

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

Manjinder Kaur,¹ Balaiya Velmurugan,¹
Alpna Tyagi,¹ Gagan Deep,¹ Suchitra Katiyar,¹
Chapla Agarwal,^{1,2} and Rajesh Agarwal^{1,2}

¹Department of Pharmaceutical Sciences, School of Pharmacy, and ²University of Colorado Cancer Center, University of Colorado-Denver, Aurora, Colorado

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 Cancer Ther 2009;8(8):2366–74]

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 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 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

Received 3/5/09; revised 5/20/09; accepted 5/27/09; published OnlineFirst 7/28/09.

Grant support: National Cancer Institute/USPHS grant R01 CA112304.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: M. Kaur and B. Velmurugan have contributed equally to this work and share first authorship.

Requests for reprints: Rajesh Agarwal, Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado-Denver, C238-P15, Research 2, Room 3121, 12700 19th Avenue, Aurora, CO 80045. Phone: 303-724-4055; Fax: 303-724-7266. E-mail: Rajesh.Agarwal@UCDenver.edu

Copyright © 2009 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-09-0304

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 apoptosis in these cancer cells 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 overnight 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 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 × 10⁵ 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 nondenaturing 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 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 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 5×10^6 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, 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: $0.5236L_1(L_2)^2$, 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 buffered 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 $4 \mu\text{m}$ sections and processed for immunohistochemical staining. Briefly, sections after deparaffinization and rehydration 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 $\times 400$ 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 $\times 400$ magnification.

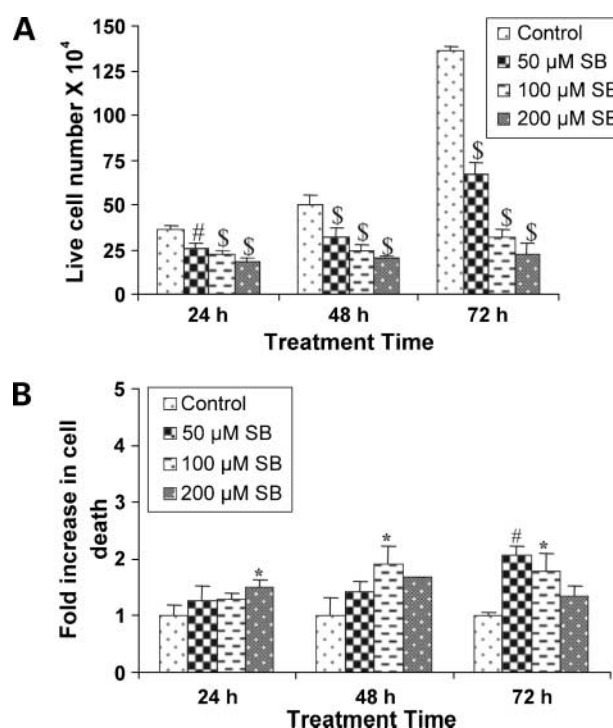


Figure 1. Silibinin inhibits the growth and decreases the viability of human colorectal cancer LoVo cells. LoVo cells were plated overnight and subsequently treated with silibinin at concentrations ranging from 0 to 200 $\mu\text{mol/L}$ in DMSO for 24 to 72 h. At the end of each treatment duration, 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 \pm SD of three samples for each treatment. Experiment was repeated three times. *, $P < 0.05$; #, $P < 0.01$; \$, $P < 0.001$.

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. $P < 0.05$ was considered statistically significant. Immunohistochemical analyses were done with a Zeiss Axioscop 2 microscope (Carl Zeiss). Microscopic images were taken by AxioCam MrC5 camera at $\times 400$ 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. Silibinin (50–200 $\mu\text{mol/L}$) treatment for 24 h reduced the growth of LoVo cells by 30% to 49% ($P < 0.01$ – 0.001 ; Fig. 1A). A further decrease in cell growth by 37% to 60% ($P < 0.001$) and 51% to 83% ($P < 0.001$) at similar silibinin concentrations was also observed following prolonged treatment durations of 48 and 72 h, respectively (Fig. 1A). Under similar treatment conditions, silibinin (50–200 $\mu\text{mol/L}$) increased the dead cell

population by ~1.5- to 2.0-fold after 24, 48, and 72 h of treatment, respectively (Fig. 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 $\mu\text{mol/L}$ silibinin was most effective in inducing cell death in these cells, although even lower concentrations of 50 and 100 $\mu\text{mol/L}$ silibinin exhibited similar but delayed effect.

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 $14 \pm 3\%$ in DMSO-treated controls to $19 \pm 2\%$ and $22 \pm 1\%$ ($P < 0.01$) after 24 h treatment with 100 and 200 $\mu\text{mol/L}$ silibinin, respectively. When the treatment time was extended to 48 h, a further increase in percent apoptotic cell population to $20 \pm 0.2\%$ ($P < 0.001$) and $38 \pm 3\%$ ($P < 0.001$) at 100 and 200 $\mu\text{mol/L}$ silibinin concentrations, respectively, was observed compared with $10 \pm 1\%$ in DMSO controls (Fig. 2A). Silibinin at lower dose of 50 $\mu\text{mol/L}$ did not induce any signifi-

cant 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 $\mu\text{mol/L}$) 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 anti-apoptotic 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 Causes Cell Cycle Arrest in Human Colorectal Cancer LoVo Cells

Because we observed a strong decrease in cell growth by 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 G_1 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 $\mu\text{mol/L}$ concentrations for 24 h (Fig. 3A). However, at the highest dose of silibinin, in addition to cells arresting at G_1 phase of cell cycle, a significant population of the cells were also observed to accumulate at G_2 -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 G_1 phase with silibinin treatment (50-200 $\mu\text{mol/L}$) compared with 43% in DMSO-treated controls ($P < 0.001$; Fig. 3B). Similarly, 17% of cells were arrested at G_2 -M phase with 200 $\mu\text{mol/L}$ dose of silibinin compared with 10% cells in control after 48 h of treatment ($P < 0.001$; Fig. 3B).

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, 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 G_1 -S transition during cell cycle progression is Rb protein (22). The unphosphorylated Rb is known to prevent cell cycle progression

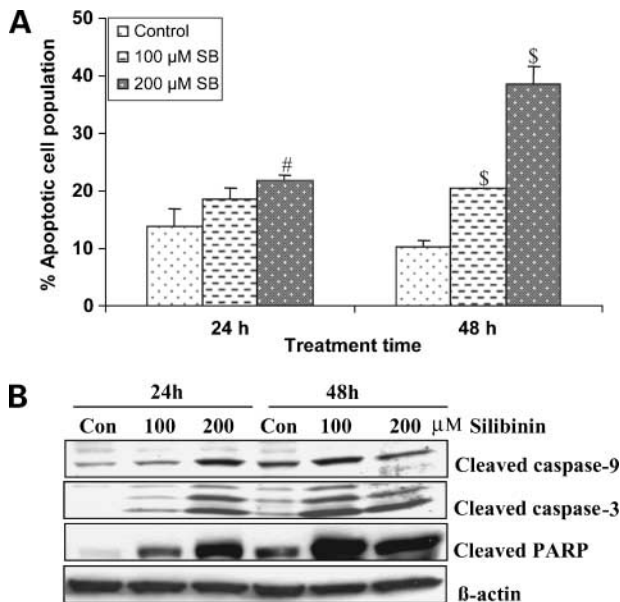


Figure 2. Silibinin induces apoptotic death in human colorectal cancer LoVo cells. LoVo cells were plated overnight and subsequently treated with 0, 100, and 200 $\mu\text{mol/L}$ silibinin in DMSO for 24 and 48 h. At the end of each treatment duration, both adherent and nonadherent cells were collected and processed. **A**, collected cells were stained with Annexin V/propidium iodide and analyzed with flow cytometer and presented as percent apoptotic death. Mean \pm SD 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, caspase-3, and cleaved poly(ADP-ribose) polymerase (PARP). Membranes were also stripped in each case and reprobed with anti- β -actin antibody to confirm equal protein loading.

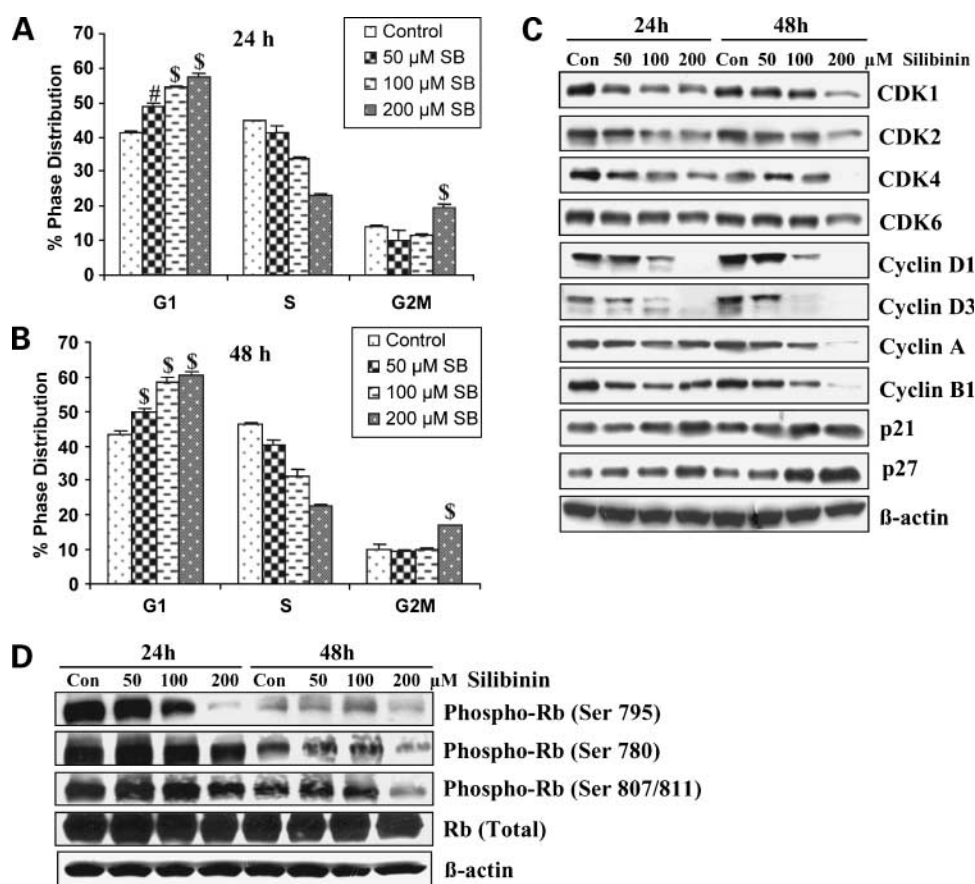


Figure 3. Silibinin causes cell cycle arrest and modulates the expression of various cell cycle regulatory molecules in human colorectal cancer LoVo cells. LoVo cells were plated overnight and subsequently treated with silibinin at concentrations ranging from 0 to 200 μ mol/L in DMSO for 24 and 48 h. At the end of treatment durations, cells were collected and either (A and B) cells were stained with saponin/propidium iodide 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, cyclins D1, D3, A, and B1, and p21, p27 and phosphorylation of Rb at Ser⁷⁹⁵, Ser⁷⁸⁰, Ser⁸⁰⁷/Ser⁸¹¹, and total Rb) by Western immunoblotting. Membranes were also stripped in each case and reprobbed with anti- β -actin antibody to confirm equal protein loading. #, $P < 0.01$; \$, $P < 0.001$.

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 Ser⁷⁹⁵ 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 μ mol/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 reprobbed 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

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 $\times 400$ magnification (Fig. 5A and C).

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

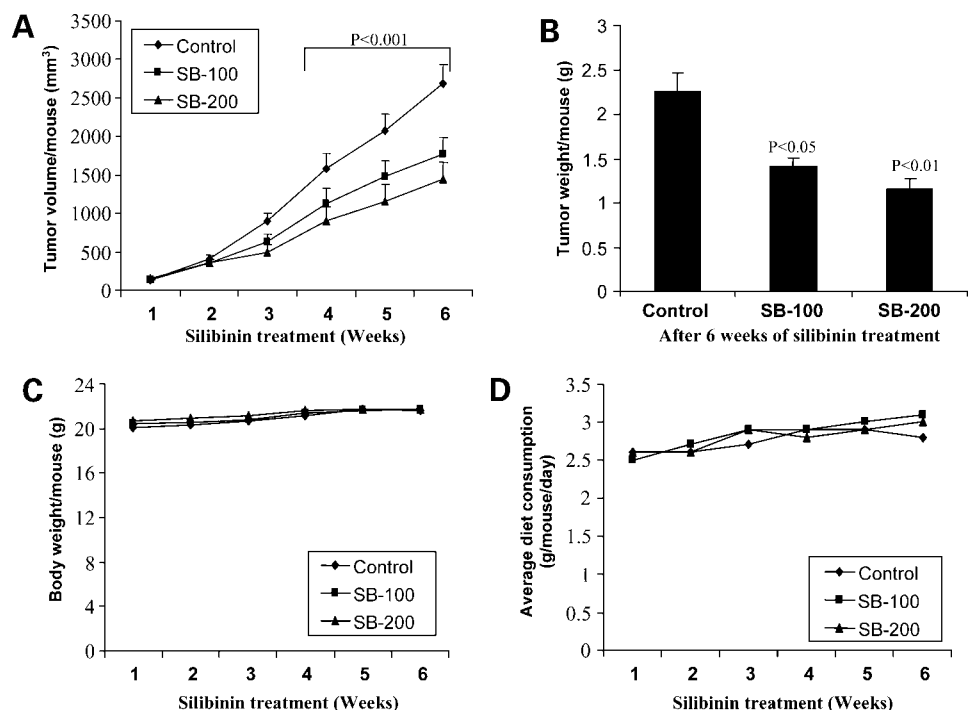
Because in our immunohistochemical studies with tumor xenograft tissue we observed that silibinin feeding exerts strong antiproliferative effect, we also examined the effect of silibinin on the expression of cell cycle regulatory mole-

cules 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

Figure 4. Silibinin inhibits human colorectal cancer 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 daily doses of silibinin for 5 d/wk for 6 wk. **A**, tumor volume (mm^3) per mouse as a function of time. **B**, tumor weight (g) per mouse at the end of study. **C**, average body weight (g) per mouse. **D**, average diet consumption (g) per mouse a day. **A** and **B**, mean \pm SE from 8 mice in each group. *SB-100*, 100 mg/kg body weight silibinin; *SB-200*, 200 mg/kg body weight silibinin.



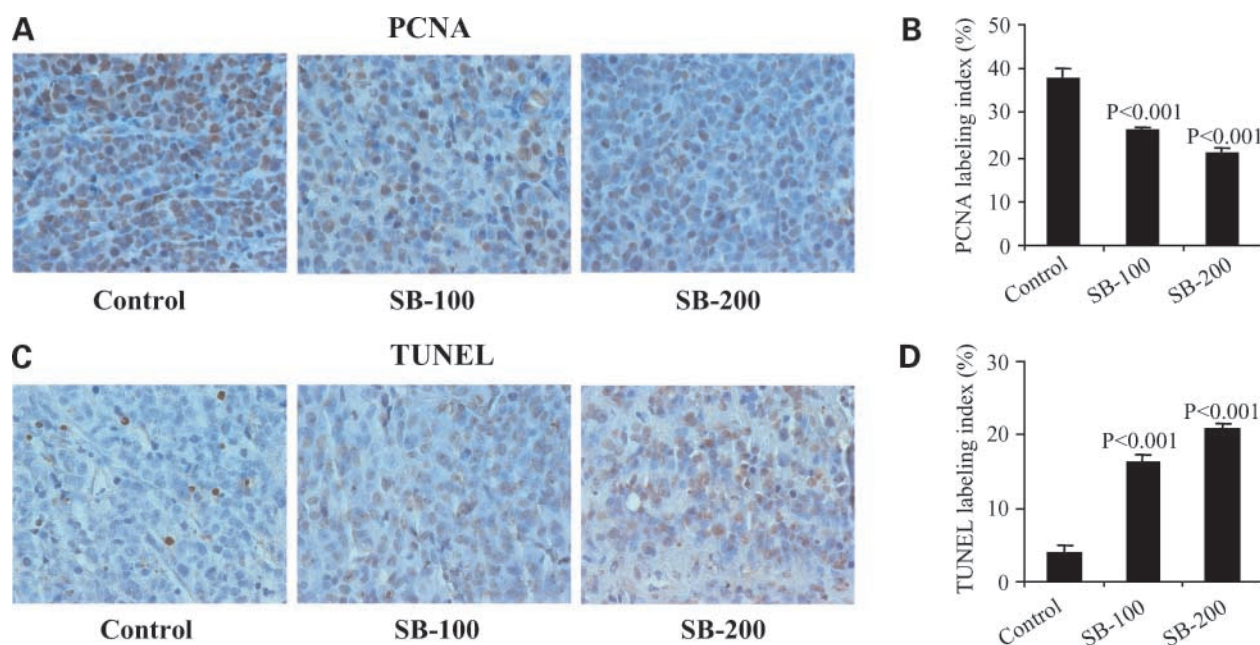


Figure 5. *In vivo* antiproliferative and proapoptotic effect of silibinin in human colorectal cancer LoVo xenograft in athymic nude mice. **A** and **B**, at the end of the experiment, mice were sacrificed and tumor tissues were analyzed for immunohistochemical staining of PCNA and photomicrographs were taken as described in Materials and Methods. Proliferation index was calculated as number of PCNA-positive cells \times 100 / total number of cells counted under \times 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 \times 100 / total number of cells counted under \times 400 magnification in five randomly selected areas in each tumor sample. Mean \pm SE from 8 mice in each group.

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 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 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 G_1 arrest was associated with a decrease in the levels of CDK2, CDK4, and CDK6 and their corresponding cyclins D1, D3, and A. Progression through G_2 -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 μ mol/L) reduces the levels of CDK1 as well as cyclins A and B1 and thereby possibly causes G_2 -M-phase arrest. The present study also showed that silibinin

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 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ 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 criteria 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 supports further preclinical testing of silibinin and its clinical use against colorectal cancer.

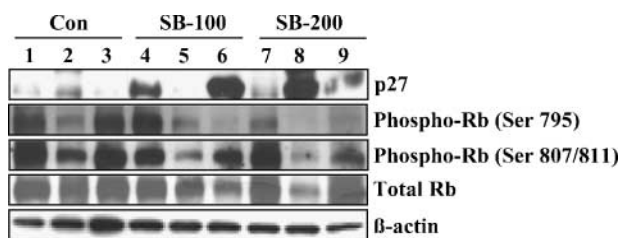


Figure 6. Silibinin modulates the expression of cell cycle regulatory molecules in human colorectal cancer LoVo xenograft in athymic nude mice. Three randomly selected samples from each group were analyzed for cell cycle regulatory molecules. Total cell lysates were prepared and Western blot analysis was done for p27, phospho-Rb (Ser⁷⁹⁵ and Ser^{807/811}), and total Rb as described in Materials and Methods. Membranes were also stripped in each case and reprobred with anti- β -actin antibody to confirm equal protein loading. Con, control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71–96.
- Millikan KW, Staren ED, Doolas A. Invasive therapy of metastatic colorectal cancer to the liver. *Surg Clin North Am* 1997;77:27–48.
- Ottaiano A, Franco R, Aiello Talamanca A, et al. Overexpression of both CXC chemokine receptor 4 and vascular endothelial growth factor predicts early distant relapse in stage II-III colorectal cancer patients. *Clin Cancer Res* 2006;12:2795–803.
- Naithani R, Huma LC, Moriarty RM, McCormick DL, Mehta RG. Comprehensive review of cancer chemopreventive agents evaluated in experimental carcinogenesis models and clinical trials. *Curr Med Chem* 2008;15:1044–71.
- Kumar N, Shibata D, Helm J, Coppola D, Malafa M. Green tea polyphenols in the prevention of colon cancer. *Front Biosci* 2007;12:2309–15.
- Pierini R, Gee JM, Belshaw NJ, Johnson IT. Flavonoids and intestinal cancers. *Br J Nutr* 2008;99:ES53–9.
- Tanaka T, Kohno H, Mori H. Chemoprevention of colon carcinogenesis by dietary non-nutritive compounds. *Asian Pac J Cancer Prev* 2001;2:165–77.
- Wu H, Dai Q, Shrubsole MJ, et al. Fruit and vegetable intakes are associated with lower risk of colorectal adenomas. *J Nutr* 2008; Epub ahead of print.
- Gescher A. Polyphenolic phytochemicals versus non-steroidal anti-inflammatory drugs: which are better cancer chemopreventive agents? *J Chemother* 2004;16 Suppl 4:3–6.
- McPartland JM, Vilgalys RJ, Cubeta MA. Mushroom poisoning. *Am Fam Physician* 1997;55:1797–1800, 1805–9, 1811–2.
- Kidd P, Head K. A review of the bioavailability and clinical efficacy of milk thistle phytosome: a silybin-phosphatidylcholine complex (Siliphos). *Altern Med Rev* 2005;10:193–203.
- Deep G, Agarwal R. Chemopreventive efficacy of silymarin in skin and prostate cancer. *Integr Cancer Ther* 2007;6:130–45.
- Ladas EJ, Kelly KM. Milk thistle: is there a role for its use as an adjunct therapy in patients with cancer? *J Altern Complement Med* 2003;9:411–6.
- Velmurugan B, Singh RP, Tyagi A, Agarwal R. Inhibition of azoxymethane-induced colonic aberrant crypt foci formation by silibinin in male Fisher 344 rats. *Cancer Prev Res (Phila PA)* 2008;1:376–84.
- Agarwal C, Singh RP, Dhanalakshmi S, et al. Silibinin upregulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells. *Oncogene* 2003;22:8271–82.
- Kohno H, Tanaka T, Kawabata K, et al. Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. *Int J Cancer* 2002;101:461–8.
- Hogan FS, Krishnegowda NK, Mikhailova M, Kahlenberg MS. Flavonoid, silibinin, inhibits proliferation and promotes cell-cycle arrest of human colon cancer. *J Surg Res* 2007;143:58–65.
- Singh RP, Gu M, Agarwal R. Silibinin inhibits colorectal cancer growth by inhibiting tumor cell proliferation and angiogenesis. *Cancer Res* 2008;68:2043–50.
- Sherr CJ. Cancer cell cycles. *Science* 1996;274:1672–7.
- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
- Murray AW. Recycling the cell cycle: cyclins revisited. *Cell* 2004;116:221–34.
- Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell* 1995;81:323–30.
- Rubin SM, Gall AL, Zheng N, Pavletich NP. Structure of the Rb C-terminal domain bound to E2F1-1: a mechanism for phosphorylation-induced E2F release. *Cell* 2005;123:1093–106.
- Deep G, Agarwal R. New combination therapies with cell-cycle agents. *Curr Opin Investig Drugs* 2008;9:591–604.
- Zi X, Grasso AW, Kung HJ, Agarwal R. A flavonoid antioxidant, silymarin, inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors, G₁ arrest, and anticarcinogenic effects in human prostate carcinoma DU145 cells. *Cancer Res* 1998;58:1920–9.
- Duriez PJ, Shah GM. Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem Cell Biol* 1997;75:337–49.
- Roy S, Kaur M, Agarwal C, Tecklenburg M, Sclafani RA, Agarwal R. p21 and p27 induction by silibinin is essential for its cell cycle arrest effect in prostate carcinoma cells. *Mol Cancer Ther* 2007;6:2696–707.
- Surh YJ. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 2003;3:768–80.
- Luper S. A review of plants used in the treatment of liver disease: part 1. *Altern Med Rev* 1998;3:410–21.
- Cheung CW, Taylor PJ, Kirkpatrick CM, et al. Therapeutic value of orally administered silibinin in renal cell carcinoma: manipulation of insulin-like growth factor binding protein-3 levels. *BJU Int* 2007;100:438–44.
- Singh RP, Tyagi A, Sharma G, Mohan S, Agarwal R. Oral silibinin inhibits *in vivo* human bladder tumor xenograft growth involving down-regulation of survivin. *Clin Cancer Res* 2008;14:300–8.
- Singh RP, Deep G, Chittezhath M, et al. Effect of silibinin on the growth and progression of primary lung tumors in mice. *J Natl Cancer Inst* 2006;98:846–55.
- Flaig TW, Gustafson DL, Su LJ, et al. A phase I and pharmacokinetic study of silybin-phytosome in prostate cancer patients. *Invest New Drugs* 2007;25:139–46.
- Yang SH, Lin JK, Huang CJ, Chen WS, Li SY, Chiu JH. Silibinin inhibits angiogenesis via Flt-1, but not KDR, receptor up-regulation. *J Surg Res* 2005;128:140–6.
- Yang SH, Lin JK, Chen WS, Chiu JH. Anti-angiogenic effect of silymarin on colon cancer LoVo cell line. *J Surg Res* 2003;113:133–8.
- Zhu XL, Liang L, Ding YQ. Overexpression of FMNL2 is closely related to metastasis of colorectal cancer. *Int J Colorectal Dis* 2008;23:1041–7.
- McLoughlin P, Roengvorapoj M, Gissel C, Hescheler J, Certa U, Sachinidis A. Transcriptional responses to epigallocatechin-3 gallate in HT 29 colon carcinoma spheroids. *Genes Cells* 2004;9:661–9.
- Pai R, Nakamura T, Moon WS, Tarnawski AS. Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors. *FASEB J* 2003;17:1640–7.
- Meeran SM, Katiyar SK. Cell cycle control as a basis for cancer chemoprevention through dietary agents. *Front Biosci* 2008;13:2191–202.
- Taylor WR, Stark GR. Regulation of the G₂-M transition by p53. *Oncogene* 2001;20:1803–15.
- Tyagi A, Agarwal C, Agarwal R. The cancer preventive flavonoid silibinin causes hypophosphorylation of Rb/p107 and Rb2/p130 via modulation of cell cycle regulators in human prostate carcinoma DU145 cells. *Cell Cycle* 2002;1:137–42.
- Loda M, Cukor B, Tam SW, et al. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med* 1997;3:231–4.
- Esposito V, Baldi A, De Luca A, et al. Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. *Cancer Res* 1997;57:3381–5.
- Porter PL, Malone KE, Heagerty PJ, et al. Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med* 1997;3:222–5.
- Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* 2006;6:369–81.
- Nickeleit I, Zender S, Kossatz U, Malek NP. p27kip1: a target for tumor therapies? *Cell Div* 2007;2:13.
- Assoian RK, Yung Y. A reciprocal relationship between Rb and Skp2: implications for restriction point control, signal transduction to the cell cycle and cancer. *Cell Cycle* 2008;7:24–7.
- Ziegler DS, Kung AL. Therapeutic targeting of apoptosis pathways in cancer. *Curr Opin Oncol* 2008;20:97–103.
- Logue SE, Martin SJ. Caspase activation cascades in apoptosis. *Biochem Soc Trans* 2008;36:1–9.
- Hoh C, Boockock D, Marczylo T, et al. Pilot study of oral silibinin, a putative chemopreventive agent, in colorectal cancer patients: silibinin levels in plasma, colorectum, and liver and their pharmacodynamic consequences. *Clin Cancer Res* 2006;12:2944–50.