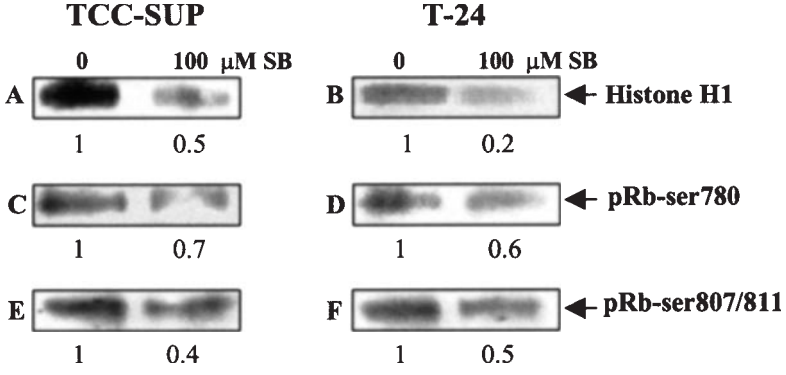

Fig. 7. Silibinin inhibits CDK2 and CDK4 kinase activity in human bladder transitional cell carcinoma TCC-SUP and T-24 cells. Cells were cultured and treated with either DMSO or 100 μM silibinin for 24 h as described in the Materials and methods. At the end of the treatments, total cell lysates were prepared. For CDK2 kinase activity (**A** and **B**), CDK2 was immunoprecipitated from total cell lysates by using specific antibody followed by kinase assay employing histone H1 as a substrate. Samples were then boiled in sample buffer followed by SDS–PAGE and autoradiography as detailed in the Materials and methods. For CDK4 kinase activity (**C**–**F**), CDK4 was immunoprecipitated from total cell lysates by using specific antibody followed by kinase assay employing Rb-fusion protein as a substrate. Samples were then boiled in sample buffer followed by SDS–PAGE and immunoblotting as detailed in the Materials and methods. Membranes were probed with anti-pRb-ser780 (C and D) and pRb-ser807/811 antibodies followed by peroxidase-conjugated secondary antibody, and visualized by ECL detection system as described in the Materials and methods. The densitometric data (arbitrary) shown under the autoradiograms or immunoblots in (A–F) are from representative autoradiograms/blots. SB, silibinin.

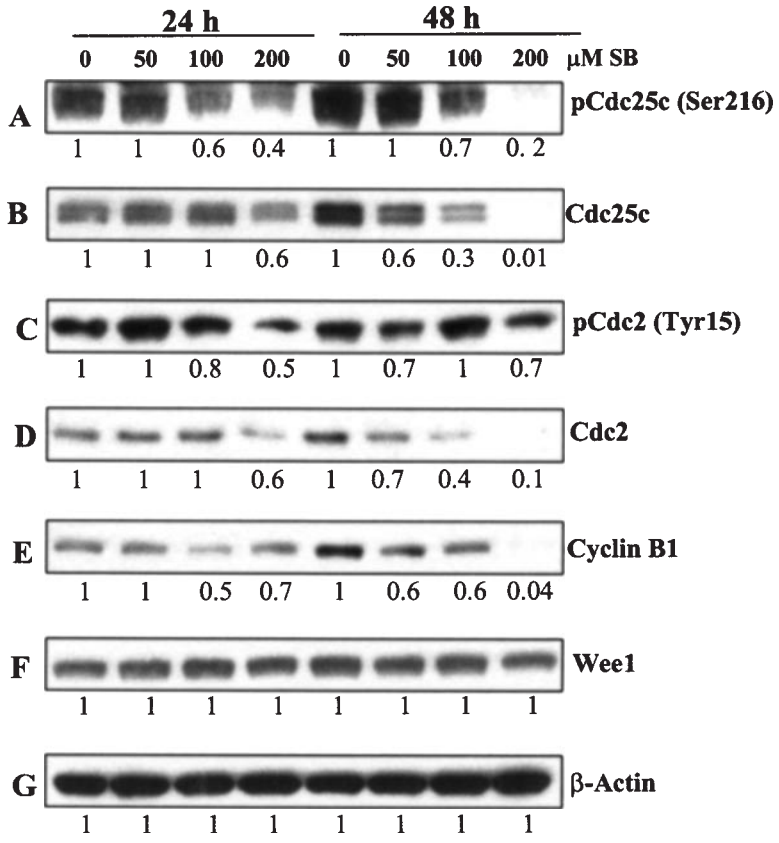

Fig. 8. Silibinin modulates the protein expression of G_2/M cell cycle regulators in TCC-SUP cells. Cells were cultured as described in the Materials and methods, and treated with either DMSO alone (control) or varying concentrations of silibinin. Different treatments are as labeled in the figure; lanes labeled as '0' denote DMSO treatment alone. At the end of the treatments, total cell lysates were prepared and subjected to SDS–PAGE followed by western immunoblotting. Membranes were probed with anti-pCdc25c (Ser216), Cdc25c, pCdc2 (Tyr15), Cdc2, Cyclin B1, Wee1 and β-actin antibodies followed by peroxidase-conjugated appropriate secondary antibodies, and visualized by ECL detection system. The densitometric data (arbitrary) shown under the immunoblots in (**A–G**) are from representative blots. SB, silibinin.

then analyzed the cells by flow cytometry following annexin V and PI staining. As shown in Figure 9A, the 200 μM concentration of silibinin effectively induced 8–18% apoptotic cell population following 24, 48 and 72 h of treatment in TCC-SUP cells compared with 5–7% apoptotic cells in controls; silibinin treatment at a 100 μM dose also resulted in 12% apoptotic cells following 72 h treatment (Figure 9A). Contrary to TCC-SUP cells, T-24 cells showed little or no apoptosis after exposure to silibinin (Figure 9B), suggesting both selectivity and specificity of silibinin-caused apoptotic induction in human bladder TCC cells.

Based on our results showing apoptosis induction by silibinin in TCC-SUP cells, we conducted additional assays involving caspase 3 and PARP cleavages by western blotting to further confirm the apoptotic response of silibinin. As shown in Figure 9C and D, silibinin treatment of TCC-SUP cells resulted in a strong cleavage of both caspase 3 and PARP in a dose- and a time-dependent manner; equal protein loading was confirmed by probing the same membrane with β-actin antibody (Figure 9E).

Discussion

The present study elucidates the biological effects of silibinin, the major biologically active component in milk thistle extract, a widely consumed dietary supplement, in human bladder TCC cells. The data support the hypothesis that silibinin could be an effective chemoprevention/intervention agent for bladder cancer. Our results clearly demonstrate that silibinin induces cell cycle arrest and growth inhibition in two different human bladder TCC cells representing different stages of human bladder cancer malignancy, and it selectively induces apoptosis in high-grade invasive TCC-SUP cells. The molecular mechanistic studies reported here demonstrate that silibinin strongly induces the protein expression of Cip1/p21 and Kip1/p27, and decreases CDK2, 4, 6 and cyclin D1, D3 and E protein expression, together with an increased interaction/binding between CDKIs and CDK possibly causing an inhibition in the kinase activity of CDKs. These mechanistic observations were in accord with an overall efficacy of silibinin in inducing a G_1 arrest in the cell cycle followed by the inhibition of cell growth (29).

Cell division depends on the activation of cyclin, which binds to CDKs to induce cell cycle progression towards S phase and later to initiate mitosis; uncontrolled CDK kinase activity is one of the major causes of cancer progression as their functions are tightly regulated by CDKIs such as the Cip1/p21 and Kip1/p27 proteins in normal cell cycle progression (29). Following anti-mitogenic signals or DNA damage, CDKIs (Cip1/p21 and Kip1/p27) bind to the cyclin–CDK complexes to inhibit their catalytic activity and thus inhibit cell cycle progression (29). The Rb protein, which is involved in the regulation of the transcription factor E2F (32), is a

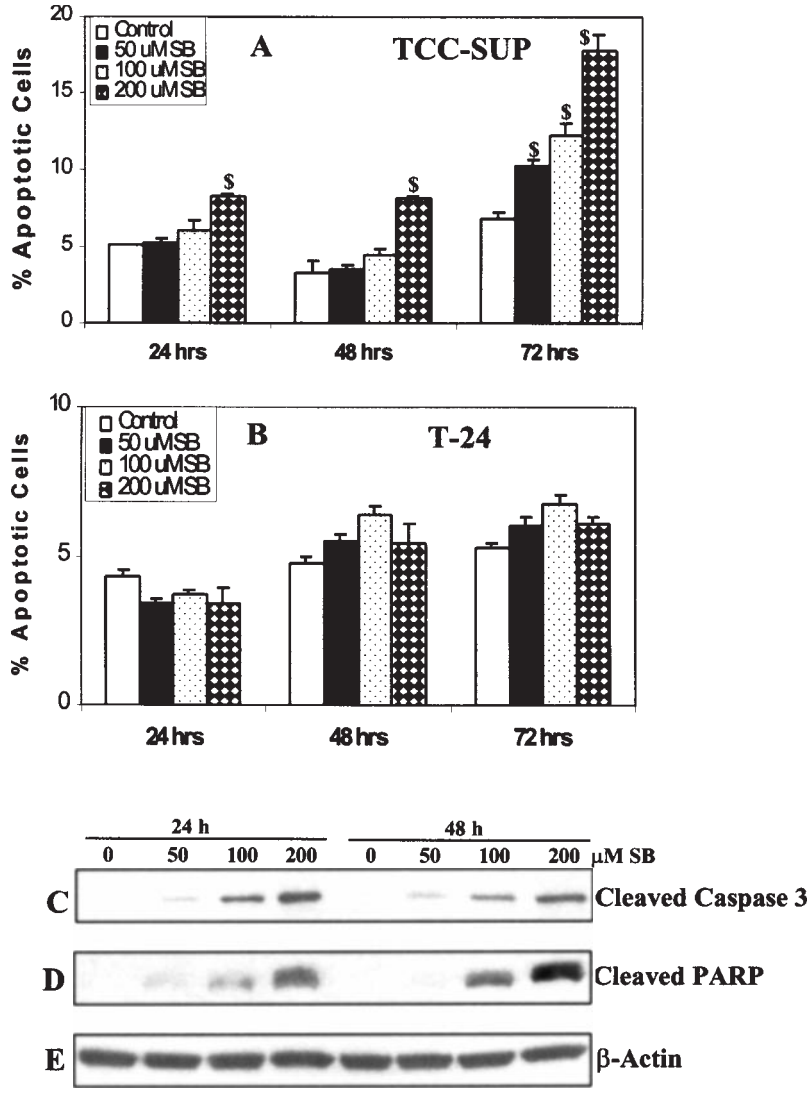

Fig. 9. Silibinin selectively induces apoptotic cell death in TCC-SUP cells. At 60% confluency, (**A**) TCC-SUP and (**B**) T-24 cells were treated with DMSO (control) or different concentrations of silibinin. Following 24, 48 and 72 h of these treatments, cells were collected by trypsinization and processed for FACS analysis following annexin V-PI staining. For western blot analysis, TCC-SUP cells were treated with DMSO (labeled as '0' for control) or varying doses of silibinin for 24 and 48 h, and total cell lysates were prepared and subjected to SDS–PAGE followed by western immunoblotting. Membranes were probed with anti-cleaved caspase 3 (**C**), cleaved PARP (**D**) and β-actin (**E**) antibodies followed by peroxidase-conjugated appropriate secondary antibodies, and visualized by ECL detection system. The immunoblots shown in (C–E) are from representative blots. $, $P < 0.05$; for differences with control group. SB, silibinin.

critical determinant for the restriction-point transition during G_1 phase (29,30), as it is phosphorylated during G_1 phase initially by CDK4 or CDK6 and is subsequently maintained in this form by CDK2 (31,32). The expression level of cyclins is also an important determinant in cell cycle progression particularly during G_1/S and G_2/M transitions (29). D-type cyclins have been shown to be important for progression through the G_1 phase, where cyclin E is expressed in late G_1 that plays an important role in the G_1 to S transition (33–36). The increased protein expression of G_1 cyclins in cancer cells has also been shown to be a major factor in driving uncontrolled growth because cancer cells either lack (with undetectable expression) CDKIs or they are non-functional (37). The increased expression of CDKIs together with decreased expression of cyclins and CDKs and decreased CDK kinase activity induced by silibinin treatment in human bladder TCC cells suggest that silibinin might be effective for the treatment or prevention of bladder cancer.

In addition to G_1 arrest and growth inhibition in TCC-SUP cells, silibinin also caused a strong G_2/M arrest at a higher dose. In the eukaryotic cells, the cyclin B/Cdc2 complex performs an important function in controlling the G_2/M phase where it rapidly phosphorylates the target protein to induce progression to the M phase (26,29). Phosphorylation and dephosphorylation of specific amino acids in Cdc2 are responsible for the control of G_2/M cell cycle progression by the cyclin B1/Cdc2 complex (26,29). More specifically, in the G_2 phase, Cdc2 is phosphorylated at Thr14 and Tyr15 by the protein kinases Myt1 and Wee1 to be converted into an inactive precursor, or it is dephosphorylated at Tyr15 and Thr14 by Cdc25c to be in an active form (38,39). The Chk1/2 kinases phosphorylate and inactivate Cdc25c, which then cannot activate Cdc2 (26,29). Consistent with these reports, in the present study, we observed that silibinin decreases the protein levels of pCdc25c (Ser216), Cdc25c, pCdc2 (Tyr15), Cdc2 and cyclin B1 in TCC-SUP cells, which might be the possible molecular

mechanism of silibinin efficacy in inducing G_2/M arrest in TCC-SUP cells. In this regard, it is important to re-emphasize that, as we did not observe any changes in Wee1 protein kinase that phosphorylates and inactivates cdc2 and since we observed a strong decrease in pCdc25c (ser 216) and total Cdc25c that dephosphorylates and activates cdc2, the observed efficacy of silibinin in inducing G_2/M arrest in TCC-SUP cells is possibly due to a decrease in cdc2 and cyclin B1 levels causing a decrease in their interaction as well as an overall decrease in cdc2 kinase activity. More studies, however, are needed in the future to further support this assumption, as well as to define the role of upstream events such as Chk1 and Chk2 and their regulators ATM, ATR and DNA-PK in the observed G_2/M arrest by silibinin in TCC-SUP cells.

Cancer develops when the balance between cell proliferation and cell death is disturbed, and the aberrant cell proliferation leads to tumor growth. It is well known that apoptosis and its related signaling pathways have a profound effect on the progression of cancer (40), suggesting that agents inducing apoptotic death of human cancer cells may play a critical role in cancer prevention/intervention including bladder cancer. In this regard, whereas there are several classes of chemotherapy drugs causing apoptotic death of cancer cells, their non-selective efficacy (toxicity) in other tissues has been a limitation in their efficacy. Our data demonstrating significant apoptotic death induction by silibinin only in TCC-SUP cells, but not in T-24 TCC cells, suggest the possibility of both selectivity and specificity in silibinin efficacy against bladder TCC cells. Our other findings showing that silibinin induces both G_1 and G_2/M arrests in TCC-SUP cells versus only G_1 arrest in T-24 cells under identical treatment conditions, further show some selectivity and specificity in its biological responses in different bladder cancer cell types. It is also important to identify here that despite the fact that silibinin causes G_2/M and apoptosis in TCC-SUP cells versus only a G_1 arrest in T-24 cells, in cell growth inhibition studies, silibinin showed better efficacy in T-24 cells versus TCC-SUP cells following 72 h of treatments at different doses. A lack of apoptotic effect of silibinin in T-24 cells suggest that there are other pathways contributing strongly in its growth inhibitory responses in this human bladder TCC cell type. One such pathway could be an inhibitory effect of silibinin on erbB1-mediated mitogenic signaling, as reported by us in human prostate carcinoma cells where it causes strong growth inhibitory effects without causing apoptotic death (17). More studies, however, are needed in future to support this assumption, and to identify the mechanism of silibinin efficacy in modulating mitogenic and survival signaling cascades in human TCC cells. In addition, the significance of the observations made in the present study need to be established in a broader context by conducting several additional studies in future employing normal and different stages of human bladder TCC cells as well as in established bladder cancer animal models. The data presented in this study, also support and warrant silibinin efficacy studies in pre-clinical bladder TCC models.

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