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 is one of the leading causes of cancer-related morbidity and mortality. The use of nontoxic phytochemicals in the prevention and intervention of colorectal cancer has been suggested as an alternative to chemotherapy. Here we assessed the anticancer efficacy of silibinin against advanced colorectal cancer 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 poly (ADP-ribose) polymerase. 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 cyclin-dependent kinases (1, 2, 4, and 6) and increases the level of cyclin-dependent kinase inhibitors (p21 and p27). Consistent with these results, silibinin treatment also decreased the phosphorylation of retinoblastoma protein at Ser780, Ser795, and Ser807/Ser811 sites without significantly affecting its total level. In animal studies, oral administration of silibinin for 6 weeks (at 100 and 200 mg/kg/d for 5 days/wk) 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 retinoblastoma phosphorylation. Together, these results suggest the potential use of silibinin against advanced human colorectal cancer. [Mol Can Ther 2009;8(8):OF1–OF9]

Introduction

Colorectal cancer is one of the leading causes of cancer-related deaths worldwide. According to American Cancer Society estimates, ∼148,810 new cases of colorectal cancer were diagnosed in 2008 and ∼49,960 patients died due to this malignancy in the United States alone (1). The high mortality among colorectal cancer patients is principally attributed to the metastasis of the neoplasm to distant organs (2, 3). The high toxicity and harmful side effects associated with chemotherapy and radiotherapy limit their potential benefits; therefore, alternative measures have been warranted to lower the burden of this malignancy. One approach to control colorectal cancer growth and metastasis is through its preventive intervention by nontoxic 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 nondietary phytochemicals against various cancers including colorectal cancer (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 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 shown the preclinical efficacy of both silybinin and its crude extract source, silymarin, against various epithelial cancers, and at least silybinin efficacy is currently being evaluated in cancer patients (12, 13). Further, both silymarin and silibinin have shown strong efficacy against colorectal cancer cells in both in vitro and in vivo studies (14–18). However, the potential efficacy of silibinin against advanced-stage colorectal cancer is largely...
unknown. Because the advanced 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 colorectal cancer LoVo cells.

One of the hallmarks of cancer cells including colorectal cancer 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 (CDK), the activity of which is controlled by binding of different cyclins and CDK inhibitors (21). The active CDK/cyclin complex phosphorylates retinoblastoma (Rb) 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 antiapoptotic and prosurvival factors is known to make cancer cells resistant toward apoptosis induction by various therapeutic drugs (20). Therefore, induction of cell cycle arrest and apoptosis by nontoxic 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 colorectal cancer LoVo cells in both cell culture and xenograft studies. Our findings show that silibinin induces cell cycle arrest and also inhibits colorectal cancer 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 colorectal cancer growth by silibinin.

Materials and Methods

Chemicals and Cell Line

Silibinin was procured from Sigma. Antibody against p21/Cip1 was from Millipore, and antibody against p27/Kip1 was from Neomarkers. Antibodies for CDK1, CDK2, CDK4, CDK6, and cyclins A, B1, D1, and D3 were from Santa Cruz Biotechnology. Anti–cleaved caspase-3, anti–cleaved caspase-9, anti–cleaved poly(ADP-ribose) polymerase, anti-Rb, and anti–phospho-Rb (Ser780, Ser795, and Ser807/Ser811) antibodies were from Cell Signaling Technology. Anti–proliferating cell nuclear antigen (PCNA) and N-Universal Negative Control mouse antibodies were from DAKO. Carboxymethyl cellulose (CMC) was purchased from Fluka-Buchs. LoVo cells were obtained from the American Type Culture Collection. Cells were grown in F-12 medium supplemented with 10% fetal bovine serum and 100 units/mL streptomycin and penicillin and maintained under standard culture conditions of 37°C, 95% humidified air, and 5% CO₂.

Cell Growth and Viability Assay

The growth and viability of cells were measured by trypan blue exclusion assay. Briefly, the cells were plated over-night at a density of 5,000/cm² of 60 mm culture dish. Subsequently, cells were treated with either DMSO alone or silibinin (50-200 μmol/L in DMSO) for 24 to 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 the 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) as per vendor's protocol. Briefly, at the end of each treatment, nonadherent and adherent cells were collected after brief trypsinization, washed once with ice cold PBS, and subsequently stained with Annexin V and propidium iodide. Stained cells were analyzed by flow cytometry using fluorescence-activated cell sorting analysis core facility of the 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 × 10⁷ cells in 0.5 mL saponin/propidium iodide solution were incubated at 4°C for 24 h in the dark (25). Cell cycle distribution was then analyzed by flow cytometry using fluorescence-activated cell sorting 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), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonflouride, 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 2× SDS-PAGE sample buffer and run on 8% to 16% Tris-glycine gels. Separated proteins were then transferred onto nitrocellulose membrane by Western blotting. After 1 h 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 h at room temperature and visualized by enhanced chemiluminescence detection system (GE Healthcare Bioscience). To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody (Sigma).

Experimental Design for Tumor Xenograft Study

Athymic (nu/nu) male nude mice were obtained from the National Cancer Institute and housed in our animal care facility at standard laboratory conditions and fed autoclaved AIN-76A rodent diet (Dyets) 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 medium. Six-week-old athymic male mice were s.c. injected with 5 × 10⁶ 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, whereas mice in control (first) group were gavaged with 0.2 mL of 0.5% (w/v) CMC daily, second and third groups were gavaged with 100 and 200 mg/kg/d doses of silibinin in 0.2 mL of 0.5% CMC 5 days/wk, 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. The tumor volume was calculated by the formula: \( V = \frac{4}{3} \pi (L_1 \times L_2)^2 \), where \( L_1 \) 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 and weighed, and a part was fixed in buffer formalin and the remaining tissue was stored at −80°C until further analysis. 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 described above.

**Immunohistochemical Staining for PCNA**

Tumor tissues fixed in 10% phosphate-buffered formalin for 10 h 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 staining. Briefly, sections after deparaffinization and rehybridization were treated with 0.01 mol/L 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 horseradish peroxidase-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 ×400 magnification.

**TUNEL Staining for Apoptotic Cells**

Apoptotic cells were detected using the DeadEnd colorimetric terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) system (Promega) following the 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 Immunohistochemical Analyses**

All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific). Statistical significance of difference between control and treated groups was determined by either Student’s t test or one-way ANOVA followed by Bonferroni t test. Differences between control and treated groups were considered statistically significant. Immunohistochemical analyses were done with a Zeiss Axioscop 2 microscope (Carl Zeiss). Microscopic images were taken by AxioCam MrC5 camera at ×400 magnification and processed by AxioVision software documentation system (Carl Zeiss).

**Results**

**Silibinin Inhibits the Growth and Decreases the Viability of Human Colorectal Cancer LoVo Cells**

We first examined the effect of silibinin on the growth and viability of human colorectal cancer LoVo cells. LoVo cells were plated overnight and subsequently treated with silibinin at concentrations ranging from 0 to 200 μmol/L in DMSO for 24 to 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 described in Materials and Methods. Mean ± SD of three samples for each treatment. Experiment was repeated three times. *, \( P < 0.05; \#, P < 0.01; \$, P < 0.001. \)

![Figure 1](image-url)
Silibinin Induces Apoptosis in Human Colorectal Cancer LoVo Cells

Because 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/propidium iodide staining. As shown in Fig. 2A, apoptotic cell population increased from 14 ± 3% in DMSO-treated controls to 20 ± 2% (P < 0.01) after 24 h treatment with 100 and 200 μmol/L 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 μmol/L silibinin concentrations, respectively, was observed compared with 10 ± 1% in DMSO controls (Fig. 2A). Silibinin at lower dose of 50 μmol/L did not induce any significant increase in apoptotic death compared with 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 μmol/L) significantly increased the levels of cleaved caspase-9 and -3 in LoVo cells, thereby indicating the involvement of these caspases in apoptosis induction. Further, silibinin treatment also increased the cleavage of poly(ADP-ribose) polymerase (Fig. 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 proapoptotic and antiapoptotic members of Bcl-2 family, that is Bax, Bcl-2, and Bcl-XL; however, no change in the protein levels of these molecules was observed on silibinin treatment (data not shown).

Silibinin Modulates the Expression of Cell Cycle Regulatory Proteins in Human Colorectal Cancer LoVo Cells

Because we observed a strong cell cycle arrest with silibinin treatment (Fig. 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 Fig. 3A, significant population of cells were arrested at G1 phase (49-57% compared with 41.5% in DMSO-treated controls; P < 0.01-0.001) on treatment with silibinin at 50, 100, and 200 μmol/L concentrations for 24 h (Fig. 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% compared with 14% in DMSO-treated controls; P < 0.001; Fig. 3A). This effect was sustained even when the treatment period was extended to 48 h, where 50% to 61% of cells were arrested in G1 phase with silibinin treatment (50-200 μmol/L) compared with 43% in DMSO-treated controls (P < 0.001; Fig. 3B). Similarly, 17% of cells were arrested at G2-M phase with 200 μmol/L dose of silibinin compared with 10% cells in control after 48 h of treatment (P < 0.001; Fig. 3B).

Silibinin Causes Cell Cycle Arrest in Human Colorectal Cancer LoVo Cells

Because 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 (Fig. 3C). Silibinin treatment also resulted in a strong dose- and time-dependent decrease in the levels of cyclins D1 and D3 and a moderate decrease in the levels of cyclins A and B1 (Fig. 3C). Next, we examined the effect of silibinin on the levels of CDK inhibitors, which are known to regulate cell cycle progression by binding to cyclin-CDK complexes (24, 27). As shown in Fig. 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 Rb protein (22). The unphosphorylated 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 at least 16 sites for CDK phosphorylation, but the significance of most of these sites is still unclear (22, 23). Rb phosphorylation at Ser295 site is known to disrupt its binding with E2F, whereas phosphorylation at various threonine and serine sites including Ser780 and Ser807/Ser811 is considered important for maintaining its inactive hyperphosphorylated state (22, 23). Because silibinin treatment induced strong G1 arrest in LoVo cells, we next examined the effect of silibinin on the phosphorylation of Rb. As shown in Fig. 3D, silibinin treatment for 24 and 48 h resulted in a significant decrease (mainly at 200 μmol/L dose) in the phosphorylation of Rb protein at Ser795, Ser780, and Ser807/Ser811 sites. Under similar treatment conditions, silibinin treatment only slightly decreased the total Rb levels at the highest concentration (Fig. 3D). In all cases, membranes were stripped and reprobed with β-actin antibody to confirm equal protein loading.

**Silibinin Inhibits Human Colorectal Cancer LoVo Xenograft Growth**

Based on our results showing strong efficacy of silibinin in advanced metastatic colorectal cancer LoVo cells in culture, next we examined the *in vivo* efficacy of silibinin against the colorectal cancer 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 for 5 days/wk for 6 weeks caused a marked time-dependent inhibition in LoVo xenograft growth in comparison with vehicle control (Fig. 4A). The average final tumor volume was reduced from 2,692 mm³/mouse in control group to 1,766 and 1,450 mm³/mouse in 100 and 200 mg/kg body weight silibinin treatment groups, respectively, which accounted for 34% and 46% decrease at the end of experimental period (*P* < 0.001; Fig. 4A). Consistent with this observation, compared with control, tumor weight in silibinin-fed groups was decreased by 38% (*P* < 0.05) and 49% (*P* < 0.01) at 100 and 200 mg/kg body weight doses, respectively (Fig. 4B). Silibinin feeding did not show any gross signs of toxicity and/or possible adverse side effects as measured by two profiles, that is, body weight and diet consumption. As shown in Fig. 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 colorectal cancer without any apparent signs of toxicity.

**Silibinin Inhibits Cell Proliferation and Induces Apoptosis in Human Colorectal Cancer LoVo Xenograft**

Next, to gain insight into the mechanism(s) underlying the *in vivo* antitumor efficacy of silibinin and to assess

![Figure 3.](image-url)
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 immunohistochemistry for PCNA, a marker for cell proliferation, and TUNEL, a marker for apoptotic response (Fig. 5). As shown in Fig. 5A, compared with 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 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 compared with CMC-fed controls (Fig. 5B). In case of TUNEL, as shown in Fig. 5C, tumor xenografts from silibinin-fed groups showed a marked increase in TUNEL positive cells compared with CMC-fed controls. The quantification of TUNEL stained samples showed that there was 4.2-fold ($P < 0.001$) and 5.4-fold 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 (Fig. 5D). Representative photographs for PCNA- and TUNEL-positive cells in control and silibinin groups are shown at ×400 magnification (Fig. 5A and C).

**Silibinin Modulates the Expression of Cell Cycle Regulatory Proteins in Human Colorectal Cancer LoVo Xenograft**

Because in our immunohistochemical studies with tumor xenograft tissue we observed that silibinin feeding exerts strong antiproliferative 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 (Fig. 6). However, no alteration in the level of p21 was observed in tumors from silibinin-fed animals compared with CMC-fed controls (data not shown). Further, silibinin treatment also decreased the levels of Rb phosphorylation at Ser795 and Ser807/Ser811 sites (Fig. 6) but did not significantly affect the Rb phosphorylation at Ser780 site (data not shown). Silibinin treatment also slightly decreased the total levels of Rb in the xenograft tissue (Fig. 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, that is, 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-induced aberrant crypt foci, 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 colorectal cancer cells by inhibiting the vascular endothelial growth factor secretion (34, 35). In a coculture assay, silibinin treatment also inhibited the chemotactic migration of endothelial cells toward the colon cancer cells (35). Recently, we also reported the strong in vitro 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 colorectal cancer, its usefulness against advanced-stage colorectal cancer remains unknown. Moreover studies evaluating the efficacy of silibinin against cancer cells representing various clinical stages of colorectal cancer are prerequisite for any future clinical trial with silibinin in colorectal cancer patients. The present study is novel in this aspect that for the first time efficacy of silibinin was examined against advanced-stage highly metastatic colorectal cancer cells (36, 38). We found that silibinin inhibits the growth and induces apoptosis in advanced human colorectal cancer 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 colorectal cancer 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 colorectal cancer LoVo cells. Silibinin-mediated G1 arrest was associated with a decrease in the levels of CDK2, CDK4, and CDK6 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 μmol/L) reduces the levels of CDK1 as well as cyclins A and B1 and thereby possibly causes G2-M-phase arrest. The present study also showed that silibinin

![Figure 5](https://example.com/figure5.png)

**Figure 5.** In vivo antiproliferative and proapoptotic effect of silibinin in human colorectal cancer 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 PCNA and photomicrographs were taken as described in Materials and Methods. Proliferation index was calculated as number of PCNA-positive cells × 100 / total number of cells counted under ×400 magnification in five randomly selected areas in each tumor sample. C and D, apoptotic cell population in tissues from various groups was analyzed by TUNEL assay as described in 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. Mean ± SE from 8 mice in each group.
OF8 Suppression of Colorectal Cancer Growth by Silibinin

Silibinin modulates the expression of cell cycle regulatory molecules in human colorectal cancer 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 done for p27, phospho-Rb (Ser795 and Ser807/Ser811), and total Rb as described in Materials and Methods. Membranes were also stripped in each case and reprobed with anti-β-actin antibody to confirm equal protein loading. Con, control.

Figure 6. Silibinin modulates the expression of cell cycle regulatory molecules in human colorectal cancer 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 done for p27, phospho-Rb (Ser795 and Ser807/Ser811), and total Rb as described in Materials and Methods. Membranes were also stripped in each case and reprobed with anti-β-actin antibody to confirm equal protein loading. Con, control.

inhibits the phosphorylation of Rb at specific serine sites in LoVo cells in both cell culture and xenograft tissue. The decrease in the phosphorylation of Rb protein might be due to a decrease in the kinase activity of CDK2, CDK4, and CDK6, which could be related to observed decrease in their expression level as well as increase in the level of CDK inhibitors especially the p27. These findings are similar to our previous findings in DU145 cells, where silibinin-mediated G1 arrest was through hypophosphorylation 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 CDK inhibitors 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 colorectal cancer 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 S-phase kinase-associated protein 2-mediated proteasomal degradation (46, 47). In our previously published study, we found that silibinin attenuates the levels of 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 poly(ADP-ribose) polymerase 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 -9 along with cleavage of poly(ADP-ribose) polymerase. 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 pharmacologic effective doses of the drug without causing any systemic toxicity. We have reported earlier that ≥150 μmol/L concentration of silibinin could be achieved in the plasma of mice without causing any toxicity (15). Hoh et al. have shown in colorectal cancer patients that 141 μmol/L concentration of silibinin could be achieved in the colorectal tissue by feeding silibinin at 1,440 mg/d silibinin for 7 days (50). In the present study, most of the effects of silibinin on the biological endpoints were significant at 100 μmol/L 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), that is, assuming a human body surface area of 1.8 m² 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 colorectal cancer 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 showed the anticancer efficacy of silibinin against advanced colorectal cancer 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 colorectal cancer LoVo cells under both in vitro and in vivo conditions. Overall, these results support further preclinical testing of silibinin and its clinical use against colorectal cancer.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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