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Silibinin suppresses growth and induces apoptotic death of human colorectal carcinoma LoVo cells in culture and tumor xenograft

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Abstract

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality. The use of non-toxic phytochemicals in the prevention and intervention of CRC has been suggested as an alternative to chemotherapy. Here we assessed the anticancer efficacy of silibinin against advance CRC LoVo cells both *in vitro* and *in vivo*. Our results showed that silibinin treatment strongly inhibits the growth of LoVo cells ($P<0.05–0.001$) and induces apoptotic death ($P<0.01–0.001$), which was associated with increased levels of cleaved caspases (3 and 9) and cleaved PARP. Additionally, silibinin caused a strong cell cycle arrest at G1 phase, and a slight but significant G2/M phase arrest at highest concentration ($P<0.01–0.001$). Molecular analyses for cell cycle regulators showed that silibinin decreases the level of cyclins (D1, D3, A and B1) and CDKs (1,2,4,6) and increases the level of CDKIs (p21 and p27). Consistent with these results, silibinin treatment also decreased the phosphorylation of Rb protein at Ser780, Ser795 and Ser807/811 sites without significantly affecting its total level. In animal studies, oral administration of silibinin for 6 weeks (at 100 and 200 mg/kg/day, 5 days per week) significantly inhibited the growth of LoVo xenograft ($P<0.001$) in athymic nude mice without any apparent toxicity. Analyses of xenograft tissue showed that silibinin treatment inhibits proliferation and increases apoptosis along with a strong increase in p27 levels but a decrease in Rb phosphorylation. Together these results suggest the potential use of silibinin against advanced human CRC.

Keywords

Colorectal cancer; silibinin; apoptosis; cell proliferation; chemoprevention

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide. According to American Cancer Society estimates, about 148,810 new cases of CRC were diagnosed in 2008 and approximately 49,960 patients died due to this malignancy in the United States alone (1). The high mortality among CRC patients is principally attributed to the

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metastasis of the neoplasm to distant organs (2,3). The high toxicity and harmful side effects associated with chemo- and radio-therapy limit their potential benefits; therefore, alternative measures have been warranted to lower the burden of this malignancy. One approach to control CRC growth and metastasis is through its preventive intervention by non-toxic phytochemicals, which target one or more events related to carcinogenesis, and thereby reduce overall cancer risk (4–6). Numerous studies have reported the strong anticancer efficacy of dietary and non-dietary phytochemicals against various cancers including CRC (5–8). In this regard, polyphenolic flavonoids are important group of phytochemicals and have received greater attention for their potential role in cancer prevention and intervention; and many of these agents are currently in various phases of clinical trials (5,6,9).

Silibinin, a 1:1 racemic mixture of two flavonolignans silybin A and silybin B, is the primary active constituent of crude extract (known as silymarin) from the seeds of milk thistle plant (*Silybum marianum*). Silibinin is known to possess strong hepatoprotective efficacy, and is used in the treatment of severe hepatotoxicity due to *Amanita phalloides* poisoning (10). Silibinin also has a long history of human use and is considered exceptionally safe as it has exhibited extremely low toxicity even at acute or chronic administration in both animals and humans (11). Several studies by others and us have clearly demonstrated the pre-clinical efficacy of both silibinin and its crude extract source, silymarin, against various epithelial cancers, and at least silibinin efficacy is currently being evaluated in cancer patients (12,13). Further, both silymarin and silibinin have shown strong efficacy against CRC cells both in *in vitro* and *in vivo* studies (14–18). However, the potential efficacy of silibinin against advance stage CRC is largely unknown. Since the advance metastatic stage of colon cancer is the chief cause of mortality, in the present study we investigated the efficacy of silibinin against advanced metastatic human CRC LoVo cells.

One of the hallmarks of cancer cells including CRC cells is the uncontrolled proliferation, which is mainly associated with cell cycle deregulation and evasion of apoptosis by cancer cells (19,20). Cell cycle is known to be regulated by various cyclin dependent kinases (CDKs), whose activity is controlled by binding of different cyclins and cyclin dependent kinase inhibitors (CDKIs) (21). The active CDK/cyclin complex phosphorylates Rb (retinoblastoma) and results in the release of E2F transcriptional factor from Rb/E2F repressor complex, which then regulates the expression of genes involved in cell cycle progression and DNA replication (22,23). The aberrant expression of these key cell cycle regulators has been directly linked with cancer growth and progression (24). Similarly, the constitutive activation of various anti-apoptotic and pro-survival factors is known to make cancer cells resistant towards apoptosis induction by various therapeutic drugs (20). Therefore, induction of cell cycle arrest and apoptosis by non-toxic phytochemicals could be an effective strategy to check the uncontrolled proliferation and survival of cancer cells. In the present study, for the first time we investigated the anticancer efficacy of silibinin and the associated mechanism/s in advanced metastatic human CRC LoVo cells, both in cell culture and xenograft studies. Our findings show that silibinin induces cell cycle arrest and apoptosis in these cancer cells and also inhibits CRC LoVo tumor xenograft growth in nude mice. Importantly, the molecular mechanistic aspects of silibinin efficacy identified in LoVo cell culture studies, were also operational in tumor xenograft studies under *in vivo* conditions, suggesting strong implications of our findings in controlling advanced human CRC growth by silibinin.

Materials and methods

Chemicals and cell line

Silibinin was procured from Sigma Chemical Co. (St. Louis, MO, USA). Antibody against p21/Cip1 was from Millipore (Jaffrey, NH, USA), and antibody against p27/Kip1 was from Neomarkers, Inc. (Fremont, CA, USA). Antibodies for CDK1, CDK2, CDK4, CDK6, cyclin

A, B1, D1 and D3 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-cleaved caspase-3, -9, anti-cleaved poly (ADP-ribose) polymerase (PARP), anti-Rb and anti-phospho-Rb (Ser 780, Ser 795 and Ser 807/811) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Anti-proliferating cell nuclear antigen (PCNA) and N-Universal Negative Control mouse antibodies were from Dako (Carpinteria, CA, USA). Carboxymethyl cellulose (CMC) was purchased from Fluka-Buchs, Switzerland. LoVo cells were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in F12 media supplemented with 10% fetal bovine serum and 100 Units/ml of streptomycin and penicillin, and maintained under standard culture conditions of 37°C, 95% humidified air and 5% CO_2.

Cell growth and viability assay

The growth and viability of cells were measured by Trypan blue exclusion assay. Briefly, the cells were plated overnight at a density of 5000 cells/cm^2 of 60 mm culture dish. Subsequently, cells were treated with either DMSO alone or silibinin (50–200 μM in DMSO) for 24–72 h. DMSO concentration did not exceed 0.1% (v/v) in any treatment. At the end of treatment durations, cells were harvested after trypsinization, and total number of viable and dead cells was counted using hemocytometer after staining with Trypan blue dye.

Apoptosis assay

Apoptotic death induced by silibinin treatment was quantified using Vybrant Apoptosis Assay Kit 2 (Molecular Probes, Eugene, OR) as per vendor's protocol. Briefly, at the end of each treatment, non-adherent and adherent cells were collected after brief trypsinization, washed once with ice cold PBS, and subsequently stained with Annexin V and PI. Stained cells were analyzed by flow cytometry using FACS analysis core facility of University of Colorado Cancer Center.

Flow cytometry analysis for cell cycle distribution

Following desired treatments, cells were harvested by brief trypsinization and washed twice with ice-cold PBS, and cell pellets were collected. Approximately 0.5×10^5 cells in 0.5ml of saponin/propidium iodide (PI) solution were incubated at 4°C for 24 h in the dark (25). Cell cycle distribution was then analyzed by flow cytometry using FACS analysis core facility of the University of Colorado Cancer Center.

Western immunoblotting

Total lysates, from cells in culture and tumors in xenograft studies, were prepared in non-denaturing lysis buffer (10 mmol/L Tris-HCl (pH 7.4), 150mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP40, and 5 units/mL aprotinin) as reported earlier (25). For western blotting, lysates (40–70 μg) were denatured in 2X SDS-PAGE sample buffer and were run on 8–16% Tris-glycine gels. Separated proteins were then transferred onto nitrocellulose membrane by western blotting. After one hour of blocking in blocking buffer at room temperature, membranes were probed with desired primary antibodies overnight at 4°C followed by appropriate peroxidase conjugated secondary antibody for 1 hour at room temperature and visualized by ECL detection system (GE Healthcare Bioscience, Piscataway, NJ). To ensure equal protein loading, each membrane was strippedand reprobed with anti-β-actin antibody (Sigma, St Louis, MO).

Experimental design for tumor xenograft study

Athymic (*nu/nu*) male nude mice were obtained from the National Cancer Institute (Bethesda, MD) and housed in our animal care facility at standard laboratory conditions and fed autoclaved

AIN-76A rodent diet (Dyets Inc., Bethlehem, PA) and water *ad libitum*. All the protocols used were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver. LoVo cells grown in culture were harvested by trypsinization, washed and resuspended in serum-free F-12 media. Six-week-old athymic male mice were s.c. injected with 5×10^6 LoVo cells mixed with matrigel (1:1) in the right flank to initiate tumor growth. Mice were then randomly divided into three groups, each having 8 mice. After 24 h, while mice in control (first) group were gavaged with 0.2 ml of 0.5% (w/v) carboxymethyl cellulose (CMC)/day, second and third groups were gavaged with 100 and 200mg/kg/day doses of silibinin in 0.2 ml of 0.5% CMC 5 days/week, respectively, for 6 weeks. Body weight and diet consumption were recorded twice weekly throughout the study. After tumors started growing, their sizes were measured twice weekly. Thetumor volume was calculated by the formula $0.5236L_1(L_2)^2$, wherein L_1 is the long axis and L_2 is the short axis of the tumor. At the end of the experiment, mice were euthanized, tumors were excised, weighed and a part was fixed in buffered formalin and the remaining tissue was stored at −80°C until further analysis. Out of the frozen tissue samples, three samples were randomly selected from each group for western blot analysis. Tissue samples were homogenized in lysis buffer and centrifuged to clear debris, and the resulting total cell lysates were used for western immunoblotting as detailed above.

Immunohistochemical staining for PCNA

Tumor tissues fixed in 10% phosphate-buffered formalin for 10 hours at 4°C were dehydrated in ascending concentrations of ethanol, cleared with xylene and embedded in PolyFin. Paraffin-embedded tissue blocks were cut with a rotary microtome into 4-μm sections and processed for immunohistochemical (IHC) staining. Briefly, sections after deparaffinization and re-hydration were treated with 0.01M sodium citrate buffer (pH 6.0) in a microwave for 30 min for antigen-retrieval. Sections were then quenched of endogenous peroxidase activity by immersing in 3% hydrogen peroxide for 5 min. Next, sections were incubated with mouse monoclonal anti-PCNA antibody (1:400 dilution) in PBS for 2 h at room temperature in a humidity chamber followed by overnight incubation at 4°C. In negative staining control, sections were incubated with N- Universal Negative Control mouse antibody under identical conditions. The sections were then incubated with biotinylated secondary antibody for 1 h at room temperature followed by 30 min incubation with HRP-conjugated streptavidin followed by incubation with 3, 3′-diaminobenzidine. Next, sections were counterstained with Harris Hematoxylin, dehydrated and mounted. Proliferating cells were quantified by counting PCNA-positive cells (brown stained) and total number of cells at five arbitrarily selected fields at 400X magnification.

TUNEL staining for apoptotic cells

Apoptotic cells were detected using the DeadEnd Colorimetric TUNEL system (Promega, Madison, WI) following manufacturer's protocol. The extent of apoptosis was evaluated by counting the TUNEL-positive cells (brown-stained) as well as the total number of cells in five randomly selected fields at 400× magnification.

Statistical and IHC analyses

All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific, San Rafael, CA). Statistical significance of difference between the control and treated groups was determined either by Student's t-test or one-way ANOVA followed by Bonferroni *t*-test. $P<0.05$ was considered statistically significant. IHC analyses were done with a Zeiss Axioscop 2 microscope (Carl Zeiss Inc, Jena, Germany). Microscopic images were taken by AxioCam MrC5 camera at 400× magnification, and processed by AxioVision software documentation system (Carl Zeiss Inc).

Results

Silibinin inhibits the growth and decreases the viability of human CRC LoVo cells

We first examined the effect of silibinin on the growth and viability of human CRC LoVo cells. Silibinin (50–200 μM) treatment for 24 h reduced the growth of LoVo cells by 30–49% ($P<0.01$–0.001) (Figure 1A). A further decrease in cell growth by 37–60% ($P<0.001$) and 51–83% ($P<0.001$) at similar silibinin concentrations was also observed following prolonged treatment durations of 48 and 72 h, respectively (Figure 1A). Under similar treatment conditions, silibinin (50–200 μM) increased the dead cell population by ~ 1.5–2.0 folds after 24, 48 and 72 h of treatment, respectively (Figure 1B). The observed lesser number of dead cells at higher doses of silibinin and at later time points could be probably due to the conversion of dead cells into debris after prolonged treatment time. From these observations, it is clear that 200 μM silibinin was most effective in inducing cell death in these cells, though even lower concentrations of 50 and 100 μM silibinin exhibited similar but delayed effect.

Silibinin induces apoptosis in human CRC LoVo cells

Since we observed a significant increase in cell death after silibinin treatment, we next assessed the effect of silibinin on the induction of apoptosis in LoVo cells by Annexin V-PI staining. As shown in Figure 2A, apoptotic cell population increased from 14 ± 3% in DMSO treated controls to 19 ± 2% and 22 ± 1% ($P<0.01$) after 24 h treatment with 100 and 200 μM silibinin, respectively. When the treatment time was extended to 48 h, a further increase in percent apoptotic cell population to 20 ± 0.2% ($P<0.001$) and 38 ± 3% ($P<0.001$) at 100 and 200 μM silibinin concentrations, respectively, was observed as compared to 10 ± 1% in DMSO controls (Figure 2A). Silibinin at lower dose of 50 μM did not induce any significant increase in apoptotic death compared to the DMSO even after 48 h of treatment (data not shown).

To study the mechanistic aspects of apoptosis induction by silibinin, we next assessed the activation of caspases by western blot analysis. Silibinin treatment (100 and 200 μM) significantly increased the levels of cleaved caspase-9 and cleaved caspase-3 in LoVo cells, thereby indicating the involvement of these caspases in apoptosis induction. Further, silibinin treatment also increased the cleavage of PARP (Figure 2B), which is a known marker of apoptosis and a downstream target of activated caspase-3 (26). We also studied the effect of silibinin treatment on the expression of various pro- and anti-apoptotic members of Bcl-2 family namely Bax, Bcl-2 and Bcl-XL; however, no change in the protein levels of these molecules was observed upon silibinin treatment (data not shown).

Silibinin causes cell cycle arrest in human CRC LoVo cells

Since we observed a strong decrease in cell growth by silibinin treatment (Figure 1A) which could be due to the induction of cell cycle arrest, next we examined the effect of silibinin on cell cycle progression of LoVo cells. As shown in Figure 3A, significant population of cells were arrested at G1 phase (49–57% as compared to 41.5% in DMSO treated controls; $P<0.01$–0.001) upon treatment with silibinin at 50, 100 and 200 μM concentrations for 24 h (Figure 3A). However, at the highest dose of silibinin, in addition to cells arresting at G1 phase of cell cycle, a significant population of the cells were also observed to accumulate at G2/M phase (19% as compared to 14% in DMSO treated controls; $P<0.001$, Figure 3A). This effect was sustained even when the treatment period was extended to 48 h, where 50–61% of cells were arrested in G1 phase with silibinin treatment (50–200 μM) compared to 43% in DMSO treated controls ($P<0.001$, Figure 3B). Similarly, 17% of cells were arrested at G2/M phase with 200 μM dose of silibinin as compared to 10% cells in control after 48 h of treatment ($P< 0.001$, Figure 3B).

Silibinin modulates the expression of cell cycle regulatory proteins in human CRC LoVo cells

Since we observed a strong cell cycle arrest with silibinin treatment, we next assessed the effect of silibinin on various cell cycle regulatory molecules. Results showed that silibinin treatment strongly decreases the protein levels of CDK1, CDK2 and CDK4 after 24 and 48 h of treatment, but its effect on CDK6 level was comparatively moderate (Figure 3C). Silibinin treatment also resulted in a strong dose- and time- dependent decrease in the levels of cyclin D1 and D3 and a moderate decrease in the levels of cyclin A and B1 (Figure 3C). Next, we examined the effect of silibinin on the levels of CDKIs, which are known to regulate cell cycle progression by binding to cyclin-CDK complexes (24, 27). As shown in Figure 3C, silibinin up regulates the levels of p21 and p27 in a dose- and time-dependent manner.

Another critical molecule regulating the G1-S transition during cell cycle progression is retinoblastoma (Rb) protein (22). The un-phosphorylated Rb is known to prevent cell cycle progression by binding with E2Fs (22,23). The ability of Rb to interact with E2Fs and to suppress transcription is regulated by its phosphorylation by CDKs (22,23). Rb is known to contain atleast 16 sites for CDK phosphorylation, but the significance of most of these sites is still unclear (22,23). Rb phosphorylation at Ser 795 site is known to disrupt its binding with E2F, while phosphorylation at various Threonine and Serine sites including Ser 780 and Ser 807/811 is considered important for maintaining its inactive hyperphosphorylated state (22, 23). Since silibinin treatment induced strong G1 arrest in LoVo cells, we next examined the effect of silibinin on the phosphorylation of Rb. As shown in Figure 3D, silibinin treatment for 24 and 48 h resulted in a significant decrease (mainly at 200 μM dose) in the phosphorylation of Rb protein at Ser 795, Ser 780 and Ser 807/811 sites. Under similar treatment conditions, silibinin treatment only slightly decreased the total Rb levels at the highest concentration (Figure 3D). In all cases, membranes were stripped and reprobed with β-actin antibody to confirm equal protein loading.

Silibinin inhibits human CRC LoVo xenograft growth

Based on our results showing strong efficacy of silibinin in advanced metastatic CRC LoVo cells in culture, next, we examined the *in vivo* efficacy of silibinin against the CRC LoVo xenograft in athymic nude mice. The administration of silibinin through oral gavage in CMC vehicle at 100 and 200 mg/kg body weight doses, 5 days/week for 6 weeks causeda marked time-dependent inhibition in LoVo xenograft growth in comparison to vehicle control (Figure 4A). The average final tumor volume was reduced from 2692 mm^3 per mouse in control group to 1766 and 1450 mm^3 per mouse in 100 and 200mg/kg body weight silibinin treatment groups, respectively, which accounted for 34%and 46% decrease at the end of experimental period ($P<0.001$, Figure 4A). Consistent with this observation, compared to control, tumor weight in silibinin-fed groups was decreased by 38% ($P<0.05$) and 49% ($P<0.01$) at 100and 200 mg/kg body weight doses, respectively (Figure 4B). Silibinin feeding did not show any gross signs of toxicity and/or possible adverse side effects as measured by two profiles, namely body weight and diet consumption. As shown in Figure 4C and D, there was no considerable difference in the body weight gain and the diet intake between control and silibinin-fed groups. These results suggest *in vivo* antitumor efficacy of oral silibinin against CRC without any apparent signs of toxicity.

Silibinin inhibits cell proliferation and induces apoptosis in human CRC LoVo xenograft

Next, to gain insight into the mechanism/s underlying the *in vivo* antitumor efficacy of silibinin and to assess whether the molecular mechanistic aspects of silibinin efficacy identified in LoVo cell culture studies are also operational in tumor xenograft studies under *in vivo* conditions, tumor xenograft tissues were analyzed by IHC for PCNA, a marker for cell proliferation and TUNEL, a marker for apoptotic response (Figure 5). As shown in Figure 5A, compared to controls, xenograft samples from silibinin-fed groups showed a marked decrease in PCNA

staining. The quantification of PCNA-positive cells in tumor sections showed that oral gavage administration of silibinin at both the doses (100 and 200 mg/kg body weight) results in 31.5% ($P<0.001$) and 44.7% ($P<0.001$) decrease in proliferation index as compared to CMC fed controls (Figure 5B). In case of TUNEL, as shown in Figure 5C, tumor xenografts from silibinin-fed groups showed a marked increase in TUNEL positive cells compared to CMC-fed controls. The quantification of TUNEL stained samples showed that there was 4.2 folds- ($P<0.001$) and 5.4 folds- increase ($P<0.001$) in the number of TUNEL-positive cells in tumor sections from animals fed with silibinin at the dose levels of 100 and 200 mg/kg body weight, respectively, over that of CMC-fed control animals (Figure 5D). Representative photographs for PCNA- and TUNEL-positive cells in control and silibinin groups are shown at 400× magnification (Figure 5A and C).

Silibinin modulates the expression of cell cycle regulatory proteins in human CRC LoVo xenograft

Since in our IHC studies with tumor xenograft tissue, we observed that silibinin feeding exerts strong anti-proliferative effect, we also examined the effect of silibinin on the expression of cell cycle regulatory molecules in these tissues by western blot analyses. Results showed that silibinin treatment significantly up regulates the level of p27 at both 100 and 200 mg/kg body weight doses (Figure 6). However, no alteration in the level of p21 was observed in tumors from silibinin-fed animals as compared to CMC-fed controls (data not shown). Further, silibinin treatment also decreased the levels of Rb phosphorylation at Ser 795 and Ser 807/811 sites (Figure 6), but did not significantly affect the Rb phosphorylation at Ser 780 site (data not shown). Silibinin treatment also slightly decreased the total levels of Rb in the xenograft tissue (Figure 6). Western blot analyses of the xenograft tissue for the levels of other cell cycle regulatory proteins such as CDKs (1, 2, 4 and 6) and cyclins (D1 and A) did not show any alterations with or without silibinin treatment (data not shown). In all cases, membranes were stripped and reprobed with β-actin antibody to confirm equal protein loading.

Discussion

In recent years, the focus of cancer control has been on the search for anticancer agents which are safer and have higher patient acceptability. Various natural agents such as phytochemicals, which are generally a part of human diet or traditional herbal medications, have been receiving attention in this regard (4,5,8). Numerous studies are available in literature now, where these agents have been proven to be effective as anticancer agents in various experimental models of human cancers, and at least few of them have reached the clinical trial stage (4,12,28). In the present study, we investigated the anticancer efficacy of one such agent i.e. silibinin against advanced colorectal cancer cells both *in vitro* and *in vivo*.

Silibinin has been traditionally used for the treatment of liver ailments throughout Europe in the form of milk thistle extract (29). Studies conducted by our group and others have revealed that in addition to its hepatoprotective effects, silibinin also exhibits strong anticancer efficacy in cell culture and animal models (preclinical) of human cancers of various organs/tissue origin such as prostate, bladder, skin, lung, colon etc (12,30–32). Based on silibinin efficacy in these cancer models, our group has extended studies to clinical level and has recently completed a phase-I clinical trial in prostate cancer patients (33). In case of colon cancer, recently we reported that dietary administration of silibinin significantly inhibits azoxymethane (AOM)-induced aberrant crypt foci (ACF), which are considered as putative precursors of colon cancer (14). Silibinin has also been reported to inhibit proliferation and to induce cell cycle arrest and apoptosis in colon cancer cells in cell culture (15,17). In other studies, silibinin decreased angiogenesis in CRC cells by inhibiting the VEGF secretion (34,35). In a co-culture assay silibinin treatment also inhibited the chemotactic migration of endothelial cells towards the

colon cancer cells (35). Recently, we also reported the strong *in vivo* anticancer effects of silibinin against human colon carcinoma HT29 cells (18), which represent the primary localized stage of colon cancer (36,37). Despite these studies showing the efficacy of silibinin against CRC, its usefulness against advance stage CRC remains unknown. Moreover studies evaluating the efficacy of silibinin against cancer cells representing various clinical stages of CRC are prerequisite for any future clinical trial with silibinin in CRC patients. The present study is novel in this aspect that for the first time efficacy of silibinin was examined against advanced stage highly metastatic CRC cells (36,38). We found that silibinin inhibits the growth and induces apoptosis in advance human CRC LoVo cells both *in vitro* and *in vivo*.

Aberrant cell cycle regulation has been recognized as one of the essential characteristics of cancer cells including CRC cells (19,24). Therefore, targeting the deregulated cell cycle through phytochemicals and dietary agents has been suggested as an attractive strategy to check uncontrolled proliferation in cancer cells (39). Results from the present study showed that silibinin treatment induces cell cycle arrest in advanced metastatic CRC LoVo cells. Silibinin mediated G-1 arrest was associated with a decrease in the levels of CDK 2, 4 and 6 and their corresponding cyclins D1, D3 and A. Progression through G2/M phase involves the sequential association of CDK1 (Cdc2) with cyclin A followed by its association with cyclin B1 (40). In our study, we found that silibinin at highest concentration (200 μM) reduces the levels of CDK1 as well as cyclins A and B1, and thereby possibly causes G2/M phase arrest. Present study also showed that silibinin inhibits the phosphorylation of Rb at specific serine sites in LoVo cells both in cell culture and xenograft tissue. The decrease in the phosphorylation of Rb protein might be due to decrease in the kinase activity of CDK 2, 4 and 6, which could be related to observed decrease in their expression level as well as increase in the level of CDKIs especially the p27. These findings are similar to our previous findings in DU145 cells, where silibinin mediated G1 arrest was through hypo-phosphorylation of Rb related proteins (41). Further, proliferative index, measured in terms of PCNA positive cells, was reduced in LoVo xenograft tissues from silibinin fed animals which also reflects the growth inhibitory effects of increased levels of p27 and the decreased phosphorylation of Rb in these tumors.

The other highlight of the present study is that silibinin treatment strongly increases the levels of CDKIs p21 and p27 in LoVo cells. These results confirm our previous findings in HT29 cells where we reported a strong increase in the expression of these molecules (15). However, the analysis of tumor xenograft tissue showed that silibinin treatment only up regulates the levels of p27 without affecting the levels of p21. These findings suggest that p27 might be the specific *in vivo* target molecule responsible for the observed anticancer efficacy of silibinin against human CRC LoVo tumor xenograft. Studies have reported that increased p27 expression is a favorable prognostic marker for the disease free and overall survival in breast and colorectal cancer patients (42–44). The expression of p27 is often deregulated in cancer cells, and in most cases, cancer progression has been correlated with decreased levels of this protein (42–45). The levels of p27 can be regulated at transcription, translation and post translation levels; however, in most of the cancers, deregulation in p27 level has been attributed to post translation mechanism involving Skp2 mediated proteasomal degradation (46,47). In our previously published study, we found that silibinin attenuates the levels of Skp2 (S-phase kinase-associated protein 2) in DU145 cells, which is an E3 ligase and is required for ubiquitylation and proteasomal degradation of p27 (27). There is a possibility that similar mechanism might be involved in the up regulation of p27 by silibinin in LoVo cells; however, more studies are required to confirm the involvement of such mechanisms.

Induction of apoptosis in cancer cells is another approach to limit their uncontrolled proliferation (48). In this process, activation of caspases is the central event (49). Once activated, the executioner caspases at the downstream of caspase cascade act on the key molecules inside the cells to orchestrate cell death (49). Cleavage of PARP by activated

caspases is considered as a marker for apoptotic death (26). In our study, we found that silibinin induces apoptotic death in LoVo cells, which was accompanied with activation of caspase-3 and caspase-9 along with cleavage of PARP. Even in LoVo cells grown as tumor xenograft in athymic nude mice, increased levels of TUNEL positive cells were observed in tumors from animals fed with silibinin, thereby implying that induction of apoptosis may be another contributing factor in the silibinin-mediated reduced tumor growth. However, further studies are required to elucidate the mechanism of apoptosis induction by silibinin.

One of the major concerns in developing cancer therapeutic or preventive drugs is to achieve pharmacological effective doses of the drug without causing any systemic toxicity. We have reported earlier that 150 μM or more concentration of silibinin could be achieved in the plasma of mice without causing any toxicity (15). Hoh et al. have shown in CRC patients that 141 μmol/L concentration of silibinin could be achieved in the colorectal tissue by feeding silibinin at 1440 mg/day silibinin for 7 days (50). In the present study most of the effects of silibinin on the biological end-points were significant at 100 μM dose; therefore these *in vitro* observations at pharmacologically achievable doses of silibinin seem clinically relevant. Furthermore, the *in vivo* doses of 100 and 200 mg/kg body weight used in the present study theoretically extrapolate to 600 and 1200 mg silibinin/person daily, following the criterions described in the study by Hoh et al. (50) i.e. assuming a human body surface area of 1.8 m^2 for a body weight of 70 kg. These extrapolated values fall in the range of silibinin doses (360–1440 mg) used in the clinical trial conducted in CRC patients by Hoh et al (50), and confirm the clinical relevance of the *in vivo* doses of silibinin used in the present study.

In summary, the present study demonstrated the anticancer efficacy of silibinin against advanced CRC LoVo cells. Molecular analyses showed that silibinin treatment induces cell cycle arrest in these cells through modulating the expression of various cell cycle regulators; and also induces apoptosis through activation of caspases. Silibinin treatment also significantly inhibited the growth of LoVo xenograft in athymic nude mice by significantly inhibiting proliferation and inducing apoptosis. Importantly the present study identified p27 as the potential molecular target of silibinin for its efficacy against CRC LoVo cells under both *in vitro* and *in vivo* conditions. Overall, these results supports further pre-clinical testing of silibinin and its clinical use against CRC.

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Abbreviations

CDK
: cyclin-dependent kinase

CDKI
: cyclin-dependent kinase inhibitor

Rb
: retinoblastoma

PARP
: poly ADP-ribose polymerase

PCNA
: proliferating cell nuclear antigen

TUNEL

terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

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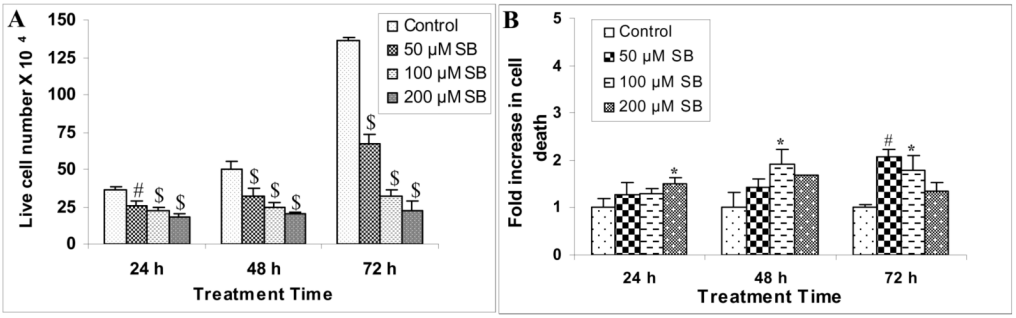

Fig. 1. Silibinin inhibits the growth and decreases the viability of human CRC LoVo cells

LoVo cells were plated overnight and subsequently treated with silibinin at concentrations ranging from 0–200 μM in DMSO for 24–72 h. At the end of each treatment duration, cells were collected and processed for the determination of live cell number (**A**) and dead cells number (**B**) as mentioned in the Materials and methods. The data shown are mean ± standard deviation of three samples for each treatment. Experiment was repeated three times. *, $P < 0.05$; #, $P < 0.01$; $, $P < 0.001$

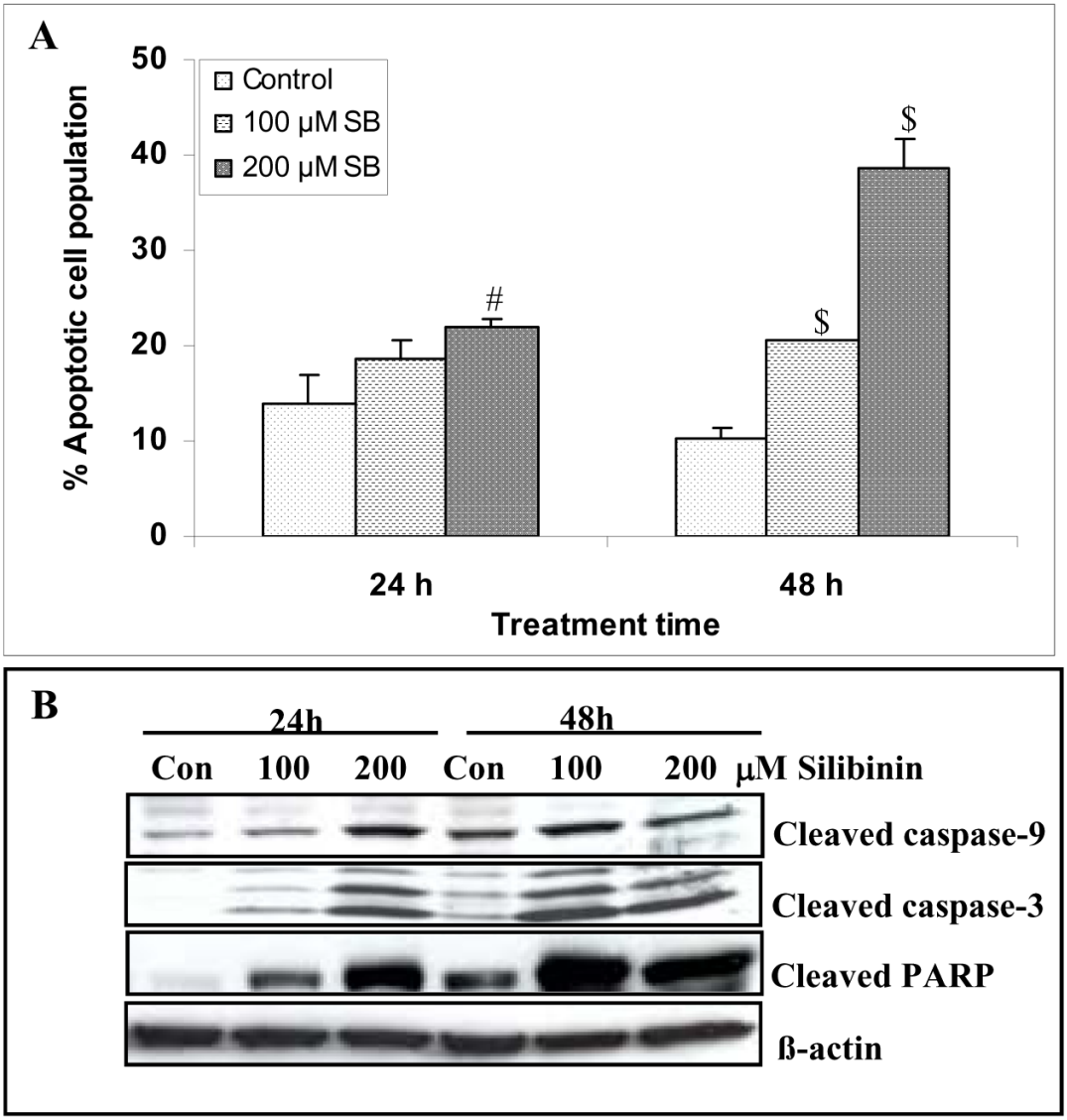

Fig. 2. Silibinin induces apoptotic death in human CRC LoVo cells

LoVo cells were plated overnight and subsequently treated with 0, 100 and 200 μM silibinin in DMSO for 24 and 48 h. At the end of each treatment duration, both adherent and non-adherent cells were collected and processed. (**A**) Collected cells were stained with Annexin V-PI and analyzed with flow cytometer and presented as percent apoptotic death. The data shown are mean ± standard deviation of three samples for each treatment. In each case experiment was repeated three times. #, $P < 0.01$; $, $P < 0.001$. (**B**) Total cell lysates were prepared at the end of the treatment as described in Materials and methods, and separated on SDS-PAGE. Separated proteins were then immunoblotted with antibodies for cleaved caspase-9, -3 and cleaved PARP. Membranes were also stripped in each case and reprobed with anti-β-actin antibody to confirm equal protein loading.

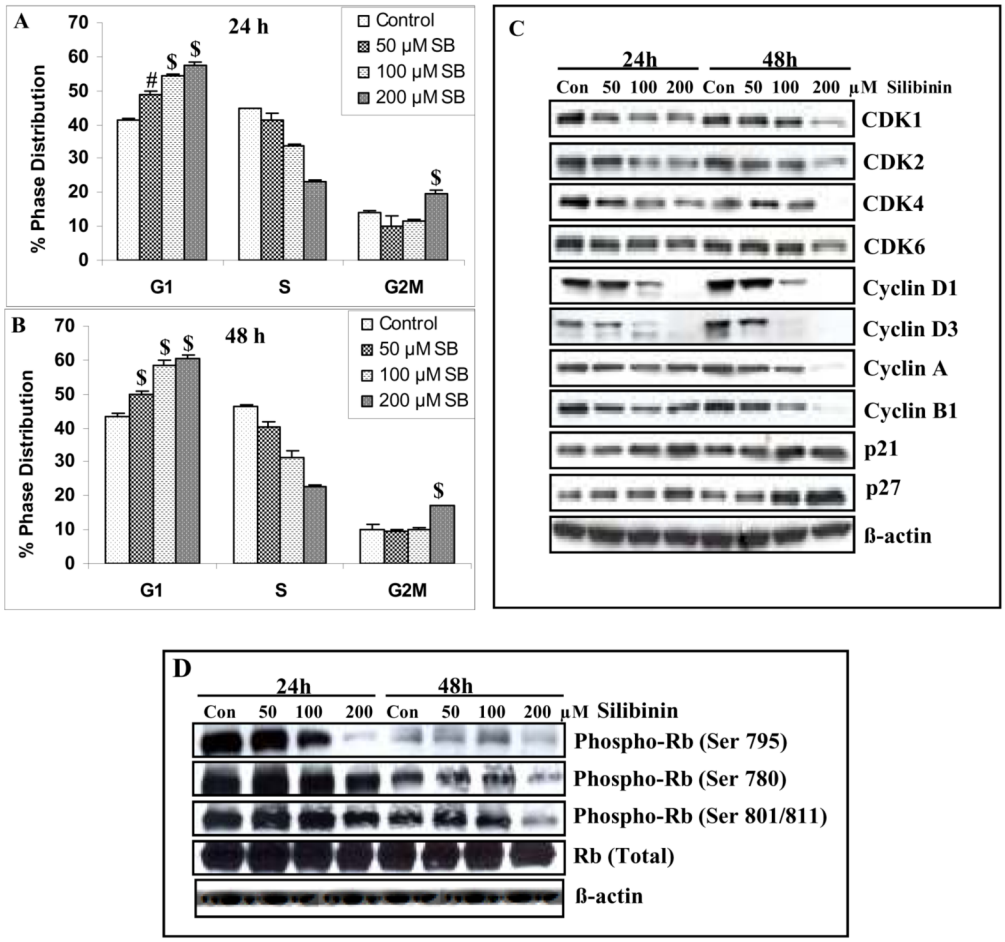

Fig. 3. Silibinin causes cell cycle arrest and modulates the expression of various cell cycle regulatory molecules in human CRC LoVo cells

LoVo cells were plated overnight and subsequently treated with silibinin at concentrations ranging from 0–200 μM in DMSO for 24 and 48 h. At the end of treatment durations, cells were collected, and were either (**A** and **B**) stained with saponin-PI and analyzed for cell cycle distribution by flow cytometry, or (**C** and **D**) total cell lysates were prepared and analyzed for various cell cycle regulatory molecules (CDK1, CDK2, CDK4, CDK6, cyclin D1, cyclin D3, cyclin A, cyclin B1, p21, p27, phosphorylation of Rb at Ser 795, Ser 780, Ser 807/811 and total Rb) by western immunoblotting. Membranes were also stripped in each case and reprobed with anti-β-actin antibody to confirm equal protein loading. #, $P < 0.01$; $, $P < 0.001$

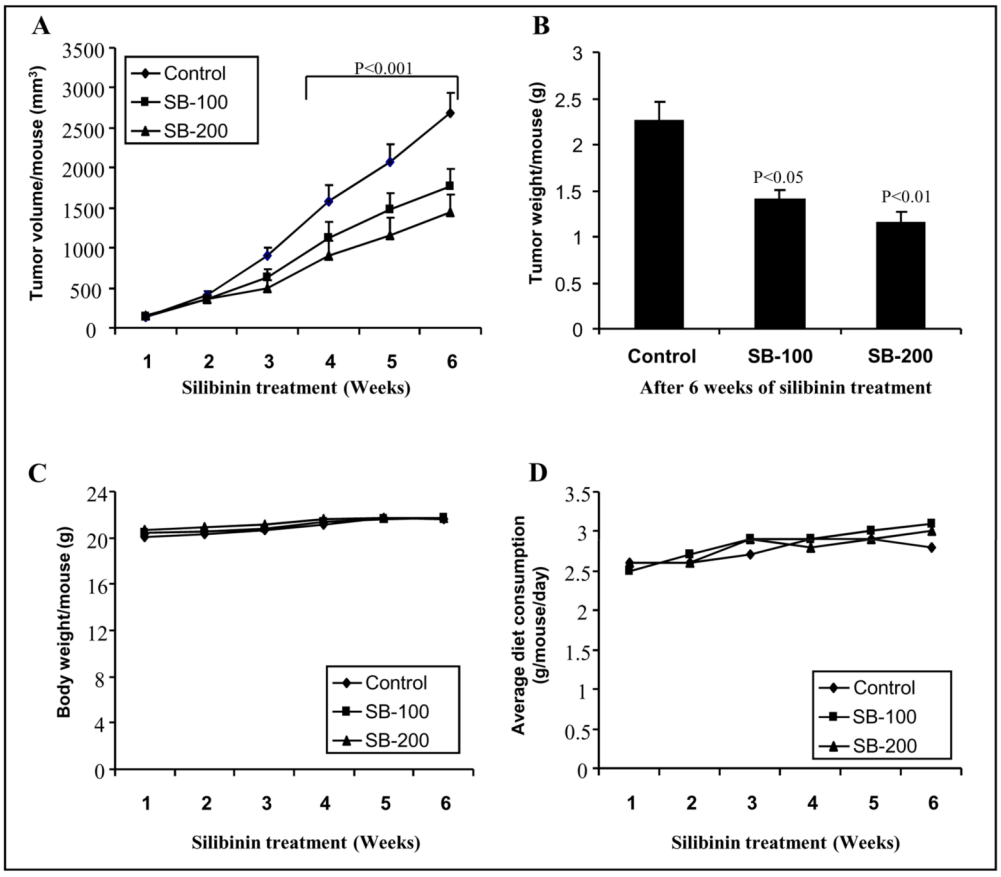

Fig. 4. Silibinin inhibits human CRC LoVo xenograft growth in athymic nude mice

Each mouse was s.c. injected with 5×10^6 LoVo cells mixed with matrigel (1:1) on the right flank. After 24 h, mice were gavaged with CMC (control group) or 100 and 200 mg/kg body weight/day doses of silibinin for 5 days/week for 6 weeks: (**A**) Tumor volume (mm^3) per mouse as a function of time. (**B**) Tumor weight (grams) per mouse at the end of study. (**C**) Average body weight (grams) per mouse. (**D**) Average diet consumption (grams) per mouse per day. In panels **A** and **B**, data shown are mean ± SE from 8 mice in each group. Abbreviations: SB-100, 100 mg/kg body weight silibinin; SB-200, 200 mg/kg body weight silibinin.

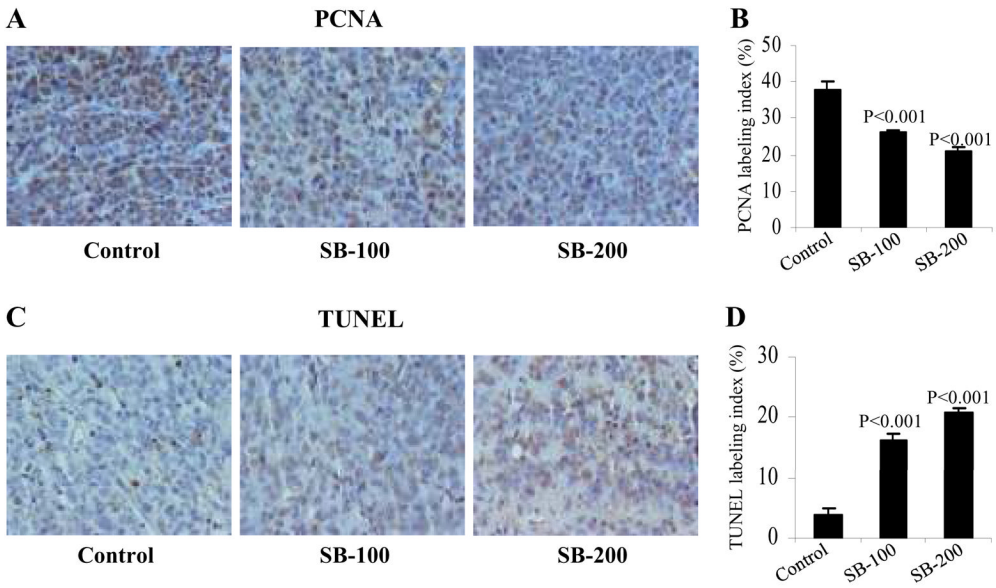

Fig. 5. ***In vivo*** **antiproliferative and pro-apoptotic effect of silibinin in human CRC LoVo xenograft in athymic nude mice**

(**A** and **B**) At the end of the experiment mice were sacrificed and tumor tissues were analyzed for immunohistochemical staining of proliferating cell nuclear antigen (PCNA) and photomicrographs were taken as detailed in the Materials and methods. Proliferation index was calculated as number of PCNA positive cells × 100/total number of cells counted under 400X magnification in 5 randomly selected areas in each tumor sample. (**C** and **D**) Apoptotic cell population in tissues from various groups was analyzed by TUNEL assay as detailed in the Materials and methods. Apoptotic index was calculated as number of positive cells × 100/total number of cells counted under 400× magnification in five randomly selected areas in each tumor sample. Data shown represents mean ± SE from 8 mice in each group. Abbreviations: SB-100, 100 mg/kg body weight silibinin; SB-200, 200 mg/kg body weight silibinin.

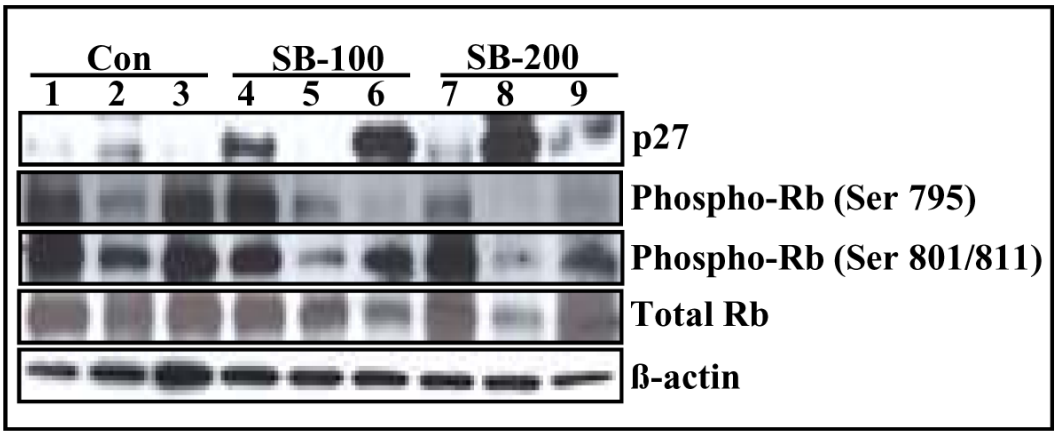

Fig. 6. Silibinin modulates the expression of cell cycle regulatory molecules in human CRC LoVo xenograft in athymic nude mice

Three randomly selected samples from each group were analyzed for cell cycle regulatory molecules. Total cell lysates were prepared and western blot analysis was performed for p27, phospho-Rb (Ser 795 and Ser 807/811) and total Rb as detailed in the Materials and methods. Membranes were also stripped in each case and reprobed with anti-β-actin antibody to confirm equal protein loading. Abbreviations: Con, Control; SB- 100, 100 mg/kg body weight silibinin; SB-200, 200 mg/kg body weight silibinin.