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Growth and Cell Cycle Regulation by Silibinin in Chang Liver Cells

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Abstract: Silibinin, the main component of silymarin, induces growth inhibition in several cancer cell types but is cytoprotective for normal hepatocytes. To ascertain whether silibinin suppresses hepatocyte proliferation, effects of this drug on viability, DNA synthesis, proliferation, the cell cycle and mitogen-activated protein kinase (MAPK) pathways were examined in cultured Chang liver cells. A biphasic growth response was observed. At low (1 and 10 μM) concentrations silibinin increased DNA synthesis, the percentage of cells entering S phase and rates of cell proliferation. At a higher (50 μM) concentration, the drug increased DNA synthesis and the percentage of cells entering S phase but no increase in cell proliferation was observed. Findings were consistent with stimulation of DNA synthesis via ERK pathways. Phosphorylations of ERKs were induced by silibinin in a dose-dependent manner and the ERK inhibitor PD98059 inhibited DNA synthesis in response to the drug. All observations reported here are therefore fully compatible with growth-regulatory action of silibinin on cultured Chang liver cells. However, the growth-regulating actions of silibinin are proposed to be highly dose-dependent, with low doses promoting and high doses suppressing growth.

Key words: Silibinin, MAPK pathways, S phase, chang liver cells

Introduction

Fruits, vegetables, herbs and plants are well-established to synthesize substances with chemopreventive effects (Park and Pezzuto, 2002). One such substance is silibinin, the main component of silymarin. Silymarin, isolated from the seeds of the milk thistle [*Silybum mrianum* (L.) Gaertn] (Mereish *et al*., 1991), is composed of silibinin (~80%, w/w, also termed silybin) and small amounts of stereoisomers such as isosilybin, dihyrosilybin, silydianin and silychristin (Wagner *et al*., 1974). The polyphenolic flavonoid skeleton of silibinin is similar to that of phytoestrogens. The growth-modulating effects of silymarin have been examined in various long-term tumorigenesis animal models as well as in cell culture systems (Singh *et al*., 2002, 2005; Bhatia *et al*., 1999; Varghese *et al*., 2005; Tyagi *et al*., 2002; Kohno *et al*., 2002). Silymarin is reported to inhibit growth, DNA synthesis and various mitogenic signals in skin, cervical, breast, prostate and colon carcinoma cells. The molecular mechanism whereby silibinin suppresses growth of prostate cancer cells has been examined in detail. In human prostate carcinoma cells this agent was found to induce G1 arrest and to suppress activation of mitogen-activated protein kinases (MAPKs, p42/p44 MAPK; also called extracellular signal–regulated kinases ERK1 and ERK2) (Zi and Agarwal, 1999; Sharma *et al*., 2001), to down-regulate TNFα-induced activation of NF-κB (Dhanalakshmi *et al*., 2002) and to up-regulate the expression of IGFBP-3 (Singh *et al*., 2002).

Silymarin and silibinin have been used clinically in Europe and Asia to treat hepatotoxicity and various other liver diseases for many years (Mereish *et al*., 1991; Salmi and Sarna, 1982; Ferenci *et al*.,

1982; Luper, 1998). In animal models, silymarin and silibinin have been shown to be nontoxic when administered in fairly large doses (Mereish *et al*., 1991; Singh *et al*., 2002; Zi *et al*., 2000). Many patients with liver diseases (31%) have used over-the-counter " alternative agents" to treat their disorders, with silymarin representing the most common of such agents (Flora *et al*., 1998). Several studies indicate that the effects of silymarin are due to its main constituent, silibinin (Bhatia *et al*., 1999; Tyagi *et al*., 2002; Dehmlow *et al*., 1996). At present, silymarin is sold as a dietary supplement and silibinin is used clinically as silipide, a lipophilic silibinin-phosphatidylcholine complex (Schandalik and Perucca, 1994).

Silibinin is reported to inhibit the growth of cancer cells. However, both silymarin and silibinin have been found clinically effective in the treatment of liver diseases and neither agent is currently indicated for treatment of cancer. Silibinin is selected for therapy of liver disease on the basis of its cytoprotective actions. These actions include reduction of cell membrane permeability (Munter *et al*., 1986), scavenging of reactive oxygen species (Mira *et al*., 1994), inhibition of leukotriene formation (Dehmlow *et al*., 1996) and suppression of DNA-binding activity of NF-κB and its consequent effects on gene expression (Saliou *et al*., 1998). However, the mechanism whereby silibinin exerts protective actions on liver cells remains to be established. The aim of the present study was to ascertain whether silibinin preserves the viability and favors the proliferation of Chang liver cells. Effects of silibinin on DNA synthesis, cell cycle distribution and activations of ERK1/2 were examined.

Materials and Methods

Materials

Silibinin, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,8-diamino-5(3-diethylaminopropyl)-6-phenylphenanthridium iodide (propidium iodide, PI), ribonuclease A (RNase A) and Triton X-100 were from Sigma Chemical Co. (St. Louis, MO). Silibinin was dissolved in 100% ethanol. PD98059 was dissolved in methanol. [^{3}H]-thymidine was from NEN Life Products, Inc. (Boston, USA). Antibodies against ERK ½ and phospho-ERK ½ were from New England Biolabs (Beverly, USA).

Cell Culture

Chang liver cells (ATCC CCL13) were cultured in monolayers in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS).

Cytotoxicity Assays

Cytotoxicity assays were performed as described earlier (Mosmann, 1983). Briefly, Chang liver cells were treated with different concentrations of silibinin (1, 10, or 50 μmol L^{-1}) for 24, 48, or 72 h at 37°C in a CO_2 (5%) incubator. Cell viability was then determined by the MTT dye uptake assay. Cells were incubated with MTT (20 μL of 5 mg mL^{-1}) for 4 h at 37°C. Lysis was performed by overnight incubation at 37°C in 20% SDS / HCl(10 mmol L^{-1}), followed by measurements of absorbance at 570 nm using a 96-well ELISA reader. Results were obtained from studies conducted on five independent occasions.

[^{3}H]-Thymidine Incorporation into DNA

Cells were exposed to 1, 10, or 50 μmol L^{-1} silibinin or were untreated for 24 , 48 or 72 h. [^{3}H]-thymidine (1 μ Ci) was added to the culture medium 16 h before harvesting. Cells were collected on filter mats using a cell harvester and the filters were washed twice with large volumes of

phosphate-buffered saline (PBS; 137 mmol L^{-1} NaCL, 1.4 mmol L^{-1} KH_2PO_4, 4.3 mmol L^{-1} Na_2HPO_4, 2.7 mmol L^{-1} KCL, pH 7.2), followed by an ethanol wash. The cell-associated radioactivity (on filter mats) was determined by liquid scintillation counting. To evaluate the effect of PD98059 on DNA synthesis in silibinin-treated cells, cultures were pretreated for 24 h with serum-free medium. PD98059 (50 ng mL^{-1}) was added 1 h prior to addition of silibinin. Findings were obtained from studies performed on five separate occasions.

Immunoblotting

Cells that had reached 80% confluence were incubated in serum-free medium for 24 h, followed by addition of silibinin (1, 10, or 50 μmol L^{-1}). Control preparations received no drug. At the end of the treatment period (30 min), cells were washed with PBS and exposed to denaturing lysis buffer as described earlier (Swarthout *et al*., 2001). For immunoblot analysis, the extracted proteins were separated by gel electrophoresis (10% polyacrylamide gels). The separated proteins were transferred to nitrocellulose filters and the phosphorylated forms of $p44^{ERK1}$ and $p42^{ERK2}$ proteins were identified with anti-phospho-ERK antibody (1:1000 in Tris-buffered saline, TBS). The amounts of $p44^{ERK1}$ and $p42^{ERK2}$ proteins present within each sample were determined with polyclonal anti-ERK1 and anti-ERK2 antibodies (1:1000 in TBS). Membranes were probed with primary antibody, following by peroxidase-conjugated secondary antibody. Proteins were visualized with the ECL Western blotting detection system (chemiluminescence) kit (Amersham, Arlington Heights, IL).

Cell Proliferation Assays

Cells were cultured in DMEM with 10% FBS and plated at 5×10^4 cells per well in 24-well plates. For measurements of cell proliferation, the procedure of Ahmad *et al*. (1998) was adopted. On day 2, medium was replenished and cells were treated with vehicle or with 1, 10 or 50 μmol L^{-1} silibinin. Each treatment condition was performed in triplicate. At days 1-3 following treatments cells were trypsinized, washed thoroughly by centrifugation and resuspension in PBS containing 0.1% formalin and collected in vials. Cell number was determined with a Coulter Counter (Coulter, Inc., Hialeah, New York).

Cell Cycle Analyses

Cell cycle analyses were performed for untreated cultures or cultures exposed to 1, 10 or 50 μmol L^{-1} silibinin for 24, 48, or 72 h. Adherent cells were detached by trypsinization and suspended cells were washed by centrifugation and resuspension in PBS and fixed in 95% ethanol for at least 4 h on ice. Fixed cells were pelleted, washed and resuspended in 1 mL staining solution containing 4 μg mL^{-1} PI, 1% Triton X-100 and 100 μg mL^{-1} RNase A in PBS. Samples were incubated for 30 min at room temperature and then subjected to FACS analysis (Becton Dickinson, Bedford, MA).

Statistical Analyses

The statistical significance of differences between control and silibinin-treated samples was evaluated by the Student's test. P values of less than 0.05 were considered statistically significant.

Results

Silibinin Increases Viability of Chang Liver Cells

Cells that had been treated with vehicle or silibinin at 1, 10, or 50 μmol L^{-1} for 24, 48, or 72 h were examined for viability (Fig. 1). After 24 or 48 h, viability was not affected at any drug

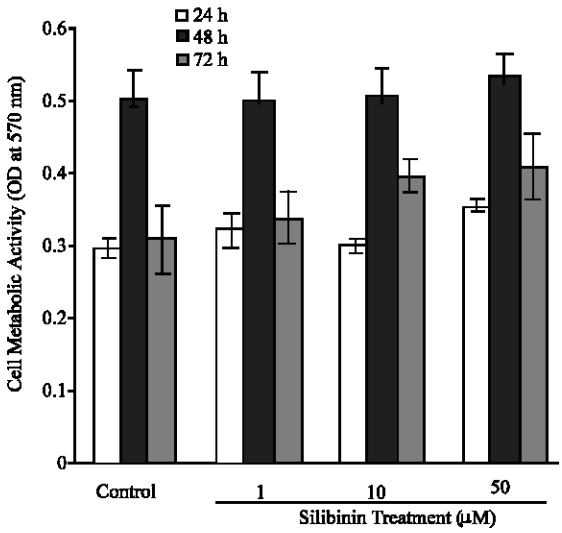

Fig. 1: Effect of silibinin on metabolic activity in Chang liver cells. The OD 570 value are expressed as the means of five independent replicates with error bars for standard deviation as the results of metabolic activity

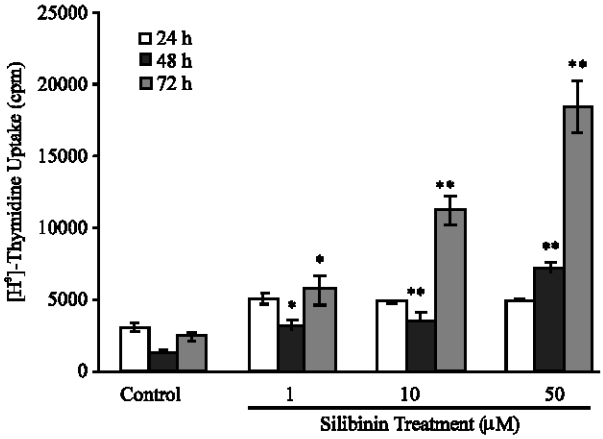

Fig. 2: Silibinin stimulates DNA synthesis in Chang liver cells. Results are expressed as the means of five independent replicates with error bars for standard deviation. *p<0.05 and **p<0.001, compared with the control

concentration. By contrast, viability was found to increase significantly and in a dose-dependent manner after 72 h of treatment with silibinin. As compared with non-treated control preparations, stimulations of viability of approximately 9, 28 and 33% were observed for silibinin treatments at 1, 10 and 50 μmol L^{-1}, respectively.

Silibinin Induces DNA Synthesis in Chang Liver Cells

The effects of vehicle and of 1, 10 and 50 μmol L^{-1} silibinin on [^{3}H]-thymidine incorporation at varying times of incubation times are shown in Fig 2. Silibinin stimulated incorporation significantly ($p<0.05$ for 1 μmol L^{-1} and $p<0.001$ for 10 and 50 μmol L^{-1}) and in a dose-dependent manner at 48 and 72 h of treatment.

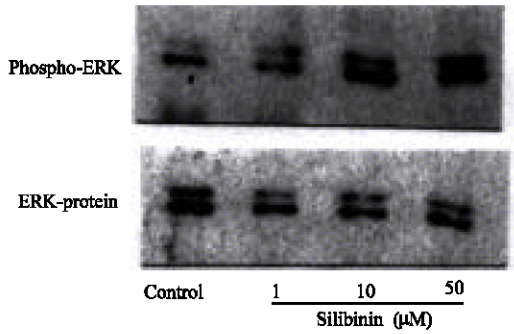

Fig. 3: Effect of silibinin on ERK activation in Chang liver cells. Cells were grown to 80% confluency and switched to DMEM supplemented with 0.1% FBS for an additional 24 hours, followed by treatment for 30 min without or with silibinin at the concentrations indicated. Equal amounts of protein were resolved by on SDS-PAGE (10%), transferred to a nitrocellulose membrane, probed with appropriate primary and secondary antibodies and visualized by the ECL detection system. The upper panel displays phospho-ERKs, and the lower panel displays total ERK proteins

Silibinin Triggers ERK Phosphorylations

Western blot analysis using protein extracts from 1, 10 and 50 μmol L^{-1} silibinin-treated cells or from untreated controls and an antibody selective for phosphorylated ERK1/2 were performed. Total amounts of ERK proteins were found to be unchanged in cells treated with various amounts of silibinin (Fig. 3). However, the amounts of activated (phosphorylated) forms of $p44^{ERK1}$ and $p42^{ERK2}$ in protein extracts of cells exposed to 10 and 50 μM silibinin increased markedly (Fig. 3). By contrast, no significant increases in these forms were detected in extracts of cells treated with 1 μmol L^{-1} drug. Under each of the treatment conditions examined, phosphorylations of JNK and p38 were not found to be affected (data not shown).

Silibinin Stimulates DNA Synthesis via the ERK Pathway

To investigate the possibility that activations of ERK1/2 are involved in the stimulation of DNA synthesis induced by silibinin, the effect of the MAPK kinase (MEK) inhibitor PD98059 on the stimulation was examined. Cells were treated with 10 or 50 μmol L^{-1} silibinin in the absence or presence of PD98059 (25 ug mL^{-1}), followed by measurements of [^{3}H]-thymidine incorporation. The stimulation of incorporation attributable to 10 and 50 μmol L^{-1} silibinin was reduced by 58 and 74%, respectively, in the presence of PD98059 ($p<0.05$ for 10 and 50 μmol L^{-1}) (Fig. 4), consistent with a role for ERKs in silibinin-dependent induction of DNA synthesis in Chang liver cells.

Effects of Silibinin on Cell Proliferation

To examine the effects of silibinin on the efficiency of cell proliferation, cell number was determined at 0, 24, 48 and 72 h of silibinin (1, 10 and 50 μmol L^{-1}) treatment. At 72 h, treatments with 1 and 10 μmol L^{-1} silibinin resulted in 18 and 36% increases in cell number, respectively, as compared with untreated controls (Fig. 5). However, treatment with 50 μmol L^{-1} silibinin was not effective in increasing cell number.

Silibinin Increases the Population of Cells in S phase

Cell cycle progression was investigated in asynchronous cultures of Chang liver cells after 24, 48 and 72 h of treatment with 1, 10, or 50 μmol L^{-1} silibinin (Table 1). Silibinin was found to significantly

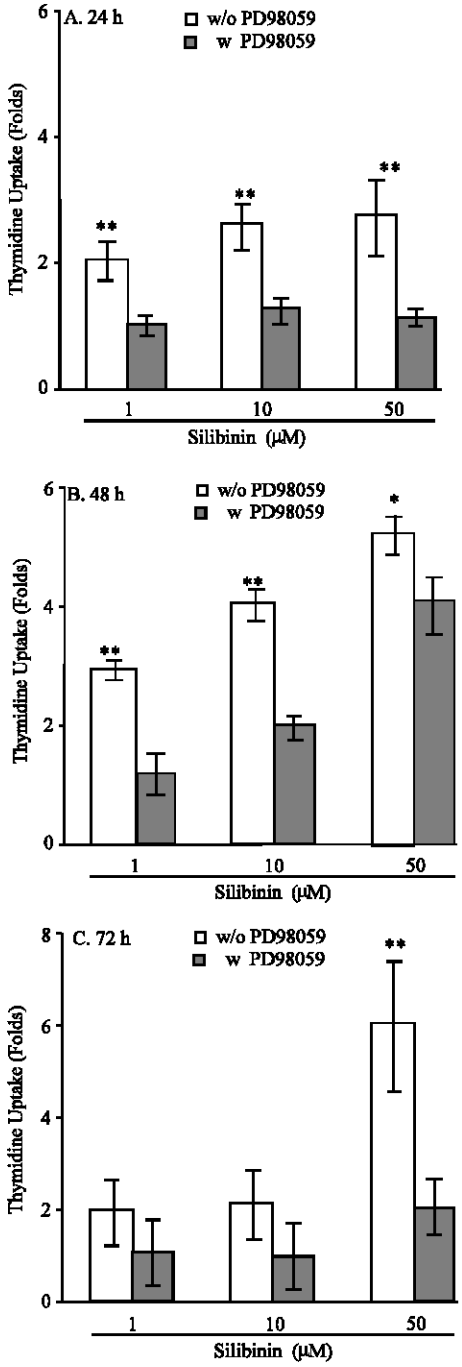

Fig. 4: Effect of PD98059 on silibinin-stimulated DNA synthesis in Chang liver cells. Findings are presented as fold increases over the control from results of three independent replicates with error bars for standard deviation. *p<0.05 and **p<0.001, compared with the PD98059 treatment

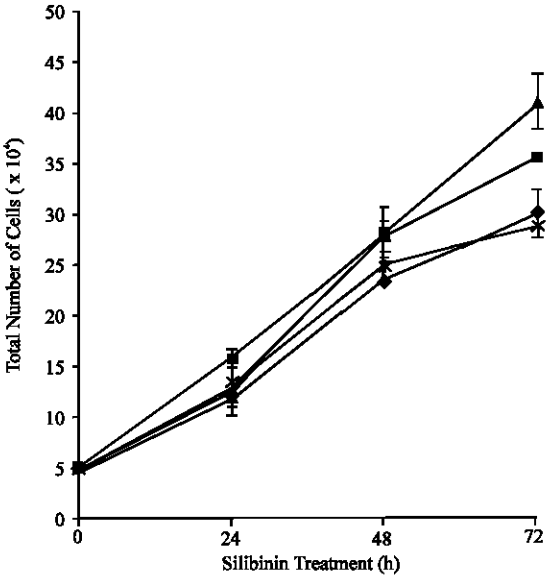

Fig. 5: Effects of silibinin on cell proliferation of Chang liver cells.
Twenty-four h after plating a density of 5×10^4 cells per well, the cells were treated without (♦) or with silibinin at doses of 1 (■), 10 (▲)or 50 (×) μmol L^{-1} for 24, 48 and or 72 h. Following trypsinization, cells were collected, washed and resuspended in PBS containing 0.1% formalin. Cell numbers was counted with an automatic Coulter Counter. Results are expressed as the means of three independent replicates with error bars for standard deviation

Table 1: Cell cycle analysis of silibinin-treated Chang liver cells. Cells were treated with 1, 10 or 50 μmol L^{-1} silibinin for 24, 48 or 72 h, then stained with propidium iodide and analyzed by FACS as described in Materials and Methods. Results are expressed as the means of three independent replicates with error bars for standard deviation

	Percentage (%) of Cell Cycle Distribution		
	G_0/G_1	S	G_2/M
A. 24 h			
Control	53.88±0.32	39.98±0.07	6.21±6.28
1 μM silibinin	55.97±2.86	39.63±2.95	4.41±0.08
10 μM silibinin	52.01±1.35	47.85±1.49*	0.15±0.15*
50 μM silibinin	53.62±0.38	46.89±0.89*	0*
B. 48 h			
Control	53.77±0.52	31.06±4.00	15.18±3.48
1 μM silibinin	41.94±5.01	54.62±8.46*	3.45±3.45
10 μM silibinin	44.33±1.99	48.64±3.49*	7.04±1.50
50 μM silibinin	39.31±0.29	60.54±0.36*	0.16±0.06*
C. 72 h			
Control	52.33±0.12	36.42±0.14	11.26±0.02
1 μM silibinin	49.40±1.20	39.84±1.80	10.77±0.61
10 μM silibinin	39.74±3.36	53.47±9.17*	6.81±5.82
50 μM silibinin	33.10±6.62	65.31±8.22*	1.60±1.60*

*$p<0.05$ compared with control cells

increase the population of cells in S phase ($p<0.05$ for 10 and 50 μmol L^{-1}) and a strong reduction in the population of cells in G2/M phases ($p<0.05$ for 50 μmol L^{-1}) was observed at each time of treatment. Additionally, a decrease in the population of cells in the G1 phase was observed after exposure to10 or 50 μmol L^{-1} silibinin for either 48 or 72 h. These results indicate that silibinin acts primarily to increase the number cells entering S phase.

Discussion

Silymarin, which has a history of almost 2000 years in the Occident as an herbal remedy, has been the focus of recent attention (Flora *et al*., 1998). The major active component of silymarin is silibinin (Wellington and Jarvis, 2001). In the present study silibinin treatment was found to promote increasing degrees of viability in Chang liver cells. The increased viability was related to stimulation of DNA synthesis in combination with a statistically significant increase in the S phase population. And, silibinin treatment resulted in activatation of ERK phosphorylation and an induction of DNA synthesis susceptible to blockade by a MAPK inhibitor. By contrast several reports indicate that silibinin inhibits MAPK/ERK1/2 activation and DNA synthesis in advanced human cancer cells (Bhatia *et al*., 1999; Tyagi *et al*., 2002; Mallikarjuna *et al*., 2004; Sharma *et al*., 2001; Chen *et al*., 2005). Findings of the present study are not in agreement with these reports. These conflicting findings may reflect differences in cell type, extent of cell transformation, or dose of silibinin. Relationships between the increased viable and proliferative effects of silibinin for cells remain to be clarified. However, the capacity of liver cells to regenerate depends on hepatocyte viability as well as increased DNA synthesis. The earlier described hepatoprotective actions of silibinin may derive from the stimulation of DNA synthesis associated with liver cell regeneration (Sonnenbichler and Zetl, 1986). All observations reported here are compatible with both growth-regulatory and cytoprotective actions of silibinin on cultured Chang liver cells.

The effects of silibinin on cell cycle distribution, like those on proliferation, were found to be dose-dependent. Lower concentrations resulted in increases in DNA synthesis and in the percentage of cells entering S phase with no change at G2/M. As a consequence increased cell proliferation, evidenced by an increase in cell number, was observed. At a higher concentration of the drug, DNA synthesis and the S phase population were both increased, but the population of cells at the G2/M transition was reduced. It is probable that this obstructed progression to G2/M was inhibitory to cell proliferation, despite prominent increases in DNA synthesis. With respect to dose of silibinin, the previous data from Agarwal *et al*. also indicated silibinin caused G0/G1 arrest at lower dose, but high dose induced G2/M arrest in human colon carcinoma cells (Agarwal *et al*., 2003). It was believed earlier that, if cells proceeded past G1, they could complete the S phase, proceed though the G2 phase and divide. However, it is now recognized that cells beyond the G1 and S phases do not always proceed to the next phase of the cell cycle (Elledge, 1996). For example, although insulin increases the population of endothelial cells in S phase, entry into the G2/M phase is blocked (Oliveira and Banerjee, 1990). In porcine smooth muscle cells, epidermal growth factor induces brisk maximal DNA synthesis without cell division due to the limited efficacy of late cell cycle (G2/M) events (Bagby *et al*., 1993). Finally, biochanin A (an isoflavone with some chemical similarity to silibinin) regulates cell cycle progression by increasing the percentage of cells entering S phase, but delays the progression from S to G2/M phases at growth inhibitory concentrations (Ying *et al*., 2002).

In conclusion, the growth-regulating actions of silibinin are proposed to be highly dose-dependent, with low doses promoting and high doses suppressing growth. Findings also support the idea that silibinin obstructed progression of the cell cycle to G2/M while preserving viability and function as evidenced by increased DNA synthesis and ERK signaling. Its relative lack of toxicity broadens silibinin's potential for therapeutic use. Nonetheless, as evidenced by this report, different doses of this drug differentially affect various stages of the cell cycle and the ability of cells to proliferate. Additionally the pharmacokinetic properties of silibinin remain to be investigated.

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