Pharm Res (2010) 27:2085–2097
DOI 10.1007/s11095-010-0207-6

RESEARCH PAPER

Silibinin Exerts Sustained Growth Suppressive Effect against Human Colon Carcinoma SW480 Xenograft by Targeting Multiple Signaling Molecules

Balaiya Velmurugan • Subhash Chander Gangar • Manjinder Kaur • Alpna Tyagi • Gagan Deep • Rajesh Agarwal

Received: 3 May 2010 / Accepted: 25 June 2010 / Published online: 14 July 2010

ABSTRACT

Purpose Earlier, we reported the strong preventive efficacy of silibinin against colorectal cancer (CRC), but its usefulness against established CRC or effect of its withdrawal on CRC growth remained unknown. Present study focused on these important issues by employing two different treatment protocols in advanced human CRC SW480 xenograft in nude mice.

Methods In the first treatment protocol, silibinin was fed for 28 days (200 mg/kg body weight, 5 days/week) to mice with growing SW480 xenograft; thereafter, tumor growth was monitored for additional 3 weeks without silibinin treatment. In the second protocol, silibinin treatment was started after 25 days of SW480 cells injection (established tumors), and tumor growth was studied 4 days, 8 days and 16 days after silibinin treatment.

Results In both treatment protocols, silibinin had strong and sustained inhibitory effect on xenograft growth. Detailed xenograft analyses showed that silibinin, in both treatment protocols, exerts anti-proliferative, pro-apoptotic and anti-angiogenic effects. Further, silibinin reduced the expression of β-catenin and phospho-GSK3β in xenograft tissues. Silibinin also targeted signaling molecules involved in CRC proliferation and survival (cyclin D1, c-Myc and survivin) as well as angiogenesis regulators (VEGF and iNOS).

Conclusions Collectively, these findings substantiate silibinin's therapeutic efficacy against CRC, advocating its translational potential.

KEY WORDS angiogenesis · β-catenin · colorectal cancer · silibinin · SW480 cells

ABBREVIATIONS

CRC	colorectal cancer
APC	adenomatous polyposis coli
CD31	cluster of differentiation 31
CMC	carboxymethylcellulose
GSK3β	glycogen synthase kinase 3 beta
PCNA	proliferating cell nuclear antigen
ANOVA	analysis of variance
SEM	standard error of mean
TCF/LEF	T-cell factor/lymphoid enhancing factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
VEGF	vascular endothelial growth factor
iNOS	inducible nitric oxide synthase
IAP	inhibitors of apoptosis

INTRODUCTION

Colorectal cancer (CRC) is one of the most common causes of cancer-related deaths worldwide, including the United States. According to American Cancer Society, 146,970 new cases and 49,920 deaths due to CRC were estimated in 2009 in the United States (1). Despite the implementation of early screening programs, almost 50% of the CRC cases are already in advanced stage at the time of diagnosis, and the five-year survival rate for patients with non-localized tumor is only 11% (1). The therapeutic options, such as

B. Velmurugan · S. C. Gangar · M. Kaur · A. Tyagi · G. Deep · R. Agarwal
Department of Pharmaceutical Sciences, School of Pharmacy
University of Colorado Denver
Aurora 80045 Colorado, USA

R. Agarwal (✉)
Department of Pharmaceutical Sciences, School of Pharmacy
University of Colorado Denver
C238—P15, Research 2, Room 3121, 12700 E 19th Avenue
Aurora, Colorado 80045, USA
e-mail: Rajesh.Agarwal@ucdenver.edu

surgery, radiotherapy and chemotherapy are mostly ineffective against advance stage CRC, moreover, these therapeutic options have high toxicity and unacceptable side effects (2–4). These alarming statistics and lack of effective treatment options against advanced CRC warrant urgent need for testing and developing new preventive and therapeutic agents against this deadly malignancy. In this regard, controlling CRC growth and progression using dietary and plant-based non-toxic agents is an exciting option. Numerous published reports support the preventive and therapeutic efficacy of these agents against CRC and other cancers (5–9).

For the last several years, we have been working on the anti-cancer potential of silibinin, a primary polyphenolic active constituent of milk thistle (*Silybum marianum*) seeds. Several studies from our laboratory and by others have demonstrated the strong pre-clinical efficacy of silibinin against various epithelial cancers including CRC (10–14). Silibinin treatment has been reported to inhibit CRC cell growth by inducing cell cycle arrest and apoptosis both *in vitro* and *in vivo* (10,11,15,16). Recently, we reported that silibinin feeding strongly inhibits spontaneous intestinal tumorigenesis in $APC^{min/+}$mouse model, a genetically predisposed animal model of human familial adenomatous polyposis (FAP) (17). In an earlier study, we described that the dietary administration of silibinin significantly inhibits azoxymethane (AOM)-induced aberrant crypt foci (ACF), which are considered as putative precursors of colon cancer (18). Similarly, in a recent study, Sangeetha *et al.* (19) reported that silibinin modulates activity of biotransforming microbial enzymes and, thereby, prevents 1, 2-dimethylhydrazine induced pre-neoplastic lesions in the colon of Wistar rats. These studies suggest the preventive efficacy of silibinin against CRC, but its therapeutic efficacy against CRC remained largely unknown. Therefore, in the present study, we aimed to understand the therapeutic efficacy of silibinin on growing as well as established CRC xenografts. Further, this study is novel as this is the first attempt to understand the effect of silibinin treatment withdrawal on the CRC growth.

Colon carcinogenesis is a multi-stage process that involves deletions, mutations, and changes in expression of genes that are critical for cancer cells' growth, angiogenesis and metastasis (20–23). The most common genetic mutations in CRC development are the mutations in the molecular players of adenomatous polyposis coli (APC)/β-catenin pathway, also called the Wnt signaling pathway, resulting in adenoma formation (23). β-Catenin is the key component of Wnt signaling pathway, and in normal cells, most of *β*-catenin is present at cell-cell junctions with very little level in cytoplasm or nucleus (24). The turnover of β-catenin is maintained primarily through its phosphorylation by a destruction complex consisting of Axin-APC-GSK-3β (24). Once phosphorylated, β-catenin is recognized by β-TrCP that promotes its ubiquitination-mediated degradation (24). However, in presence of active Wnt signaling due to the presence of various ligands like frizzled or due to APC mutation/deletion, *β*-catenin degradation is inhibited (24,25). In such cases, excessive *β*-catenin is accumulated in the cytoplasm, and subsequently in the nucleus, where it functions as a co-transcription activator factor of T-cell factor/lymphoid enhancing factor (TCF/LEF) and leads to increased expression of many target genes responsible for proliferation, survival, angiogenesis and metastasis of CRC (24,25). Therefore, in the present study, besides focusing on the therapeutic efficacy of silibinin, we also examined silibinin's effect on β-catenin and various other signaling molecules regulated on this axis using SW480 xenograft as a model. SW480 cells over-express β-catenin due to mutant APC (26) and, therefore, offer a rational choice for present study. Our results clearly showed the sustained therapeutic effects of silibinin against CRC growth through its anti-proliferative, pro-apoptotic and anti-angiogenic effects, and revealed its strong inhibitory effect on β-catenin expression and associated signaling pathway.

MATERIALS AND METHODS

Cell Line and Reagents

Human CRC SW480 cells were procured from American Type Culture Collection (Manassas, VA) and cultured (until used for xenograft implantation) in Leibovitz's L-15 Medium with 10% fetal bovine serum under standard culture conditions. Silibinin (purity≥98% by HPLC analysis) and carboxymethylcellulose (CMC) were from Sigma (St. Louis, MO). Matrigel was from BD Biosciences (New Bedford, MA). Survivin antibody was from Novus Biologicals (Littleton, CO). Antibodies for iNOS, CD31 and VEGF were from Abcam (Cambridge, MA). Phospho-GSK3β (pGSK3β) antibody was from Cell Signaling Technology (Beverly, MA). Antibodies for cyclin D1, β-catenin and c-Myc, and normal goat serum and biotinylated anti-rabbit secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling) assay kit was from Promega Corporation (Madison, WI). Antibody for proliferating cell nuclear antigen (PCNA), streptavidin, biotinylated anti-mouse secondary antibody and N-universal negative control antibody were from Dako (Carpinteria, CA). 3, 3′-diaminobenzidine kit was from Vector Laboratories (Burlingame, CA). All other reagents were obtained in their commercially available highest purity grade.

Animals and Experimental Design for Tumor Xenograft Study

Six-week-old athymic (*nu/nu*) male nude mice were obtained from the National Cancer Institute (Bethesda, MD), housed in an 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 were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Colorado Denver. SW480 cells were harvested by trypsinization, washed and re-suspended in serum-free Leibovitz's medium. Thereafter, cells were mixed with matrigel (1:1), and approximately 5×10^6 SW480 cells were subcutaneously injected in the right flank of each mouse to initiate tumor growth. Two different protocols were followed for silibinin treatment in the present study. In Protocol-I (Fig. 1a), 24 h after tumor cell inoculation, mice were randomly divided into two groups. In the first group (control), mice were gavaged with 0.2 ml of 0.5% (w/v) carboxymethyl cellulose (CMC)/day, whereas in the second group, mice were gavaged with 200 mg/kg body weight, 5 days/week of silibinin (0.2 ml in 0.5% CMC) for a total of 28 days, and these treatments were started 1 day after xenograft implantation. Thereafter, both the groups were gavaged only with 0.5% CMC, and eight mice from each group were euthanized after 0, 7 and 21 days (Fig. 1a). In Protocol-II (Fig. 1b), mice were gavaged with 0.2 ml of 0.5% CMC, and xenograft was allowed to grow for 25 days. On the 25th day, six mice were euthanized (day 0), and remaining mice were randomly divided into two groups. Mice in one group continued on 0.5% CMC, while mice in the other group were gavaged with silibinin (200 mg/kg body weight, 5 days/week). Thereafter, six mice from each group were euthanized after 4, 8 and 16 days of silibinin treatment, which corresponds to 29, 33 and 41 day of the experiment, respectively (Fig. 1b; Protocol-II). Throughout the study, body weight and diet consumption of mice were recorded twice weekly, and after 7 days of cell inoculation, tumor size was measured twice weekly in two dimensions using a digital caliper. The tumor volume was calculated by the formula $0.5236\ L_1(L_2)^2$, where L_1 is the long axis, and L_2 is the short axis of the tumor. At the termination of each study, mice were euthanized; tumors were excised and weighed, and a part was fixed in 10% phosphate-buffered formalin for immunohistochemical (IHC) analyses.

Immunohistochemical Staining

IHC staining was performed as described earlier (11), with the following primary antibody dilutions: anti-PCNA antibody (1:400 dilution), anti-cyclin D1 antibody (1:250 dilution), anti-survivin antibody (1:100 dilution), anti-iNOS antibody (1:100 dilution), anti-CD31 antibody (1:50 dilution), anti-VEGF antibody (1:100 dilution), anti-β-catenin antibody (1:100 dilution), anti-pGSK3β antibody (1:100 dilution) or anti-c-Myc antibody (1:100 dilution). In negative control, sections were incubated with N-universal negative control antibody under identical conditions. Quantification of nuclear staining was done by counting brown-positive cells and total number of cells at ten randomly selected fields at 400X magnification. Cytoplasmic staining was quantified by immunoreactivity (represented by intensity of brown staining) and scored as 0 (no staining), +1 (very weak staining), +2 (weak staining), +3 (moderate staining), and +4 (strong staining).

TUNEL Staining for Apoptotic Cells

Apoptotic cells in xenografts were identified by DeadEnd Colorimetric TUNEL system as per vendor's protocol.

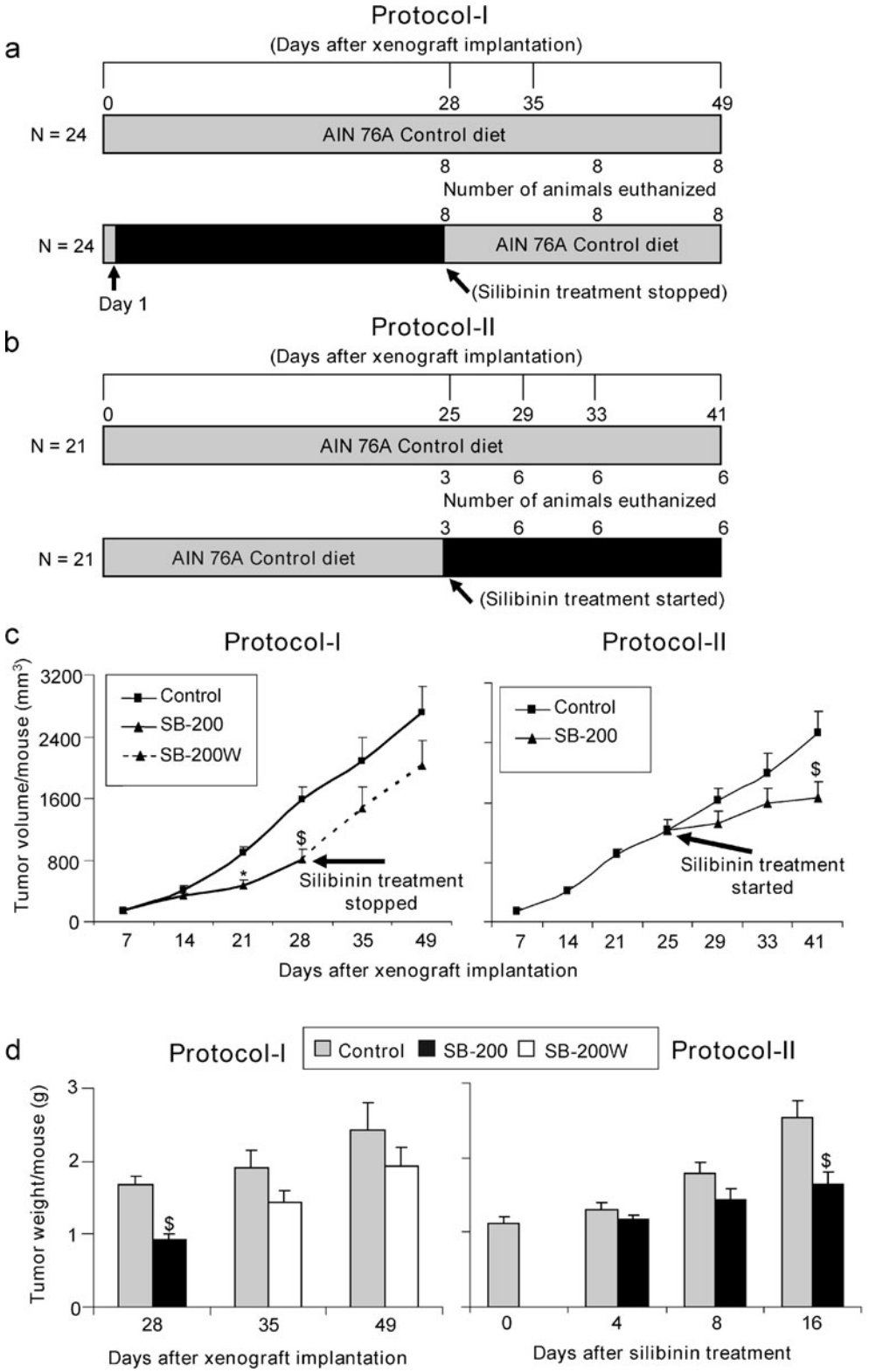
Statistical and Microscopic Analyses

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, and $p\leq0.05$ was considered statistically significant. All microscopic analyses of stained sections were performed using Zeiss Axioscope 2 microscope (Carl Zeiss Inc., Jena, Germany). Photographs were captured with Carl Zeiss AxioCam MrC5 camera at 400X magnification and processed using the AxioVision Rel 4.5 software (Carl Zeiss Inc., Jena, Germany).

RESULTS

Silibinin Inhibits Human CRC SW480 Xenograft Growth

In Protocol-I, effect of silibinin on growing tumors and outcome of its withdrawal on tumor growth was examined (Fig. 1c; Protocol-I). In this protocol, silibinin treatment (200 mg/kg body weight in 0.5% CMC, 5 days/week) was started 1 day after xenograft implantation and continued for the next 28 days, which resulted in a strong decrease in tumor volume (49.1%; $p\leq0.001$) after 28 days (Fig. 1c; Protocol-I). This experimental design also revealed that even after 7 and 21 days of silibinin withdrawal, a decrease in tumor volume sustains by 29.8 and 25.8%, respectively, compared to respective controls (Fig. 1c; Protocol-I). Similarly, tumor weight also decreased by 45% ($p\leq0.001$) after 28 days of silibinin treatment, and by 25 and 21% in mice sacrificed 7


a
Protocol-I
(Days after xenograft implantation)
0
28
35
49
N = 24
AIN 76A Control diet
8
8
8
Number of animals euthanized
8
8
8
N = 24
AIN 76A Control diet
Day 1
(Silibinin treatment stopped)
b
Protocol-II
(Days after xenograft implantation)
0
25
29
33
41
N = 21
AIN 76A Control diet
3
6
6
6
Number of animals euthanized
3
6
6
6
N = 21
AIN 76A Control diet
(Silibinin treatment started)
c
Protocol-I
Protocol-II
Tumor volume/mouse (mm3)
3200
2400
1600
800
0
Control
SB-200
SB-200W
$
*
Silibinin treatment stopped
Silibinin treatment started
7
14
21
28
35
49
25
29
33
41
Days after xenograft implantation
d
Protocol-I
Control
SB-200
SB-200W
Protocol-II
Tumor weight/mouse (g)
3
2
1
0
$
28
35
49
Days after xenograft implantation
0
4
8
16
Days after silibinin treatment

◀ **Fig. 1** Silibinin inhibits human CRC SW480 xenograft growth. (**a** & **b**) Experimental designs for the xenograft experiments. (**c**) Male athymic nude mice were subcutaneously injected with 5×10^6 SW480 cells mixed with matrigel. In Protocol-I, 1 day after xenograft implantation, the mice were administered vehicle control, *i.e.* 0.2 ml of 0.5% (w/v) CMC/day or silibinin (200 mg/kg/day in 0.2 ml of 0.5% CMC 5 days/week), and treatment continued for next 28 days. Silibinin treatment was stopped after 28 days, and eight mice from each group were euthanized after 0, 7 and 21 days. In Protocol-II, xenograft was allowed to grow for 25 days, three mice were euthanized, and remaining mice were randomly divided into two groups; vehicle control group mice were administered 0.2 ml of 0.5% (w/v) CMC/day, while in other group mice were administered 200 mg/kg/day dose of silibinin in 0.2 ml of 0.5% CMC 5 days/week. Six mice from each group were euthanized after 4, 8 and 16 days of silibinin treatment. Tumor growth was monitored and presented as tumor volume per mouse (mm^3) as a function of time from each group. (**d**) In both the treatment protocols, at the termination of the study, the mean tumor weight from each group was calculated and presented as tumor weight/mouse. In each case, data are presented as mean ± SEM. SB-200: Silibinin 200 mg/kg body weight in 0.2 ml of 0.5% CMC, 5 days/week; SB-200 W: Silibinin treatment withdrawn; *; $p \leq 0.05$, $; $p \leq 0.001$.

and 21 days, respectively, after silibinin withdrawal (Fig. 1d; Protocol-I). In Protocol-II, therapeutic potential of silibinin was evaluated on established SW480 xenograft by starting its treatment in animals 25 days post-xenograft implantation, a time when average tumor volume was above 1,000 mm^3 (Fig. 1c; Protocol II). Mice were sacrificed 0, 4, 8 and 16 days post-silibinin feeding, and as shown in Fig. 1c (Protocol II), tumor volume decreased with silibinin treatment at all the studied time-points as compared to respective controls; however, it was statistically significant only after 16 days of silibinin treatment (34%; $p \leq 0.001$). Consistent with this, we observed a similar trend towards a decrease in tumor weight with silibinin only after 16 days of its post-treatment (35.5%; $p \leq 0.001$) (Fig. 1d; protocol II).

In both the protocols, administration of silibinin by oral gavage did not cause any change in diet consumption and body weight gain in all groups during experimental period (data not shown). Furthermore, at necropsy, gross examination did not show any pathological alterations in vital organs, including liver, lung, heart and kidney.

Overall, these results suggested the strong and sustained therapeutic efficacy of silibinin against CRC. Further, to understand the underlying mechanism(s) responsible for the tumor growth inhibition after silibinin treatment, we analyzed the tumor tissues for various biomarkers and signaling molecules as described next.

Silibinin Inhibits CRC SW480 Xenograft Growth Through Anti-proliferative, Pro-apoptotic and Anti-angiogenic Effects

First, we analyzed xenograft tissues for silibinin's effect on established biomarkers for proliferation (PCNA), apoptosis (TUNEL) and angiogenesis (CD-31) by IHC. PCNA is a non-histone nuclear protein that functions as a co-factor for DNA polymerase delta, and its level of synthesis directly correlates with the rates of cellular proliferation and DNA synthesis (27). Quantification of PCNA staining in the xenografts from first protocol showed 37% ($p \leq 0.001$) decrease in proliferation index 28 days after silibinin treatment (Fig. 2a; Protocol-I). Even after silibinin withdrawal, there was 21% ($p \leq 0.01$) and 12% (statistically non-significant) decrease in PCNA positive cells at 7^{th} and 21^{st} days, respectively (Fig. 2a; Protocol-I). In Protocol-II, in established tumors, silibinin treatment for 8 and 16 days decreased the number of PCNA positive cells by 16% ($p \leq 0.01$) and 33.4% ($p \leq 0.001$), respectively, but did not significantly affect the proliferation index after 4 days of its treatment (Fig. 2a; Protocol-II).

Accumulating evidence demonstrates that natural agents offer great potential in the fight against cancer by inhibiting the carcinogenesis process through induction of apoptosis (11,28). In apoptosis analysis, we observed that silibinin treatment for 28 days increases the apoptotic cell population by 1.6-fold ($p \leq 0.001$) (Fig. 2b; Protocol-I). Further, a significant difference in the apoptotic cell population between control and silibinin-treated groups at 7^{th} day after treatment withdrawal was also observed ($p \leq 0.01$), but there was no significant effect at the 21^{st} day after the withdrawal of silibinin treatment (Fig. 2b; Protocol-I). In established tumors, silibinin treatment for 4 days increased the apoptotic cell death, but this increase did not achieve statistical significance. However, silibinin treatment for 8 and 16 days significantly increased the apoptotic cell population by 2.2- ($p \leq 0.001$) and 2.3-fold ($p \leq 0.001$), respectively (Fig. 2b; Protocol-II).

Next, we analyzed the xenograft tissue for CD31 staining, which is an established biomarker for microvessel density (10,14). In Protocol-I, IHC analyses of tumor tissues showed that silibinin treatment for 28 days decreases microvessel density by 43% ($p \leq 0.001$) (Fig. 2c; Protocol-I). Further, there was a significant difference in the microvessel density at 7^{th} day after treatment withdrawal ($p \leq 0.01$), but the difference in CD31 staining was statistically non-significant at the 21^{st} day after silibinin withdrawal (Fig. 2c; Protocol-I). In Protocol-II, silibinin only slightly decreased the microvessel density after 4 days of the treatment, but its effect on decreasing tumor vasculature was significant after 8 days (22%; $p \leq 0.05$) and 16 days (31%, $p \leq 0.001$) of treatment (Fig. 2c; Protocol-II).

Overall, these results showed strong *in vivo* anti-proliferative, pro-apoptotic and anti-angiogenic potential of silibinin, supporting its strong therapeutic efficacy against SW480 xenograft tumor growth (shown in Fig. 1c and d).

Silibinin Decreases Expression of β-catenin and Phosphorylation of its Upstream Regulator GSK-3β in SW480 Xenograft

As mentioned earlier, aberrant activation of β-catenin pathway due to loss of *APC* function is considered one of

the most important events in colon cancer growth and progression to an advanced untreatable stage (24–26). We, therefore, next analyzed the xenograft tissues for the effect of silibinin on β-catenin expression. Quantification of IHC staining in Protocol-I showed that silibinin treatment for 28 days decreases β-catenin expression by 42% ($p \leq 0.001$) (Fig. 3a; Protocol-I). Even at the 7th day after the withdrawal of silibinin treatment, a 42% ($p \leq 0.001$) reduction in β-catenin expression was observed; however, the decrease in β-catenin expression was not statistically

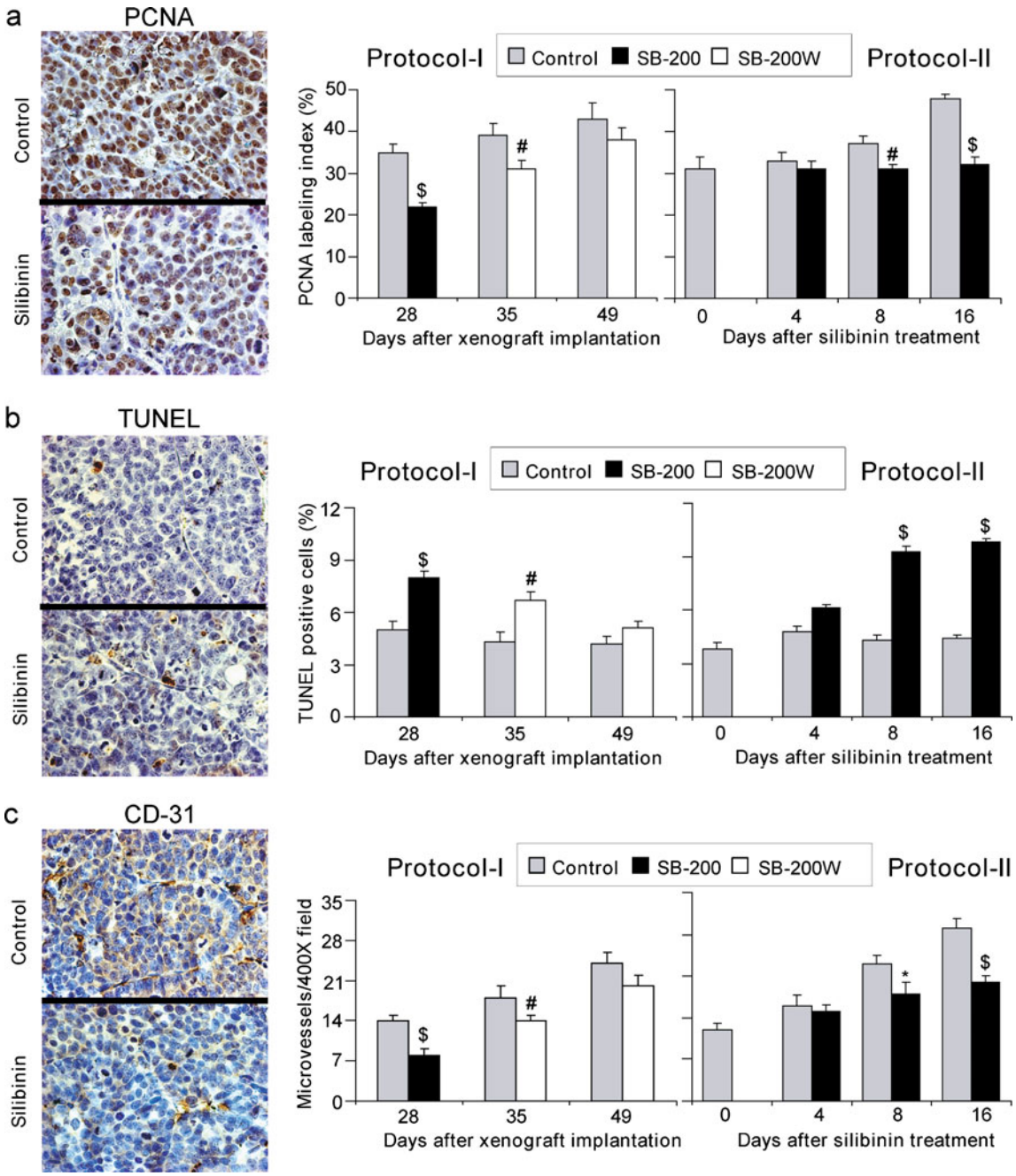


Fig. 2 *In vivo* anti-proliferative, pro-apoptotic and anti-angiogenic effects of silibinin in CRC SW480 xenografts in athymic nude mice. At the end of the study in each treatment protocol, tumor tissues were collected, processed and analyzed for immunohistochemistry staining of (**a**) PCNA, (**b**) TUNEL and (**c**) CD31 following the procedures detailed in "Materials and Methods." All representative images are from silibinin treatment (5 days/week) for 28 days (Protocol-I) tissues and were captured at 400X magnification. Percentage of PCNA and TUNEL positive cells were calculated as number of positive cells x 100/total number of cells counted at 400X magnification in ten randomly selected areas in each tumor sample. For microvessel density, CD31 positive microvessels were counted at 400X magnification in ten randomly selected areas in each tumor sample. Data shown in the bar diagrams represent mean ± SEM value from all the samples in each group. SB-200: SB 200 mg/kg body weight in 0.2 ml of 0.5% CMC, 5 days/week; SB-200 W: Silibinin treatment withdrawn; *; $p \leq 0.05$, #; $p \leq 0.01$, $; $p \leq 0.001$.

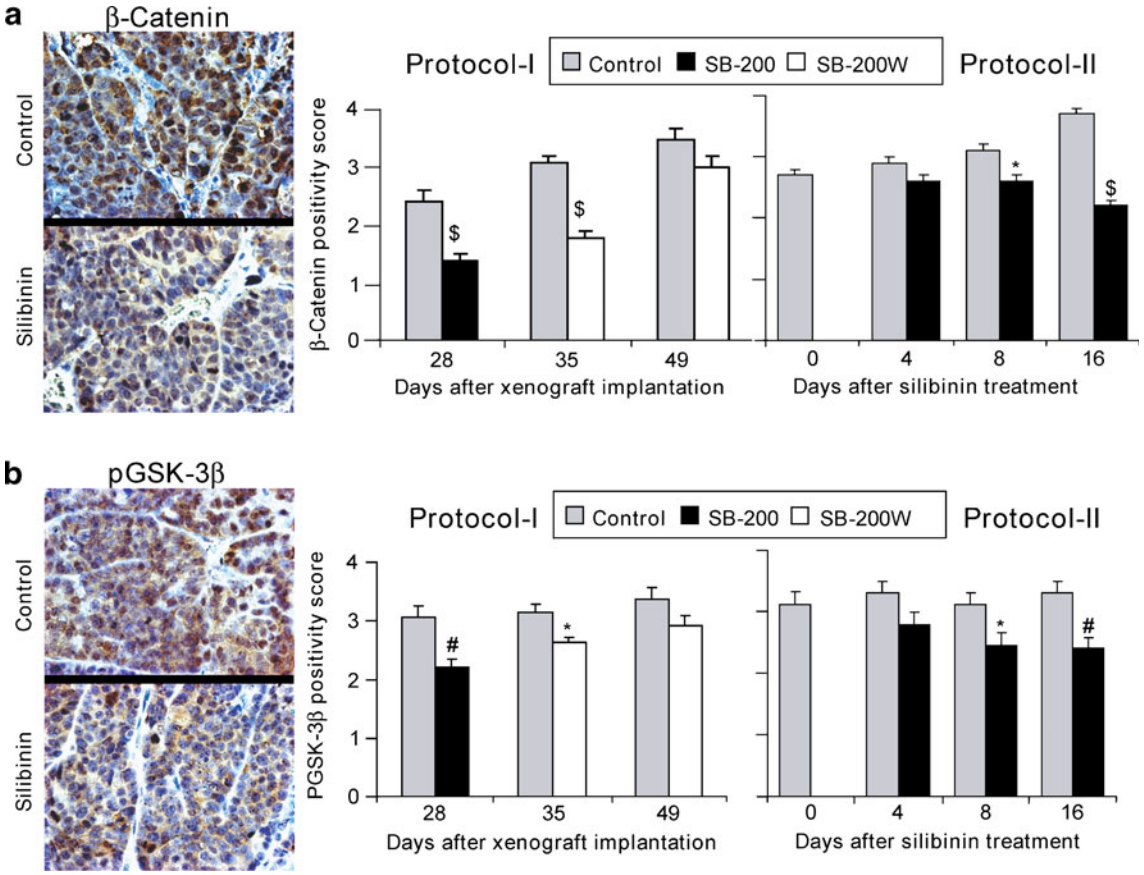


Fig. 3 Silibinin decreases β-catenin and pGSK-3β expression in CRC SW480 xenografts. At the end of the study in each treatment protocol, tumor tissues were collected, processed and analyzed for immunohistochemistry staining of (**a**) β-catenin and (**b**) pGSK-3β following the procedures detailed in "Materials and Methods." All representative images are from 28 days (Protocol-I) tissues and were captured at 400X magnification. β-catenin or pGSK-3β immunoreactivity (represented by brown staining) was analyzed in ten random areas for each tumor tissue and was scored as 0 (no staining), +1 (very weak staining), +2 (weak staining), +3 (moderate staining), and +4 (strong staining). Data shown in the bar diagrams represent mean ± SEM value from all the samples in each group. SB-200: Silibinin 200 mg/kg body weight in 0.2 ml of 0.5% CMC, 5 days/week; SB-200 W: Silibinin treatment withdrawn; *; $p \leq 0.05$, #; $p \leq 0.01$, $; $p \leq 0.001$.

significant at 21st day after silibinin withdrawal (Fig. 3a; Protocol-I). In Protocol-II, in established tumors, silibinin treatment for 4 days resulted in only a small and statistically non-significant decrease in β-catenin expression, whereas 8 days and 16 days of silibinin treatment caused 16% ($p \leq 0.05$) and 41% ($p \leq 0.001$) decrease in β-catenin expression, respectively (Fig. 3a; Protocol-II).

Next, we analyzed the xenograft tissues for the level of phosphorylated GSK-3β, which is a critical molecule in β-catenin phosphorylation and subsequent degradation (24,25). Quantification of pGSK-3β (*i.e.* its inactive state) IHC staining showed that silibinin treatment for 28 days decreases its expression by 27% ($p \leq 0.01$) (Fig. 3b; Protocol-I). Even at 7th day after the withdrawal of silibinin treatment, there was a 16% ($p \leq 0.05$) reduction in pGSK-3β expression; however, the observed decrease in pGSK-3β expression was not statistically significant after 21 days (Fig. 3a; Protocol-I). In Protocol-II, silibinin treatment for 8 days and 16 days caused 23% ($p \leq 0.05$) and 41% ($p \leq 0.01$) decrease, respectively, in pGSK-3β expression (Fig. 3b; Protocol-II), while silibinin effect on pGSK-3β expression after 4 days of treatment was statistically non-significant.

Together, these results clearly demonstrated the strong effect of silibinin in decreasing β-catenin expression in CRC cells *in vivo*, which could be related to silibinin-mediated activation of GSK-3β (as we observed a decrease in the inactive state of this molecule). Based upon this observation, next we analyzed the xenograft tissue for various β-catenin regulated molecules that are known to control CRC growth, survival and angiogenesis (29–33).

Silibinin Decreases the Expression of Signaling Molecules Critical for Growth and Survival in SW480 Xenografts

First, we analyzed the xenograft tissues for the expression of cyclin D1, c-Myc and survivin; known downstream target

genes of β-catenin (29,32,34). These three molecules are critical for the growth and survival of cancer cells (24,25,29,32,34–37).

Cyclin D1 is an important regulator of cell cycle progression and is a known biomarker for cell proliferation (38,39). Analyses of xenograft tissues from Protocol-I showed 35% ($p \leq 0.01$) decrease in cyclin D1-positive cells after 28 days of silibinin treatment (Fig. 4a; Protocol-I). Even at the 7th day after the withdrawal of silibinin treatment, there was a 14% ($p \leq 0.05$) decrease in cyclin D1-positive cells; however, the decrease in cyclin D1-positive cells was not statistically significant after 21 days (Fig. 4a; Protocol-I). In Protocol-II, in established tumors, silibinin treatment for 4 days resulted in only a small and statistically non-significant decrease in the number of cyclin D1-positive cells, but 8 and 16 days of silibinin treatment caused 17% ($p \leq 0.01$) and 30% ($p \leq 0.001$) decrease, respectively, in cyclin D1-positive cells (Fig. 4a; Protocol-II).

C-Myc is a transcriptional factor that plays a critical role in cell growth and is known to be over-expressed in several types of cancers, including CRC (29,36). Quantification of IHC staining for c-Myc showed that silibinin treatment for 28 days decreases its expression by 37% ($p \leq 0.001$) (Fig. 4b; Protocol-I). Even at the 7th day after the withdrawal of silibinin treatment, there was a 14% ($p \leq 0.05$) reduction in c-Myc expression; however, the decrease was insignificant at the 21st day after the withdrawal of silibinin treatment (Fig. 4b; Protocol-I). In Protocol-II, in established tumors, silibinin treatment for 8 days and 16 days caused 28% ($p \leq 0.05$) and 36% ($p \leq 0.001$) decrease, respectively, in c-Myc expression (Fig. 4b; Protocol-II), while silibinin effect on c-Myc after 4 days of treatment was insignificant.

Survivin is a member of inhibitors of apoptosis (IAP) gene family and is highly expressed in CRC cells (32,34,37,40). Tissue analyses showed that silibinin treatment for 28 days decreases survivin expression by 25% ($p \leq 0.001$) (Fig. 4c; Protocol-I). Even at the 7th day after the withdrawal of silibinin treatment, there was a significant decrease in survivin expression ($p \leq 0.05$); however, the decrease in survivin expression was not statistically significant after 21 days (Fig. 4c; Protocol-I). In Protocol-II, in established tumors, silibinin treatment for 8 days and 16 days caused 24% ($p \leq 0.05$) and 38% ($p \leq 0.001$) decrease, respectively, in survivin expression (Fig. 4c; Protocol-II), while silibinin effect on survivin expression after 4 days of treatment was insignificant.

Silibinin Decreases the Expression of Signaling Molecules Critical for Tumor Angiogenesis in SW480 Xenografts

Tumor cells produce and secrete various pro-angiogenic factors that are essential for neo-vascularization and subsequent growth and progression (41–43). In the present study, we observed strong anti-angiogenic effect of silibinin (Fig. 2c); therefore, next we analyzed the silibinin effect on the expression of two important angiogenesis regulators namely VEGF and iNOS.

VEGF is an important signaling protein involved in both vasculogenesis (*de novo* formation of blood vessels from endothelial progenitor cells) and angiogenesis (the growth of blood vessels from pre-existing vasculature) (41–43); advanced CRC has been shown to over-express this molecule (44,45). As shown in Fig. 5a, Protocol-I, silibinin treatment for 28 days decreased VEGF expression by 24% ($p \leq 0.001$). Even at the 7th and 21st days after the withdrawal of silibinin treatment, there was 21% ($p \leq 0.001$) and 12% ($p \leq 0.01$) reduction in VEGF expression, respectively (Fig. 5a; Protocol-I). In the second protocol, in established tumors, 8 days and 16 days of silibinin treatment caused 26% ($p \leq 0.01$) and 32% ($p \leq 0.001$) decrease, respectively, in VEGF expression (Fig. 5a; Protocol-II).

iNOS is another important regulator of tumor angiogenesis in various malignancies including CRC (46,47); therefore, we next studied the effect of silibinin treatment on iNOS expression in the xenograft tissues. The IHC analysis of tumor tissues showed that silibinin treatment for 28 days decreases iNOS expression by 32% ($p \leq 0.01$) (Fig. 5b; Protocol-I). The decrease in iNOS expression at the 7th and 21st days after the withdrawal of silibinin treatment was insignificant (Fig. 5b; Protocol-I). In Protocol-II, in established tumors, silibinin treatment for 16 days caused 43% ($p \leq 0.001$) decrease in iNOS expression (Fig. 5b; Protocol-II), while silibinin effect on iNOS expression after 4 and 8 days of treatment was insignificant.

DISCUSSION

Despite recent advances in chemotherapeutic regimens, CRC remains one of the leading causes of cancer-related death in the United States (1). Moreover, most of the synthetic chemotherapeutic agents are cytotoxic and immuno-suppressive and cause a variety of undesired side effects to the normal organs of the body, and in most cases the outcome of therapy is worse than disease itself (2–4). Prevention and intervention using non-toxic phytochemicals is emerging as an effective and practically applicable strategy to control the incidence of CRC. The process of development of CRC has a natural history of transition from a precursor lesion, *i.e.* adenomatous polyp, to invasive adenocarcinoma which spans several years, providing an extended opportunity for intervention and cancer prevention (48). Lately, several chemopreventive agents, mostly those isolated from natural sources, have been evaluated for their anti-cancer efficacy using a variety of biological/pre-

clinical assays (5–9). Silibinin, the primary active constituent present in milk thistle seed extract, is widely used to protect liver from drug or alcohol-related injuries (7). In addition to the reports on its hepato-protective effects, preclinical studies have confirmed the chemopreventive efficacy of silibinin against various epithelial malignancies, and its efficacy is currently being evaluated at least in prostate cancer patients (10–12,18,19,49,50). It has been reported to

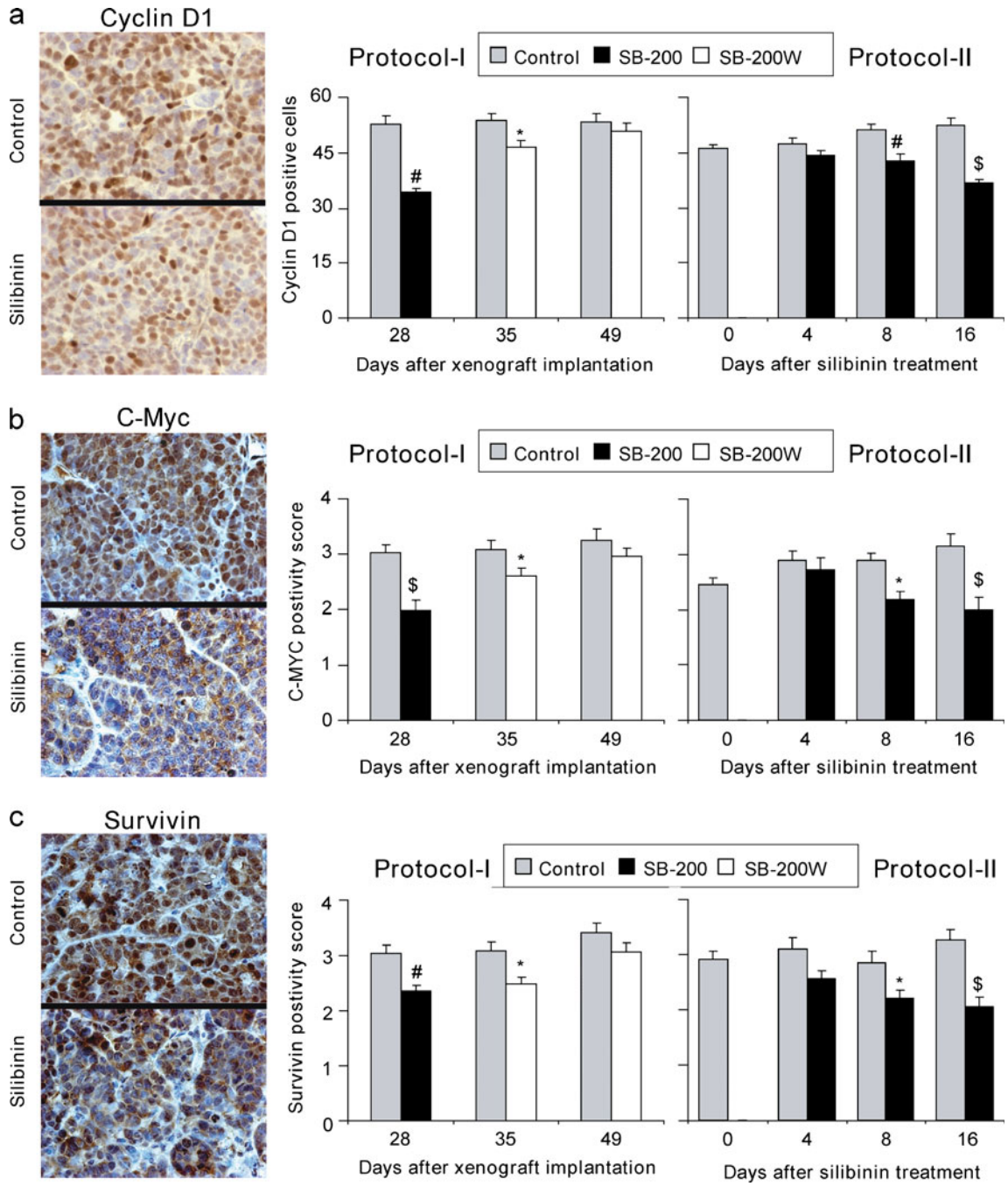


Fig. 4 Silibinin decreases the expression of signaling molecules critical for growth and survival in CRC SW480 xenografts. At the end of the study in each treatment protocol, tumor tissues were collected, processed and analyzed for immunohistochemistry staining of (**a**) cyclin D1, (**b**) c-Myc and (**c**) survivin following the procedures detailed in "Materials and Methods." The representative images (magnification of 400X) are from stained tissues of mice sacrificed at 28 days (Protocol-I). Percentage cyclin D1 positive cells were calculated as number of cyclin D1 positive cells x 100/total number of cells counted at 400X magnification in ten randomly selected areas in each tumor sample. C-Myc or survivin immunoreactivity (represented by brown staining) was analyzed in ten random areas for each tumor tissue and was scored as 0 (no staining), +1 (very weak staining), +2 (weak staining), +3 (moderate staining), and +4 (strong staining). Data shown in the bar diagrams represent mean ± SEM value from all the samples in each group. SB-200: Silibinin 200 mg/kg body weight in 0.2 ml of 0.5% CMC, 5 days/week; SB-200 W: Silibinin treatment withdrawn; *; $p \leq 0.05$, #; $p \leq 0.01$, $; $p \leq 0.001$.

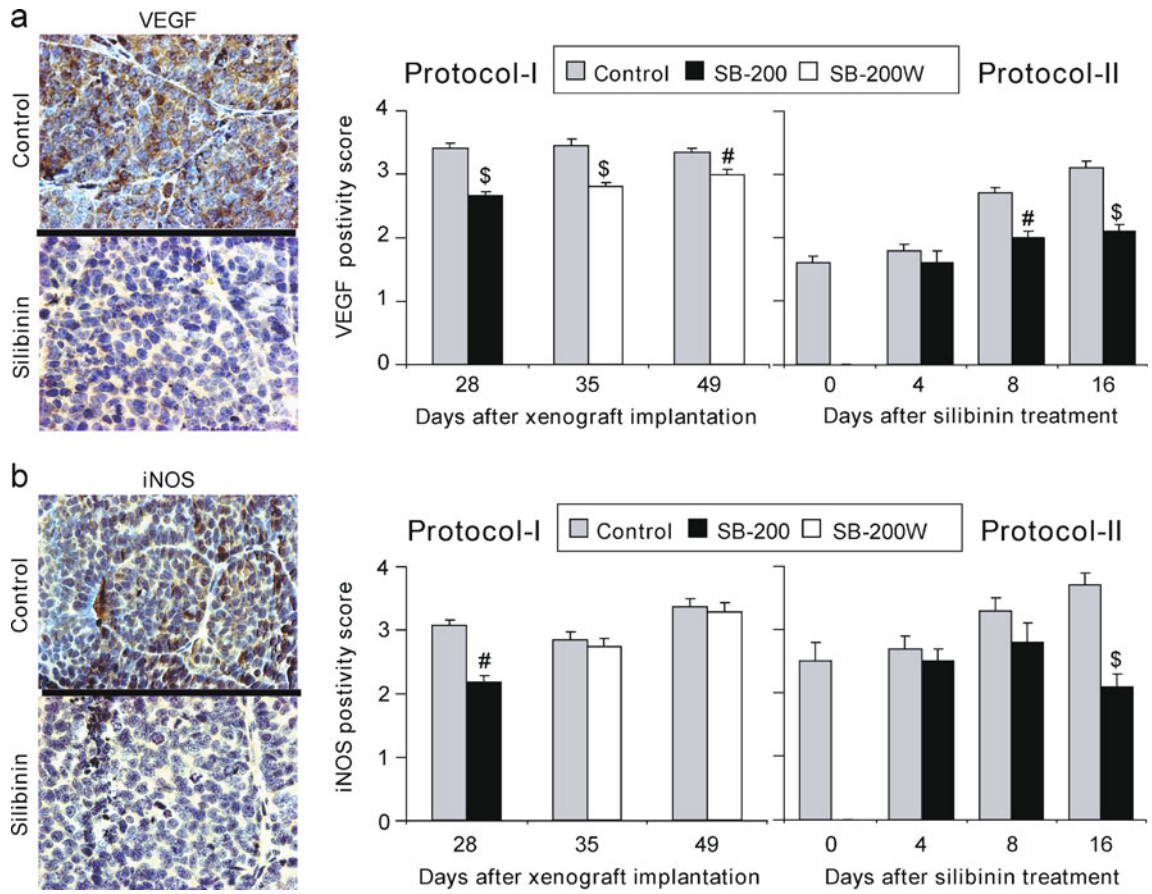


Fig. 5 Silibinin decreases the expression of important angiogenesis regulators in CRC SW480 xenografts. At the end of the study in each treatment protocol, tumor tissues were collected, processed and analyzed for immunohistochemistry staining of (**a**) VEGF and (**b**) iNOS following the procedures detailed in "Materials and Methods." The representative images (magnification of 400X) are from stained tissues of mice sacrificed at 28 days (Protocol-I). VEGF or iNOS immunoreactivity (represented by brown staining) was analyzed in ten random areas for each tumor tissue and was scored as 0 (no staining), +1 (very weak staining), +2 (weak staining), +3 (moderate staining), and +4 (strong staining). Data shown in the bar diagrams represent mean ± SEM value from all the samples in each group. SB-200: Silibinin 200 mg/kg body weight in 0.2 ml of 0.5% CMC, 5 days/week; SB-200 W: Silibinin treatment withdrawn; #; $p \leq 0.01$, $; $p \leq 0.001$.

be safe, well-tolerated, and readily available when administered orally. The bioavailability of silibinin has been documented both under pre-clinical as well as clinical settings. In a pre-clinical model, we showed that silibinin concentration of ≥150 μM could be achieved in the plasma of mice without any apparent toxicity (15). In colorectal cancer patients, 20–141 n mol/g of silibinin could be detected in the colorectal tissue when patients were given 360–1,440 mg/d silipide, a formulation of silibinin, for 7 days (50). In another clinical study involving patients with localized prostate cancer, the patients were given 13 g of silybin-phytosome daily for 14–31 days (mean study period of 20 days) prior to surgery (51). In this study, silibinin levels up to 19.7 μM were detected in blood at 1 hr after the first silybin-phytosome dose. Collectively, these studies suggest that significant bioavailability of silibinin can be achieved in plasma as well as colorectal tissue.

The main focus of the present study was to investigate the therapeutic efficacy of silibinin against advanced CRC growth using SW480 cells xenograft as a model. Briefly, the major findings from the present study are as follows. (a) Feeding of silibinin either immediately after xenograft implantation or after the establishment of xenograft suppressed the tumor growth without any apparent toxicity. More interestingly, even after withdrawal of silibinin treatment, the tumor growth in the silibinin-treated group remains decreased, suggesting the sustained therapeutic effects of silibinin against CRC. (b) The tumor inhibitory efficacy of silibinin was associated with its strong anti-proliferative, pro-apoptotic and anti-angiogenic effects. (c) In mechanistic studies, silibinin was observed to exert suppressive effects on β-catenin and its upstream signaling component pGSK3β expression. Importantly, silibinin also targeted various β-catenin-regulated signaling molecules responsible for CRC cells growth (c-Myc and cyclin D1), survival (survivin) and angiogenesis (VEGF and iNOS).

The predominant event and the most characterized mechanism in CRC development is the disruption of a

functional APC complex due to a mutation of APC or β-catenin gene leading to aberrant activation of the Wnt signaling pathway (22,23,25,26). β-Catenin, which was originally discovered as a cadherin-binding protein, has been proved to function as a co-transcriptional activator when complexed with members of the TCF family of DNA binding proteins (24,26). Nuclear accumulation of β-catenin results in the induction of a variety of oncogenic target genes which promote cell proliferation, survival and angiogenesis (29–32). Wnt signaling facilitates phosphorylation-mediated inactivation of GSK3β, thereby contributing to the increased cytoplasmic and nuclear accumulation of β-catenin and consequent activation of its target genes (20,33). In the present study, we used human CRC SW480 cells that are known to over-express β-catenin (26), and silibinin in both treatment protocols significantly decreased the expression of β-catenin consistent with a decrease in the inactive state (pGSK-3β) of upstream regulator GSK-3β. Further, silibinin treatment decreased the expression of key molecules regulated by β-catenin, namely cyclin D1, c-Myc and survivin. In view of the known importance of these molecules in CRC growth and progression (29,37,40), we believe that the modulation of β-catenin signaling might be an important mechanism responsible for the observed anti-proliferative and pro-apoptotic effect of silibinin as well as for its overall observed sustained therapeutic efficacy against CRC. These results are also supported by our recent publication wherein we reported the important role of β-catenin in the preventive efficacy of silibinin in APC $^{min/+}$ mouse model (17). However, more studies are required in the future to provide credence to the mentioned important role of β-catenin in silibinin's therapeutic efficacy.

Another possible mechanism for silibinin's therapeutic efficacy could be related to its strong anti-angiogenic potential. Neo-angiogenesis is one of the vital processes in the growth and progression of tumor (41). To grow beyond 1 to 2 mm in diameter, a tumor needs an independent blood supply, which is acquired by expressing growth factors such as VEGF that helps in recruiting new vasculature from existing nearby blood vessels (41–43). Currently, targeting tumor angiogenesis is considered the most important strategy against cancer, and various angiogenesis inhibitors have already been approved for clinical use against various cancers, including CRC (41). In the present study, silibinin, in both the protocols, significantly reduced the tumor microvessel density, confirming its strong anti-angiogenic efficacy against CRC. Correspondingly, silibinin treatment also decreased the expression of important angiogenesis regulators, namely VEGF and iNOS, in the xenograft tissues. As both VEGF and iNOS are regulated by β-catenin (30,31), the observed decrease in VEGF and iNOS could also be related to inhibitory effect of silibinin on β-catenin signaling. These results are further supported by our previous findings, wherein silibinin exerted similar strong anti-angiogenic effects in a variety of cancer models (11,12,14,17).

In conclusion, our study demonstrated the sustained *in vivo* therapeutic efficacy of silibinin against human CRC

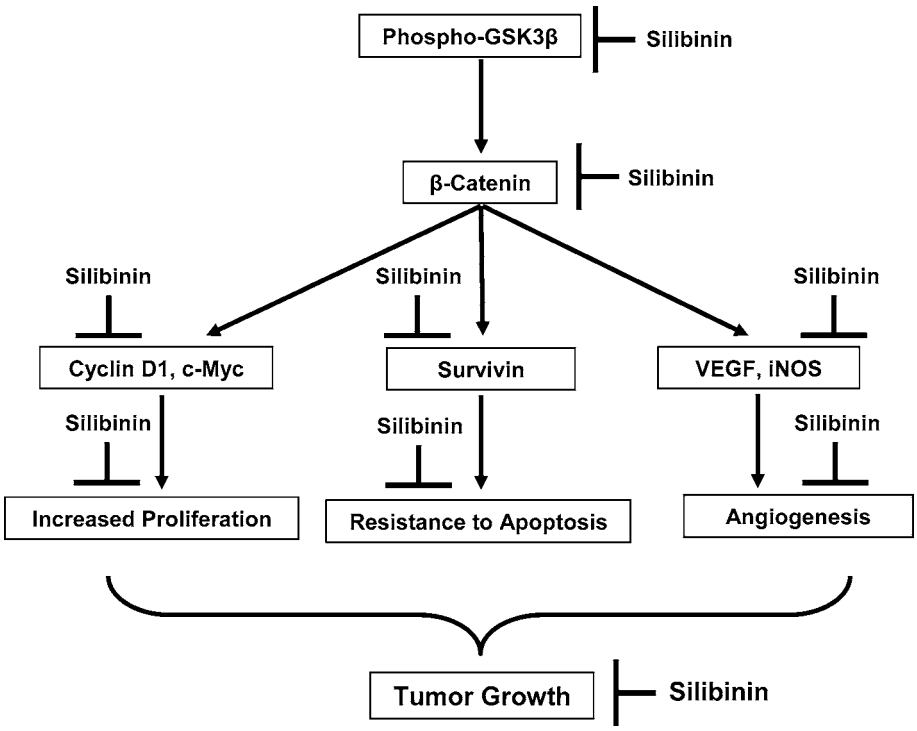


Fig. 6 Proposed mechanisms for silibinin's growth-suppressive effect against human colon carcinoma SW480 xenograft. Silibinin modulates the expression of various signaling molecules involved in the regulation of proliferation, apoptosis and angiogenesis.

SW480 xenografts without causing any adverse effects or toxicity. The observed tumor inhibitory efficacy of silibinin was associated with its anti-proliferative, pro-apoptotic and anti-angiogenic effects as well as through inhibitory effect of silibinin on β-catenin and its downstream target signaling molecules, which are known to be responsible for CRC growth, survival and angiogenesis (Fig. 6). Overall, the findings of the present investigation add to the mechanistic evidence for silibinin efficacy against advanced CRC and rationally recommend its translational utilization in controlling this deadly malignancy.

ACKNOWLEDGEMENTS

This work was supported by NCI RO1 grant CA112304.

REFERENCES

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin. 2009;59:225–49.
2. Diwanay S, Chitre D, Patwardhan B. Immunoprotection by botanical drugs in cancer chemotherapy. J Ethnopharmacol. 2004;90:49–55.
3. Boursi B, Arber N. Current and future clinical strategies in colon cancer prevention and the emerging role of chemoprevention. Curr Pharm Des. 2007;13:2274–82.
4. Lee BM, Park KK. Beneficial and adverse effects of chemopreventive agents. Mutat Res. 2003;523–524:265–78.
5. Tanaka T, Kohno H, Mori H. Chemoprevention of colon carcinogenesis by dietary non-nutritive compounds. Asian Pac J Cancer Prev. 2001;2:165–77.
6. Rajamanickam S, Agarwal R. Natural products and colon cancer: current status and future prospects. Drug Dev Res. 2008;69:460–71.
7. Deep G, Agarwal R. Chemopreventive efficacy of silymarin in skin and prostate cancer. Integr Cancer Ther. 2007;6:130–45.
8. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer. 2003;3:768–80.
9. Wu H, Dai Q, Shrubsole MJ, Ness RM, Schlundt D, Smalley WE, *et al.* Fruit and vegetable intakes are associated with lower risk of colorectal adenomas. J Nutr. 2009;139:340–4.
10. Singh RP, Gu M, Agarwal R. Silibinin inhibits colorectal cancer growth by inhibiting tumor cell proliferation and angiogenesis. Cancer Res. 2008;68:2043–50.
11. Kaur M, Velmurugan B, Tyagi A, Deep G, Katiyar S, Agarwal C, *et al.* Silibinin suppresses growth and induces apoptotic death of human colorectal carcinoma LoVo cells in culture and tumor xenograft. Mol Cancer Ther. 2009;8:2366–74.
12. Singh RP, Raina K, Sharma G, Agarwal R. Silibinin inhibits established prostate tumor growth, progression, invasion, and metastasis and suppresses tumor angiogenesis and epithelial-mesenchymal transition in transgenic adenocarcinoma of the mouse prostate model mice. Clin Cancer Res. 2008;14:7773–80.
13. Verschoyle RD, Greaves P, Patel K, Marsden DA, Brown K, Steward WP, *et al.* Evaluation of the cancer chemopreventive efficacy of silibinin in genetic mouse models of prostate and intestinal carcinogenesis: relationship with silibinin levels. Eur J Cancer. 2008;44:898–906.
14. Singh RP, Deep G, Chittezhath M, Kaur M, Dwyer-Nield LD, Malkinson AM, *et al.* Effect of silibinin on the growth and progression of primary lung tumors in mice. J Natl Cancer Inst. 2006;98:846–55.
15. Agarwal C, Singh RP, Dhanalakshmi S, Tyagi AK, Tecklenburg M, Sclafani RA, *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.
16. 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.
17. Rajamanickam S, Kaur M, Velmurugan B, Singh RP, Agarwal R. Silibinin suppresses spontaneous tumorigenesis in APC min/+ mouse model by modulating beta-catenin pathway. Pharm Res. 2009;26:2558–67.
18. 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.
19. Sangeetha N, Felix AJ, Nalini N. Silibinin modulates biotransforming microbial enzymes and prevents 1, 2-dimethylhydrazine-induced preneoplastic changes in experimental colon cancer. Eur J Cancer Prev. 2009;18:385–94.
20. Herbst A, Kolligs FT. Wnt signaling as a therapeutic target for cancer. Methods Mol Biol. 2007;361:63–91.
21. Ahmed FE. Colon cancer: prevalence, screening, gene expression and mutation, and risk factors and assessment. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2003;21:65–131.
22. Yamada Y, Mori H. Multistep carcinogenesis of the colon in Apc (Min/+) mouse. Cancer Sci. 2007;98:6–10.
23. de Lau W, Barker N, Clevers H. WNT signaling in the normal intestine and colorectal cancer. Front Biosci. 2007;12:471–91.
24. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, *et al.* The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell. 2002;111:241–50.
25. Schneikert J, Behrens J. The canonical Wnt signalling pathway and its APC partner in colon cancer development. Gut. 2007;56:417–25.
26. Yang J, Zhang W, Evans PM, Chen X, He X, Liu C. Adenomatous polyposis coli (APC) differentially regulates beta-catenin phosphorylation and ubiquitination in colon cancer cells. J Biol Chem. 2006;281:17751–7.
27. Shimazaki N, Yazaki T, Kubota T, Sato A, Nakamura A, Kurei S, *et al.* DNA polymerase lambda directly binds to proliferating cell nuclear antigen through its confined C-terminal region. Genes Cells. 2005;10:705–15.
28. Prasad V, Chandele A, Jagtap JC, Sudheer Kumar P, Shastry P. ROS-triggered caspase 2 activation and feedback amplification loop in beta-carotene-induced apoptosis. Free Radic Biol Med. 2006;41:431–42.
29. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, *et al.* Identification of c-MYC as a target of the APC pathway. Science. 1998;281:1509–12.
30. Easwaran V, Lee SH, Inge L, Guo L, Goldbeck C, Garrett E, *et al.* beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. Cancer Res. 2003;63:3145–53.
31. Du Q, Park KS, Guo Z, He P, Nagashima M, Shao L, *et al.* Regulation of human nitric oxide synthase 2 expression by Wnt beta-catenin signaling. Cancer Res. 2006;66:7024–31.
32. Zhang T, Otevrel T, Gao Z, Ehrlich SM, Fields JZ, Boman BM. Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res. 2001;61:8664–7.
33. Huang H, He X. Wnt/beta-catenin signaling: new (and old) players and new insights. Curr Opin Cell Biol. 2008;20:119–25.

34. Liang QL, Wang BR, Li GH. DcR3 and survivin are highly expressed in colorectal carcinoma and closely correlated to its clinicopathologic parameters. J Zhejiang Univ Sci B. 2009;10:675–82.
35. Nusse R. Wnt signaling in disease and in development. Cell Res. 2005;15:28–32.
36. Schmidt EV. The role of c-myc in cellular growth control. Oncogene. 1999;18:2988–96.
37. Gianani R, Jarboe E, Orlicky D, Frost M, Bobak J, Lehner R, *et al.* Expression of survivin in normal, hyperplastic, and neoplastic colonic mucosa. Hum Pathol. 2001;32:119–25.
38. Liao D, Johnson RS. Hypoxia: a key regulator of angiogenesis in cancer. Cancer Metastasis Rev. 2007;26:281–90.
39. Bemis DL, Katz AE, Buttyan R. Clinical trials of natural products as chemopreventive agents for prostate cancer. Expert Opin Investig Drugs. 2006;15:1191–200.
40. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res. 1998;58:5071–4.
41. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov. 2007;6:273–86.
42. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000;407:249–57.
43. Karamysheva AF. Mechanisms of angiogenesis. Biochemistry (Mosc). 2008;73:751–62.
44. Ellis LM, Takahashi Y, Liu W, Shaheen RM. Vascular endothelial growth factor in human colon cancer: biology and therapeutic implications. Oncologist. 2000;5 Suppl 1:11–5.
45. Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. J Clin Invest. 1995;95:1789–97.
46. Cullis ER, Kalber TL, Ashton SE, Cartwright JE, Griffiths JR, Ryan AJ, *et al.* Tumour overexpression of inducible nitric oxide synthase (iNOS) increases angiogenesis and may modulate the anti-tumour effects of the vascular disrupting agent ZD6126. Microvasc Res. 2006;71:76–84.
47. Cianchi F, Cortesini C, Fantappie O, Messerini L, Sardi I, Lasagna N, *et al.* Cyclooxygenase-2 activation mediates the proangiogenic effect of nitric oxide in colorectal cancer. Clin Cancer Res. 2004;10:2694–704.
48. Correa Lima MP, Gomes-da-Silva MH. Colorectal cancer: lifestyle and dietary factors. Nutr Hosp. 2005;20:235–41.
49. 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.
50. Hoh C, Boocock D, Marczylo T, Singh R, Berry DP, Dennison AR, *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.
51. Flaig TW, Glodé M, Gustafson D, van Bokhoven A, Tao Y, Wilson S, *et al.* A study of high-dose oral silybin-phytosome followed by prostatectomy in patients with localized prostate cancer. Prostate. 2010;70:848–55.