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Silibinin upregulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells

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Silymarin, a defined mixture of natural flavonoid, has recently been shown to have potent cancer chemopreventive efficacy against colon carcinogenesis in rat model; however, the mechanism of such efficacy is not elucidated. Here, using pure active agent in silymarin, namely silibinin, we show its antiproliferative and apoptotic effects, and associated molecular alterations in human colon carcinoma HT-29 cells. Silibinin treatment of cells at 50–100 μg/ml doses resulted in a moderate to very strong growth inhibition in a dose- and a time-dependent manner, which was largely due to a G0/G1 arrest in cell cycle progression; higher dose and longer treatment time also caused a G2/M arrest. In mechanistic studies related its effect on cell cycle progression, silibinin treatment resulted in an upregulation of Kip1/p27 and Cip1/p21 protein as well as mRNA levels, and decreased CDK2, CDK4, cyclin E and cyclin D1 protein levels together with an inhibition in CDK2 and CDK4 kinase activities. In other studies, we observed that G2/M arrest by silibinin was associated with a decrease in cdc25C, cdc2/p34 and cyclin B1 protein levels, as well as cdc2/p34 kinase activity. In the studies assessing biological fate of silibinin-treated cells, silibinin-induced cell cycle arrest and growth inhibition were not associated with cellular differentiation, but caused apoptotic death. The quantitative apoptosis analysis showed up to 15% apoptotic cell death after 48 h of silibinin treatment. Interestingly, silibinin-induced apoptosis in HT-29 cells was independent of caspases activation, as all caspases inhibitor did not reverse silibinin-induced apoptosis. This observation was further confirmed by the findings showing a lack in caspases activity increase and caspases and PARP cleavage as well as a lack in cytochrome *c* release in cytosol following silibinin treatment of HT-29 cells. Additional studies conducted in mice showed that silibinin doses found effective in HT-29 cells are achievable in plasma, which increases the significance of the present findings and their possible translation in *in vivo* anticancer efficacy of silibinin against colon cancer. Together, these results identify molecular mechanisms of silibinin efficacy as a cell cycle regulator and apoptosis inducer in human colon carcinoma HT-29 cells, and justify further studies to investigate potential usefulness of this nontoxic agent in colon cancer prevention and intervention.
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Introduction

Several studies have demonstrated the potential of flavonoids for the prevention and/or intervention of various cancers, but mechanistic evidences are still lacking in most cases. One such naturally occurring flavonoid is silibinin (Figure 1), which is the major biologically active constituent in milk thistle (*Silybum marianum*) extract. Silibinin has high human acceptance being used clinically and consumed as dietary supplement around the world for its strong antihepatotoxic efficacy (Flora *et al*., 1998; Wellington and Jarvis, 2001). Silibinin is well tolerated and largely free of any adverse effects; nontoxic in acute, subchronic and chronic tests even at large doses; and there is no known LD_{50} for silibinin in animal studies (Hahn *et al*., 1968; Vogel *et al*., 1975; Flora *et al*., 1998; Wellington and Jarvis, 2001). A wide range of studies by us and others have shown strong cancer chemopreventive and anticancer efficacy of silibinin (or its crude form known as silymarin) in different animal tumor bioassay systems as well as in cell culture models of epithelial cancers such as skin (Katiyar *et al*., 1997; Zi *et al*., 1997; Ahmad *et al*., 1998; Lahiri-chatterjee *et al*., 1999), prostate (Zi *et al*., 1998b; Zi and Agarwal, 1999a; Dhanalakshmi *et al*., 2002; Singh *et al*., 2002a), breast (Zi *et al*., 1998a; Bhatia

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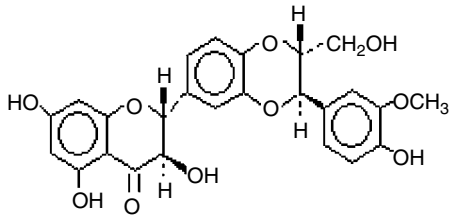

Figure 1 Chemical structure of silibinin. Silibinin is a bioactive flavanone and a major component of milk thistle extract isolated from *Silybum marianum*

et al., 1999) and cervical (Bhatia *et al*., 1999) cancers. With regard to colon cancer, a recent study has shown cancer preventive effect of silymarin against azoxymethane-induced colon carcinogenesis in male F344 rats (Kohno *et al*., 2002); however, the underlying mechanism of action remains to be elucidated. In the present study, we employed human colon carcinoma HT-29 cells to investigate the anticancer effects of silibinin and associated molecular events.

Colorectal cancer is the third most common malignancy in the world including the United States. Recent statistics suggest that 148 300 new cases of colon cancer were identified in 2002 alone, of which 56 600 patients will die of the disease (Jemal *et al*., 2002). Accordingly, colon cancer is a major public health concern around the world. In general, mutation in adenomatous polyposis coli (APC) and a loss in tumor suppressor gene p53 are widely studied genetic defects as colon cancer progresses (Kinzler and Vogelstein, 1996; Samowitz *et al*., 2002). Apart from genetic alterations, the epigenetic events such as loss of controlled cell cycle progression, increased cell survival signaling and repression of apoptosis are common in colon cancer progression (Sebolt-Leopold *et al*., 1999; Torrance *et al*., 2000; Gupta and DuBois, 2001). These reports suggest that agents, which could overcome these epigenetic defects, could be potentially effective in controlling the growth of colon cancer. Our completed studies show that silibinin has both promise and potential in inhibiting mitogenic and cell survival signaling, and altering deregulated cell cycle progression (Zi *et al*., 1998a, b; Zi and Agarwal, 1999b). Here, for the first time we report that silibinin exerts antiproliferative and apoptotic effects in human colon carcinoma HT-29 cells that involves the modulation of CDKIs (cyclin-dependent kinase inhibitors), CDKs (cyclin-dependent kinases), cyclins and other CDK regulators leading to a G0/G1 or G2/M arrest.

Results

Silibinin inhibits growth of human colon carcinoma HT-29 cells and causes G0/G1 and G2/M arrests in cell cycle progression

We first examined the dose- and time-response effect of silibinin on HT-29 cell growth. Silibinin doses used in the study were 50, 75 and 100 μg/ml dissolved in carrier DMSO, which was equally (0.1% v/v) present in all the treatments including control, for 24, 48 and 72 h. At the end of each treatment time, determination of total cell number showed that silibinin inhibits cell growth in both dose- and time-dependent manner (Figure 2a). The silibinin doses used in the study resulted in 8–39% ($P<0.05$), 35–75% ($P<0.01$–0.001) and 57–91% ($P<0.01$–0.001) inhibition in HT-29 cell growth after 24, 48 and 72 h of treatments, respectively (Figure 2a).

Based on this highly significant to almost complete inhibition of HT-29 cell growth by silibinin, next we examined its effect on cell cycle progression. We reasoned that silibinin-caused cell growth inhibition might be accompanied by modulation of cell cycle progression. To investigate this possibility, following silibinin treatment, cells were stained with saponin/propidium iodide (PI) and then subjected to FACS analysis. Cell cycle distribution analysis showed that all the silibinin doses (50–100 μg/ml) used result in an increase in G0/G1 population in a dose-dependent manner at the expense of S phase population after 24 and 48 h of treatment, except 50 μg/ml dose of silibinin for 24 h, which did not show any change in cell cycle distribution (Figure 2b and c). In addition to a G0/G1 arrest, 100 μg/ml dose of silibinin also resulted in a G2/M arrest as compared to DMSO control (Figure 2b and c). Based on the data showing that silibinin causes G0/G1 (both lower and higher doses) as well as G2/M (higher dose) arrests, next we examined the molecular mechanism of the observed cell cycle arrests.

Silibinin induces Kip1/p27 and Cip1/p21 protein and mRNA expression in HT-29 cells

The Kip/Cip family of proteins has been shown to be causally involved in the regulation of cell cycle restriction points (Grana and Reddy, 1995). First, we examined the protein level of CDKI, Kip1/p27, which is one of the key players in the regulation of G0/G1 phase of cell cycle, and Kip1/p27 is upregulated in response to antiproliferative signals that mainly leads to a G0/G1 arrest. Consistent with these reports, western blot analysis showed that silibinin strongly upregulates Kip1/p27 protein expression (Figure 3a), which was in accord with the observed cell cycle arrest by silibinin (Figure 2b and c). The protein level of Kip1/p27 increased in a dose-dependent manner after 24 h of silibinin treatment; however, in case of 48 h treatment, 100 μg/ml dose of silibinin caused a slightly lower increase in Kip1/p27 than that at 75 μg/ml dose (Figure 3a). Taken together, these results show a possible involvement of Kip1/p27 induction in silibinin-induced G0/G1 arrest in HT-29 cells. Next, we examined the effect of silibinin on Cip1/p21, which is a universal inhibitor of cell cycle progression, and has been shown to be upregulated in G0/G1 as well as in G2/M arrests (Grana and Reddy, 1995). Consistent with these reports and our cell cycle arrest data with silibinin, we observed that silibinin causes a very strong increase in Cip1/p21 protein expression in a dose-dependent manner; however, its effect was comparable at 24 and

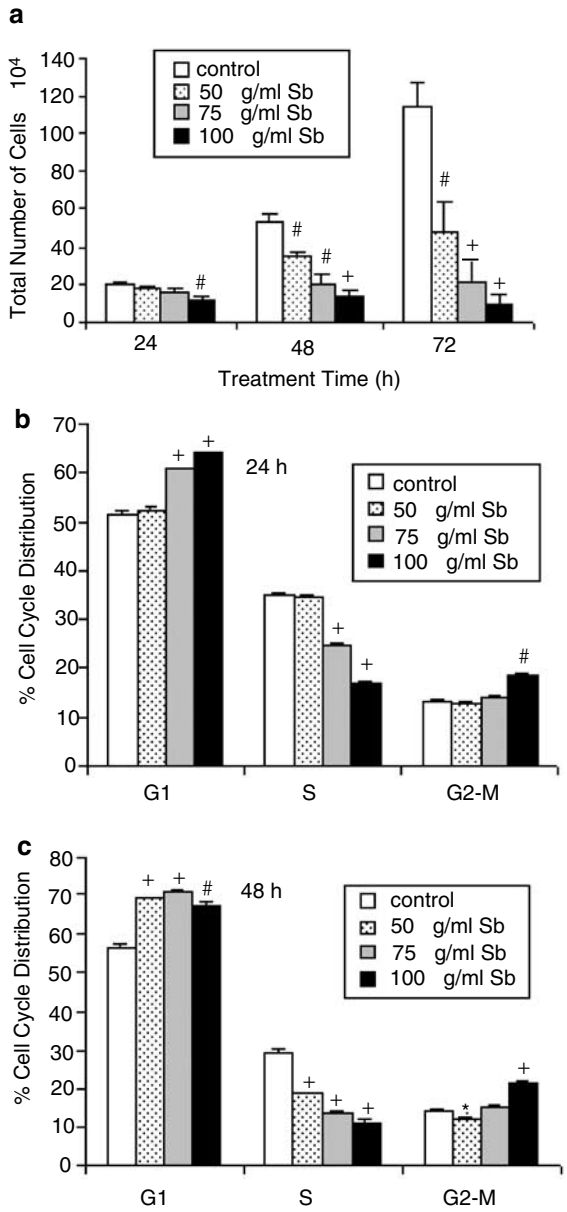

Figure 2 Silibinin inhibits growth and induces cell cycle arrest in human colon carcinoma HT-29 cells. (**a**) For the studies assessing the effect of silibinin on exponentially growing HT-29 cell growth, cells were treated with either DMSO (control) or different doses of silibinin (50, 75 and 100 μg/ml, final concentrations in medium) in DMSO. After 24, 48 and 72 h of these treatments, total cells were collected and counted using hemocytometer. The cell growth data shown are mean$\pm$s.e. of three experiments, each performed in duplicate or triplicate. In similar silibinin treatments for 24 and 48 h, cell cycle distribution was analysed by flow cytometry as detailed in Materials and methods. (**b**) Percent cell cycle distribution after 24 h of silibinin treatment. (**c**) Percent cell cycle distribution after 48 h of silibinin treatment. The data are mean$\pm$s.e. of three samples for each treatment. These results were similar in two independent experiments. *, $P<0.05$; #, $P<0.01$; $^{+}$, $P<0.001$ compared with control. Sb, silibinin

48 h treatments (Figure 3b). An increase in both CDKIs was further confirmed by reprobing the same membranes with anti-β-actin antibody as a loading control (Figure 3c).

Based on our findings showing that silibinin strongly increases Kip1/p27 and Cip1/p21 protein levels in HT-29 cells, next we examined whether silibinin transcriptionally upregulates CDKIs using Northern blot analysis. The data shown in Figures 3d and e support transcriptional increase in Kip1/p27 and Cip1/p21 by silibinin, respectively. These increases in mRNA levels were further confirmed by measuring β-actin mRNA as a loading control (Figure 3f). When the magnitude of Kip1/p27 induction by silibinin was compared at both protein and mRNA levels, the two were comparable; however, in case of Cip1/p21 induction, the observed increase in protein at 100 μg/ml silibinin dose was much stronger than that for mRNA levels (Figure 3b versus e). One possible explanation for this difference could be Cip1/p21 protein stabilization by silibinin via a post-translational mechanism; more studies, however, are needed in future to decipher such anticipation. Overall, these results suggest that silibinin-induced expression of Kip1/p27 and Cip1/p21 might be a critical event leading to an alteration in CDK–cyclin complexes and other related events followed by cell cycle arrest in HT-29 cells, and formed the basis of further investigation.

Silibinin modulates G0/G1 cell cycle regulators and decreases kinase activity of CDKs

CDKs and cyclins are important determinants in the regulation of cell cycle progression. In most cancer cells, high expression of these molecules accompanied by increased CDK activity is causally involved in deregulated and unchecked cell proliferation (Grana and Reddy, 1995; Morgan, 1995). Accordingly, we next analysed whether silibinin modulates the expression of G0/G1 CDKs and cyclins in HT-29 cells. Western blot analysis showed that silibinin treatment at 50–100 μg/ml doses results in a moderate to strong decrease in the expression of CDK2, CDK4, cyclin D1 and cyclin E (Figure 4a–d) in a dose- and a time-dependent manner; specifically, 48 h of silibinin treatment at higher doses showed 60–90% decrease in the protein levels of these G0/G1 cell cycle regulators (Figure 4a–d). Reprobing of the membranes with anti-β-actin antibody confirmed equal protein loading in each case (data not shown).

The induced expression of CDKIs normally leads to an increase in its binding to and subsequent decrease in the kinase activity of CDK–cyclin complex. Accordingly, we next investigated the effect of silibinin on CDK2 and CDK4 kinase activity. As shown in Figure 4e and f, silibinin treatment of HT-29 cells at 100 μg/ml dose for 24 and 48 h resulted in a strong decrease in histone H1-associated CDK2 (90–98% decrease) and Rb (retinoblastoma)-associated CDK4 kinase (60% decrease) activities, which could be due to a decrease in protein levels of CDK2 and CDK4 as well as their increased binding with CDKIs. Together, these results suggest that silibinin-caused G0/G1 arrest in HT-29 cells could be via an induction of CDKIs and a decrease in the levels of G0/G1 cell cycle regulators leading to a decrease in the kinase activity of CDKs.

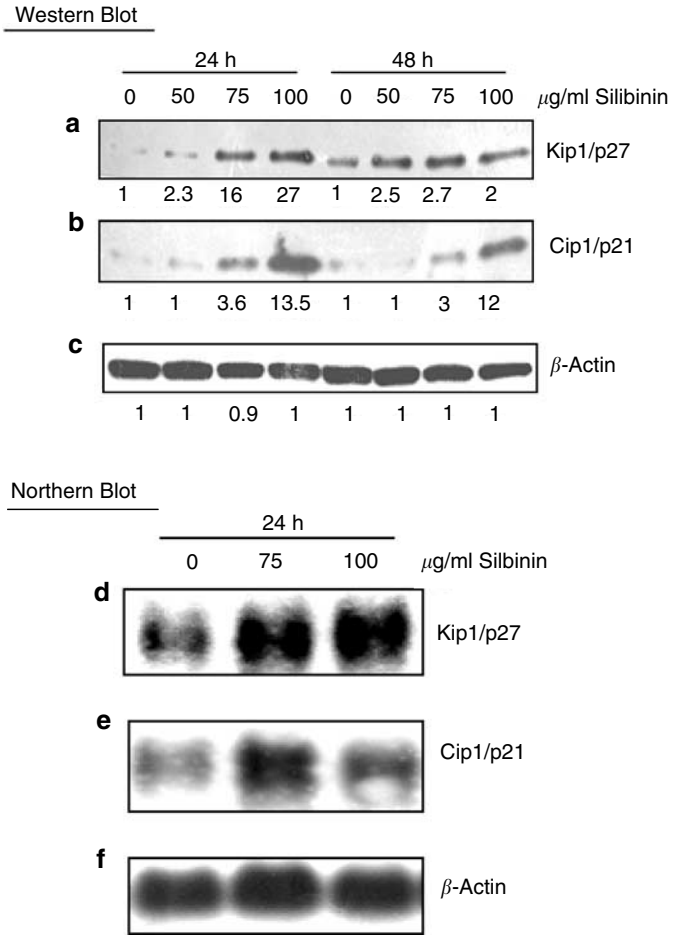

Figure 3 Effect of silibinin on cyclin-dependent kinase inhibitors. HT-29 cells were treated with DMSO or 50, 75 and 100 μg/ml doses of silibinin in complete medium for 24 and 48 h. At the end of each treatment time, cell lysates were prepared in nondenaturing lysis buffer as mentioned in Materials and methods. A measure of 80 μg of protein lysates per sample was used for SDS–PAGE and Western blotting. Membranes were probed with anti-Kip1/p27 and Cip1/p21 primary antibodies followed by peroxidase-conjugated appropriate secondary antibody, and visualized by ECL detection system. Membrane was stripped and reprobed with anti-β-actin antibody for protein loading correction. (**a**) Kip1/p27, (**b**) Cip1/p21 and (**c**) β-actin. The densitometric data are presented below each band in fold change as compared to their respective controls. RNA (25 μg) from cells treated with 0, 75 or 100 μg/ml doses of silibinin in complete medium for 24 h was isolated and Northern blot analysis was performed as described in Materials and methods. Northern blots were hybridized with ^{32}P-labled (**d**) Kip1/p27, (**e**) Cip1/p21 or (**f**) β-actin cDNAs to detect mRNA and then visualized by autoradiography. Blots shown are representative of at least three independent experiments in each case

Silibinin modulates G2/M cell cycle regulators and decreases cdc2/p34 kinase activity

Based on the observation that silibinin treatment at 100 μg/ml dose for 48 h also causes a strong G2/M arrest in the cell cycle progression of HT-29 cell, we next assessed the effect of silibinin on G2/M cell cycle regulators, namely wee1, chk1, chk2, cdc25C, cdc2/p34 and cyclin B1. In Western blot analysis, silibinin did not show any effect on wee1, chk1 and chk2 protein levels (data not shown); however, it decreased the protein levels of cdc25C (Figure 5a), cdc2/p34 (Figure 5b) and cyclin B1 (Figure 5c). Densitometric analysis of the blots showed 40, 70 and 80% decrease in the protein levels of these molecules following 48 h silibinin treatment of cells at 100 μg/ml dose, respectively. Protein loading was checked by reprobing the membranes with β-actin antibody, which did not show any change in the protein level (Figure 5d). Consistent with the decreases in these G2/M regulators, as well as a G2/M arrest, 100 μg/ml dose of silibinin treatment for 48 h also resulted in a strong decrease in histone H1-associated cdc2/p34 kinase activity (Figure 5e).

Silibinin does not induce differentiation of HT-29 cells

An alteration in cell cycle regulators causing checkpoint arrests has also been associated with cellular differentiation (Ding *et al*., 1998; Kitamura *et al*., 1999); however, in the present study, silibinin-induced G0/G1 arrest in HT-29 cells did not result in any morphological changes suggestive of cellular differentiation (data not shown). To further support this observation, we also performed activity assay for alkaline phosphatase, which is one of the most investigated differentiation markers in colon carcinoma cells (Ding *et al*., 1998; Kitamura *et al*., 1999). Consistent with a lack of morphological changes,

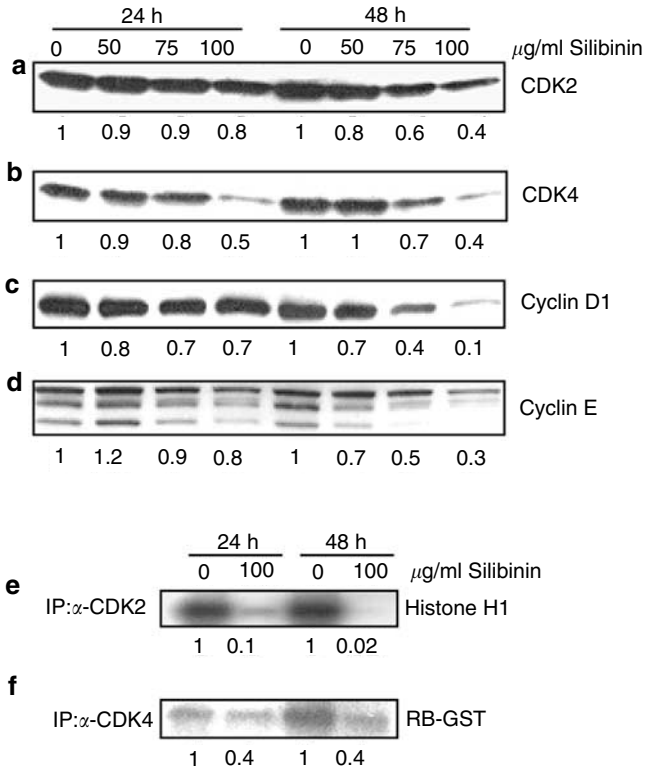

Figure 4 Effect of silibinin on G1 cyclin-dependent kinases and cyclins. HT-29 cells were treated with DMSO or 50, 75 and 100 μg/ml doses of silibinin in complete medium for 24 and 48 h. At the end of each treatment time, cell lysates were prepared in nondenaturing lysis buffer as mentioned in Materials and methods. A measure of 40–80 μg of protein lysates per sample was used for SDS–PAGE and Western blotting. Membranes were probed for CDK2, CDK4, cyclin D1 and cyclin E, and stripped for reprobing with anti-β-actin antibody for protein loading correction. (**a**) CDK2, (**b**) CDK4, (**c**) cyclin D1 and (**d**) cyclin E. For kinase studies, cells were treated with either DMSO or 100 μg/ml dose of silibinin in complete medium for 24 and 48 h. CDK2-associated H1 histone and CDK4-associated Rb kinase activities were determined as described in Materials and methods. (**e**) Histone H1-associated CDK2 kinase activity and (**f**) Rb-GST-associated CDK4 kinase activity. The densitometric data are presented below each band in fold change as compared to their respective controls. Blots shown are representative of three independent experiments in each case

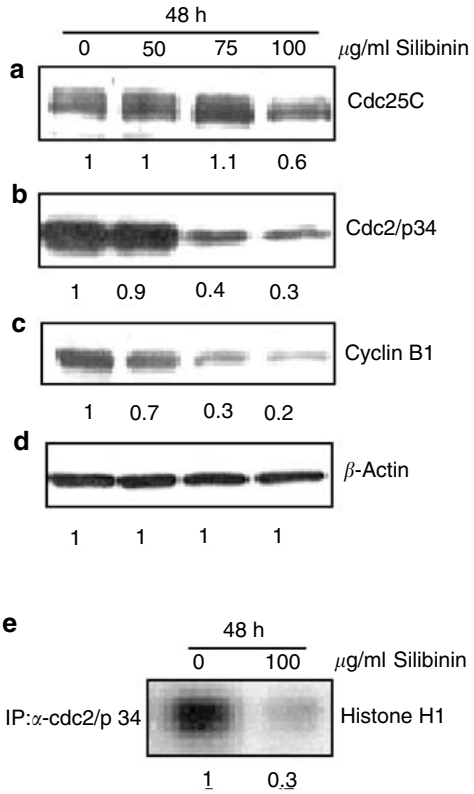

Figure 5 Effect of silibinin on G2/M cell cycle regulators in HT-29 cells. Cells were treated with DMSO or 50, 75 and 100 μg/ml doses of silibinin in complete medium for 48 h. At the end of the treatment, cell lysates were prepared in nondenaturing lysis buffer as mentioned in Materials and methods. (**a**–**d**) A measure of 40–80 μg of protein lysates per sample was used for SDS–PAGE and Western blotting, and membranes were probed for cdc25C, cdc2/p34 and cyclin B1, respectively. Membranes were also stripped and reprobed with anti-β-actin antibody for protein loading correction. (**a**) cdc25C, (**b**) cdc2/p34, (**c**) cyclin B1 and (**d**) β-actin. (**e**) Histone H1-associated kinase activity of cdc2/p34 was measured as described in Materials and methods. The densitometric data are presented below each band in fold change as compared to their respective controls. Blots shown are representative of three independent experiments in each case

silibinin treatment for 24, 48 and 72 h did not result in an increase in alkaline phosphatase activity (Table 1), suggesting that silibinin-caused cell cycle arrests and cell growth inhibition do not associate with differentiation induction.

Table 1 Effect of silibinin on the alkaline phosphatase activity in HT-29 cells

	Control	*50 μg/ml Sb*	*75 μg/ml Sb*	*100 μg/ml Sb*
24 h	0.082±0.007	0.074±0.001	0.069±0.002	0.068±0.004
48 h	0.103±0.009	0.107±0.002	0.097±0.001	0.109±0.020
72 h	0.130±0.004	0.128±0.008	0.157±0.006	0.120±0.011

Alkaline phosphatase activity is presented as mean±s.e.m. of $OD_{405\,nm}$ of three samples for each treatment

Silibinin induces apoptotic death of HT-29 cells independent of caspase pathway

In addition to its cell growth and cell cycle checkpoint effects, we also assessed whether silibinin induces apoptotic death in HT-29 cells using annexin V and PI staining followed by flow cytometric analysis. Silibinin treatment for 24 h at 50–100 μg/ml doses did not cause any significant apoptosis in HT-29 cells (data not shown); however, similar treatments for 48 h resulted in an increase in apoptotic cell population in a dose-dependent manner (Figure 6a). Quantification of these data showed that 50, 75 and 100 μg/ml doses of silibinin result in 8.3 ($P<0.05$), 11.2 ($P<0.001$) and 15.2% ($P<0.001$) apoptotic cells after 48 h of treatment as compared to control showing 3.5% apoptotic cells (Figure 6a). These results clearly identify apoptosis induction as another anticancer mechanism of silibinin in HT-29 cells, based on which further studies were

performed to investigate the possible involvement of caspase-cascade activation by silibinin in its apoptotic response. Surprisingly, use of 100 μM dose of all caspases inhibitor (Z-Val-Ala-Asp-OMe-FMK) did not reverse silibinin-caused apoptosis in HT-29 cells (Figure 6a). In another set of similar experiments, all caspases inhibitor together with silibinin showed only a slight reduction in silibinin-induced G2/M arrest at 100 μg/ml dose (Figure 6b).

Based on these unanticipated results in terms of a lack of caspases involvement in silibinin-induced apoptosis, we performed additional experiments to confirm that silibinin-induced apoptotic death of HT-29 cells is independent of caspase pathway. First, we did caspase activity assays using specific chromogenic substrates for caspase 3 and 9. As shown in Figure 6c, silibinin treatment did not show any increase in the activities of these caspases. To further validate these findings, Western blot analysis was carried out for caspase 3 and 9, and PARP. We did not observe any cleaved fragments of caspase 3 and PARP, except a very slight increase in cleaved caspase 9 after 48 h of silibinin treatment, which seems to be insufficient in making a significant contribution in silibinin-induced apoptosis in HT-29 cells (Figure 6d). We also assessed cytochrome *c* release in cytosol from mitochondria, an upstream event in caspases activation. Again, there was no decrease in mitochondrial cytochrome *c* levels, as well as there was a lack of its release in cytosol in silibinin-treated HT-29 cells (Figure 6d). Together, these findings clearly indicate the involvement of caspase-independent pathway(s) in silibinin-caused apoptotic death of HT-29 cells, and suggest more studies in future to identify them.

Pharmacologically achievable levels of silibinin and their relevance to the present study

To establish an *in vivo* relevance of the above-described cell culture findings, achieving pharmacological levels of silibinin in similar dose ranges are highly desirable. In this regard, our earlier studies with silibinin- or

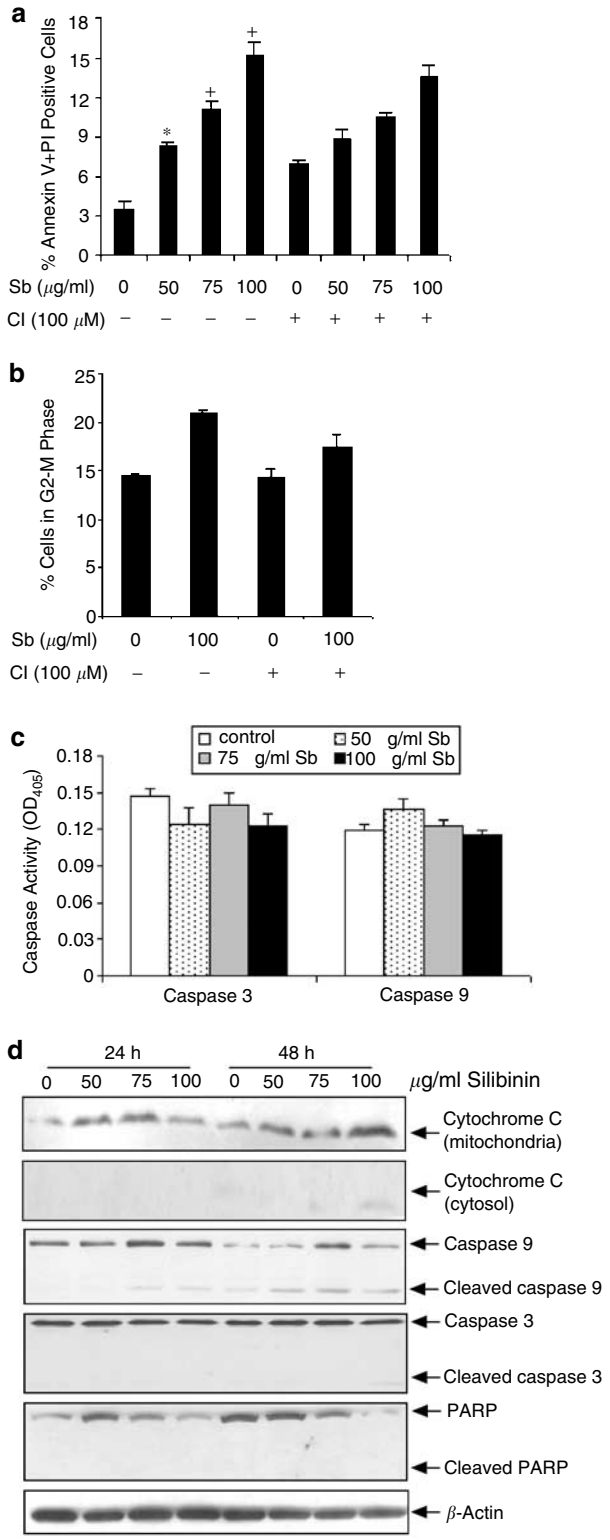

Figure 6 Silibinin induces caspase-independent apoptosis in HT-29 cells. (**a**) Cells were treated with DMSO, or 50–100 μg/ml doses of silibinin, either alone or in combination with all caspases inhibitor (100 μM) in complete medium for 48 h. At the end of the treatment, both floating and attached cells were pooled and subjected to annexin V/PI staining followed by flow cytometric analysis as described in Materials and methods. Quantitative data are presented as mean $\pm$ s.e. of percent apoptotic cells of triplicate samples in each treatment group. (**b**) Under similar treatment conditions as described above, but using only 100 μg/ml silibinin dose treatment, cell cycle analysis was performed and quantitative data for percent cells in G2–M phase are presented as mean $\pm$ s.e. of triplicate samples in each treatment group. (**c**) Cells were treated with either DMSO or 75 and 100 μg/ml doses of silibinin in complete medium for 48 h. At the end of the treatment, cell lysates were prepared and activities of caspase 3 and 9 were measured using specific substrates as detailed in Materials and methods. Data are presented as mean $\pm$ s.e. of triplicate samples from each treatment. (**d**) Cells were treated with DMSO or 50–100 μg/ml doses of silibinin for 24 and 48 h. At the end of the treatment, total cell lysates as well as mitochondrial and cytosolic fractions were prepared followed by SDS–PAGE and Western blotting as mentioned in Materials and methods. Membranes were probed for cytochrome *c*, caspase 9, caspase 3 and PARP. Membranes were stripped and reprobed with anti-β-actin antibody for protein loading correction. *, $P<0.05$; $^{+}$, $P<0.001$ compared with control. Sb, silibinin; CI, all caspases inhibitor. Blots shown are representative of two–three independent experiments in each case

silymarin-fed mice have shown that silibinin is physiologically available in all the organs investigated including plasma without any apparent signs of toxicity (Singh *et al*., 2002a, b). In the present study, however, we again made an effort to address the question whether silibinin levels found effective in HT-29 cell cultures are pharmacologically achievable? The mice were fed different doses of silibinin in increasing concentrations by oral gavage, and concentrations of silibinin in plasma and colonic mucosa were estimated. In addition, we also assessed any adverse effects of silibinin feeding on animal body weight gain profiles and diet consumption. Silibinin feeding at the doses of 333, 666, 1000 and 2000 mg/kg mouse body weight for 16 days resulted in a dose-dependent increase in silibinin levels in plasma and colonic mucosa (Figure 7a and b). The circulating level of silibinin was achieved up to $74.0 \pm 3.0\,\mu$g/ml in plasma (Figure 7a) and $25.5 \pm 1.6\,\mu$g/g in colonic mucosa (Figure 7b). It is important to emphasize here that silibinin level in plasma and colonic mucosa did not reach a saturation level following its feeding up to 2000 mg/kg dose, indicating the possibility that silibinin feeding at the doses higher than those in the present study could result in its higher levels in both plasma and colonic mucosa. More importantly, consistent with our earlier studies and other reports, we did not observe any apparent signs of toxicity of silibinin including diet consumption (Figure 7c) and body weight gain (Figure 7d) in mice throughout the experiment. Together, these data show that silibinin doses (50–100 μg/ml) used in cell culture study are achievable in plasma under *in vivo* condition, and therefore increase the significance of the present findings and their possible translation in *in vivo* anticancer efficacy of silibinin against colon cancer.

Discussion

The findings of the present study show that silibinin exerts strong anticancer effects against colon carcinoma HT-29 cells, and that this effect involves significant alterations in cell cycle regulators resulting in both G0/G1 and G2/M arrests and cell growth inhibition as well as apoptotic death. The detailed mechanistic studies showed that silibinin increases both mRNA and protein levels of Kip1/p27 and Cip1/p21, and strongly inhibits CDK2 and CDK4 kinase activity in HT-29 cells. In

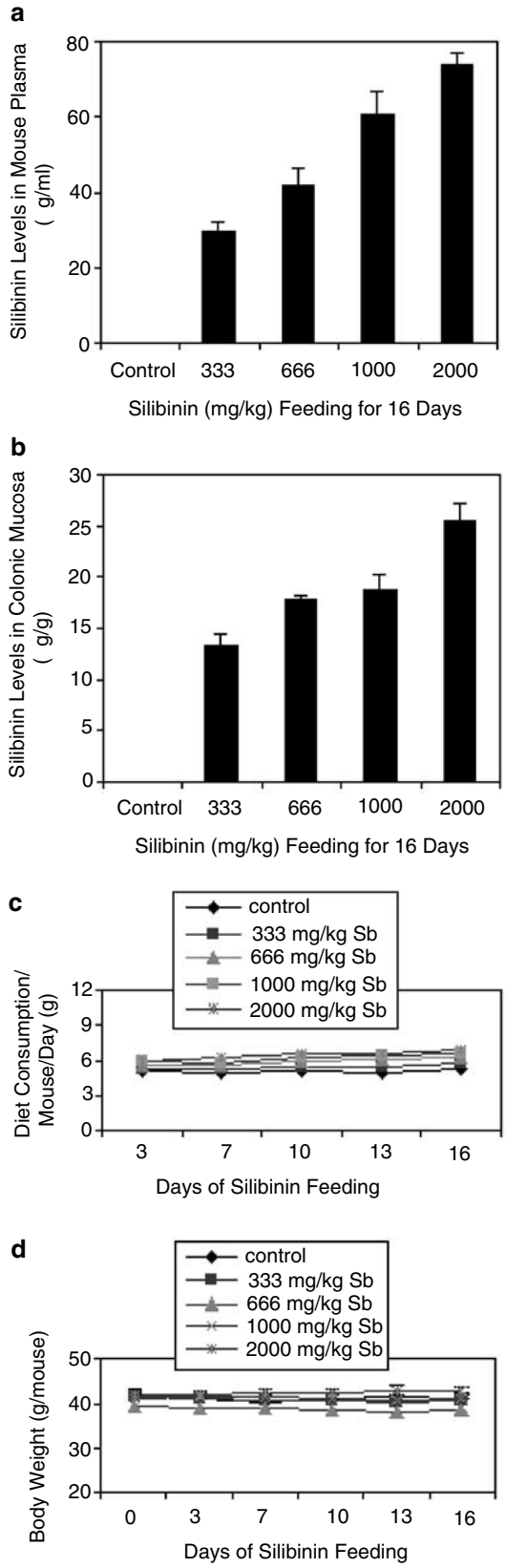

Figure 7 Effect of silibinin feeding on its bioavailability in plasma and colonic mucosa as well as on body weight and diet consumption in SENCAR male mice. Mice were fed daily with different doses of silibinin (saline control, 333, 666, 1000 and 2000 mg/kg silibinin) by oral gavage for 16 days. Body weight and diet consumption were monitored during the experiment. At the end of the treatment, mice were killed, and blood and colon were harvested and processed for the estimation of silibinin concentration by HPLC as detailed in Materials and methods. (**a**) Silibinin concentrations in mouse plasma (μg/ml); and (**b**) in colonic mucosa (μg/g). (**c**) Average diet consumption (g)/mouse/day plotted as a function of days of silibinin feeding. (**d**) Body weight (g)/mouse plotted as a function of days of silibinin feeding. Data in (**a**), (**b**) and (**d**) are presented as mean $\pm$ s.e. of six mice in each group. Sb, silibinin

terms of its effect on G1-phase-associated CDKs and cyclins, silibinin decreased CDK2, CDK4, cyclin D1 and cyclin E protein levels. In other studies assessing the effect of silibinin on G2/M cell cycle regulators, silibinin decreased the protein expression of cdc25C, cdc2/p34 and cyclin B1 together with up to 70% decrease in cdc2/p34 kinase activity. Similar to its antiproliferative effect, silibinin also resulted in apoptotic death of HT-29 cells, which was independent of caspase pathway. Further, study of bioavailability of silibinin in mice fed at different doses indicated that silibinin concentrations used in the cell culture study could be achieved in plasma.

The Kip/Cip family of CDKIs regulates the activity of CDK–cyclin complex and subsequently cell cycle restriction point (Hunter and Pines, 1994; Grana and Reddy, 1995; Morgan, 1995). The important Kip family members include Kip1/p27, which is shown to be upregulated in response to antiproliferative signals (Polyak *et al*., 1994a). Kip1/p27 is an important member of CDKIs, which shows high levels of expression in quiescent cells (Polyak *et al*., 1994b; Mori *et al*., 1997). Kip1 gene is also identified as a tumor suppressor gene where the loss of one copy of the gene is shown to be sufficient to contribute to cancer (Fero *et al*., 1998). Kip1/p27 has also been shown to have prognostic significance in various cancers including colon cancer (Catzavelos *et al*., 1997; Esposito *et al*., 1997; Guo *et al*., 1997; Jordan *et al*., 1998; Lloyd *et al*., 1999). CDKIs regulate the cell cycle progression by binding with active CDK–cyclin complexes and thereby inhibiting their kinase activities (Hunter and Pines, 1994; Grana and Reddy, 1995; Morgan, 1995). Cip1/p21 is a universal inhibitor of CDKs whose expression is mainly regulated by the p53 tumor suppressor protein (Xiong *et al*., 1993; Grana and Reddy, 1995). The increased expression of G1 cyclins in cancer cells provides them an uncontrolled growth advantage because most of these cells either lack CDKI, possess nonfunctional CDKI, or have low expression of CDKI (Sherr, 1994; Sherr and Roberts, 1999). Consistent with these facts, silibinin increased both mRNA and protein expression of Kip1/p27 and Cip1/p21. Additionally, our data suggest that silibinin-caused CDKIs upregulation involves p53-independent pathway, as HT-29 cells lack functional p53.

Generally, cell cycle checkpoints are controlled by CDKs; the holoenzymes contain cyclin as regulatory subunit and CDK as catalytic subunit (Grana and Reddy, 1995; Morgan, 1995; Sherr and Roberts, 1999). Cyclin D- and E-dependent kinases are largely involved in controlling G1/S restriction point (Grana and Reddy, 1995; Morgan, 1995; Sherr and Roberts, 1999). CDK4 interacts with cyclin D1, in which CDK4 has relatively longer half-life as compared to cyclin D1 (Hunter and Pines, 1994; Sherr and Roberts, 1999). Persistent mitogenic signaling, which happens to be the case in majority of the cancer cells, is required for the induction, synthesis and assembly of cyclin D1 with its catalytic partners (Hunter and Pines, 1994; Grana and Reddy, 1995; Sherr and Roberts, 1999). Consistent with these reports, silibinin decreased the protein expression of CDK4 and cyclin D1 as well as kinase activity (Rb-GST phosphorylation) associated with CDK4. This could be in part due to an increased expression of Kip1/p27 and its binding to the CDK4–cyclin D1 complex. A noncatalytic role of cyclin D-dependent CDKs has also been reported where they sequester Kip/Cip family of proteins in a mitogen-dependent manner, thereby facilitating the activation of CDK2–cyclin E complex (Sherr and Roberts, 1999). CDK2–cyclin E complex formation is largely independent of extracellular mitogenic stimulation, which in part accounts for the loss of the dependency on extracellular growth factors at G1/S restriction point (Dulic *et al*., 1992; Tsai *et al*., 1993; Sherr, 1994; Sherr and Roberts, 1999; Hanahan and Weinberg, 2000). CDK2–cyclin E complex peaks at G1/S transition and phosphorylates several proteins including retinoblastoma. CDK2–cyclin E activity is also linked to replication origin firing (Dulic *et al*., 1992; Tsai *et al*., 1993; Sherr, 1994; Hanahan and Weinberg, 2000). In the present study, we observed that silibinin decreases the protein expression of CDK2 and cyclin E as well as histone H1 kinase activity associated with CDK2. The inhibitory effect of silibinin on these molecular parameters associated with G1–S transition could have led to the G1 arrest of HT-29 cells as observed in cell cycle analysis. We also observed that silibinin-induced G0/G1 arrest does not drive the cells into differentiation, as we did not see any differentiation morphology, which was further confirmed by the activity assay for alkaline phosphatase. Based on these findings, further studies are needed to define the biological role of silibinin-induced expression of Kip1/p27 and Cip1/p21 in HT-29 cells.

The ability of chemopreventive or chemotherapeutic agents to suppress the growth of cancer cells is also associated with blocking the cell cycle progression at G2/M checkpoint. G2/M transition is regulated by the sequential activation and deactivation of CDK-regulatory proteins and cyclin complexes (Taylor and Stark, 2001). Protein tyrosine phosphatase cdc25C functions as a mitotic activator by dephosphorylating cdc2/p34, which forms complex with cyclin B1 and drives the cell from G2 to M phase. Due to various genetic and epigenetic alterations, cdc2/p34 kinase activity is enhanced in most human cancers (Graves *et al*., 2000; Taylor and Stark, 2001). Consistent with these facts, silibinin downregulated cdc25C, cdc2/p34 and cyclin B1 with a concomitant decrease in cdc2/p34 kinase activity in HT-29 colon carcinoma cells. These molecular alterations by silibinin could have led to a G2/M arrest in these cells. Therefore, the regulation of G1/S and G2/M transition could be an effective target to control the growth and proliferation of human colon cancer cells by silibinin. However, this needs to be tested in other human colon cancer cell lines.

Apoptosis and associated cellular events have profound effect on the progression of benign to malignant phenotype and can be targeted for the therapy of various malignancies including colon cancer (Thompson, 1995; Lowe and Lin, 2000). Accordingly, our results showing induction of apoptotic death of HT-29

cells by silibinin could be of greater significance in identifying another (together with growth inhibitory) anticancer effect of silibinin in colon cancer. We also observed that silibinin induces cell detachment at high dose and longer treatment time. This effect is in accord with silibinin-induced apoptosis in HT-29 cells, and could be related with the dose and treatment time of the compound, together with the selection of a subpopulation of the cells. Since cell cycle arrest has been reported in anticancer agent-induced apoptosis in human cancer cells (Chinni *et al*., 2001; Moragoda *et al*., 2001), the silibinin-caused G0/G1 or G2/M arrest in HT-29 cells may be in part responsible for the observed apoptotic death. Interestingly, we observed that silibinin-induced apoptosis is independent of caspases activation pathway. The all caspases inhibitor used in the present study did not reverse silibinin-caused apoptosis, G0/G1 (data not shown) arrest and G2/M arrest in HT-29 cells. Consistent with these findings, silibinin also neither released cytochrome *c* from mitochondria to cytosol, nor caused caspases activation and PARP cleavage. These observations suggest that additional investigations are needed in future to assess the molecular events, independent of caspase pathway, involved in silibinin-caused apoptosis in HT-29 cells.

In several mechanistic studies being performed in recent years, there are major issues with regard to achieving pharmacologically effective doses of the available chemotherapeutic anticancer drugs without any toxicity. Systemic toxicity of these anticancer drugs limits their use as well as success in treating the cancer patients. Furthermore, for the translation of *in vitro* anticancer effects of a compound and associated mechanisms into *in vivo* condition, pharmacologically achievable doses of that compound in circulating plasma are highly desirable. Consistent with these facts, in an animal study, we observed that silibinin doses used in the present cell culture study could be achieved in mouse plasma by oral feeding of silibinin without any apparent signs of toxicity in terms of diet consumption and body weight gain. These findings support the possible translation of mechanism-based *in vitro* anticancer efficacy of silibinin in *in vivo* condition.

In summary, our data indicate that silibinin-caused G0/G1 arrest in HT-29 cells is associated with an upregulation of both mRNA and protein levels of Kip1/p27 and Cip1/p21. Silibinin also decreased G1 CDKs and cyclins as well as CDKs-associated kinase activities. At higher dose, silibinin induces G2/M arrest accompanied with a decrease in protein levels of cdc25C, cdc2/p34 and cyclin B1 as well as cdc2/p34 kinase activity. Longer silibinin treatment also results in apoptotic death of HT-29 cells, which was independent of caspase pathway. Animal study with silibinin treatment by oral gavage showed that silibinin doses used in cell culture are achievable in blood circulation without any apparent systemic toxicity. Together, these findings suggest that silibinin has multiple mechanisms driving its anticancer effects in HT-29 cells, and that more detailed studies are needed to further assess and establish both cancer preventive and therapeutic efficacy of silibinin in other colon cancer cells as well as in preclinical colon cancer models.

Materials and methods

Cells and antibodies

Human colon carcinoma cell line HT-29 was obtained from American Type Culture Collection (Manassas, VA, USA). Silibinin was from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). DMEM and other culture materials were from Life Technologies, Inc. (Gaithersburg, MD, USA). Anti-Cip1/p21 antibody was from Calbiochem (Cambridge, MA, USA) and anti-Kip1/p27 antibody was from Neomarkers, Inc. (Fremont, CA, USA). Antibodies to CDK2, CDK4, cdc25C, cdc2/p34, wee1, chk1, chk2, cyclin D1, cyclin E, cyclin B1 and RB-GST fusion protein were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody for β-actin was from Sigma. Antibodies for caspase 9 and 3 were from Cell Signaling Technology (Beverely, MA, USA) and for PARP was from BD Pharmingen (San Diego, CA, USA). Histone H1 was from Boehringer Mannheim Corp. (Indianapolis, IN, USA). [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) and ECL detection system were from Amersham Corporation (Arlington Heights, IL, USA). [α-^{32}P]dCTP (specific activity 3000 Ci/mmol) was from Perkin-Elmer (Boston, MA, USA). Other chemicals were obtained in their commercially available highest purity grade.

Cell culture

HT-29 cells were cultured in DMEM with 10% fetal bovine serum and 1% penicillin–streptomycin under standard mammalian cell culture conditions. Cells were then treated with different doses of silibinin (50, 75 and 100 μg/ml, final concentrations in medium) dissolved in DMSO for the desired time periods (24–72 h). An equal amount of DMSO (vehicle) was present in all the treatment groups including control. Cell lysates were prepared in nondenaturing lysis buffer as reported recently (Zi *et al*., 1998b).

Cell growth assay

Cells were plated at 5000 cells/cm^2, and after 24 h, fed with fresh medium and treated with different doses of silibinin (50, 75 and 100 μg/ml) in complete medium. After 24, 48 and 72 h of these treatments, total cells were collected by brief trypsinization, and washed with PBS. Total cell number was determined by counting each sample in duplicate using a hemocytometer under an inverted microscope. Each treatment and time point had three independent plates. The data shown in this study are the mean of three independent experiments.

FACS analysis for cell cycle distribution

HT-29 cells were grown at similar confluency as in cell growth assay, and treated with desired doses of silibinin (50, 75 and 100 μg/ml) in complete medium for 24 and 48 h. In other experiments, cells were pretreated with 100 μM dose of all caspases inhibitor (Z-Val-Ala-Asp-OMe-FMK, from Enzyme Systems Products, ICN Pharmaceuticals, Inc., Aurora, OH, USA) for 2 h followed by similar silibinin treatment for 48 h. At the end of each treatment time, cells were collected after a brief incubation with trypsin–EDTA followed by processing for cell cycle analysis as reported earlier (Zi *et al*., 1998b). Briefly, 0.5×10^5 cells were suspended in 0.5 ml of saponin/PI

solution (0.3% saponin (w/v), 25 μg/ml PI (w/v), 0.1 mM EDTA and 10 μg/ml RNase (w/v) in PBS), and incubated over night at 4°C in dark. Cell cycle distribution was then analysed by flow cytometry using FACS analysis core facility of University of Colorado Cancer Center.

Assay for alkaline phosphatase

HT-29 cells were treated with desired doses of silibinin for 24, 48 and 72 h as described above. Cell lysates were prepared (in lysis buffer without sodium fluoride and sodium orthovanadate) and 100 μg protein was used from each sample to measure alkaline phosphatase activity. Briefly, alkaline phosphatase activity was assayed by *p*-nitrophenol phosphate hydrolysis (Sigma Diagnostics, St Louis, MO, USA) after incubation at 37°C for 15 min. The optical density of the solution was measured by multiwell plate reader at 410 nm. Protein estimation was performed by the Lowry method using the Bio-Rad DC protein assay kit (Bio Rad Laboratories, Hercules, CA, USA).

Western immunoblotting

At the end of each treatment, cell lysates were prepared in nondenaturing lysis buffer as reported earlier by us (Zi *et al*., 1998b). As needed, 40–80 μg of protein lysates per sample was denatured in 2 × SDS–PAGE sample buffer and subjected to SDS–PAGE on 8, 12 or 16% Tris–glycine gel. The separated proteins were transferred onto nitrocellulose membrane followed by blocking with 5% nonfat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature or overnight at 4°C. Membranes were probed for the protein levels of desired molecules using specific primary antibodies followed by peroxidase-conjugated appropriate secondary antibody, and visualized by ECL detection system.

Immunoprecipitation and kinase assays

CDK2- and cdc2/p34-associated H1 histone kinase activity was determined as described by us recently (Zi *et al*., 1998b; Tyagi *et al*., 2002) with some modifications. Briefly, 200 μg of protein lysates from each sample was precleared with protein A/G-plus agarose beads, and CDK2 and cdc2/p34 protein were immunoprecipitated using anti-CDK2 and cdc2/p34 antibodies (2 μg) and protein A/G-plus agarose beads. The beads were washed three times with lysis buffer and finally once with kinase assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 30 μl of 'hot' kinase solution (0.25 μl (2.5 μg) of histone H1, 0.5 μl of γ-^{32}P-ATP, 0.5 μl of 0.1 mM ATP and 28.75 μl of kinase buffer) for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analysed by 12% SDS–PAGE, and the gel was dried and subjected to autoradiography.

Similarly, to determine the CDK4-associated retinoblastoma (Rb) kinase activity, CDK4 protein was immunoprecipitated using specific antibody, and beads conjugated with antibody and proteins were washed three times with Rb-lysis buffer (50 mM HEPES–KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 80 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 0.1% Tween 20, 10% glycerol, 1 mM PMSF, and 10 μg/ml leupeptin and aprotinin) and once with Rb kinase assay buffer (50 mM HEPES–KOH, pH 7.5, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, 10 mM $MgCl_2$, 0.1 mM sodium orthovanadate and 1 mM DTT). Phosphorylation of Rb was measured by incubating the beads with 30 μl of 'hot' Rb kinase solution (0.25 μl (2 μg) of Rb-GST fusion protein, 0.5 μl of γ-^{32}P-ATP, 0.5 μl of 0.1 mM ATP and 28.75 μl of Rb kinase buffer) for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. Samples were analysed by SDS–PAGE, and the gel was dried and subjected to autoradiography.

Northern blot analysis

Total RNA was isolated from HT-29 cells after 24 h of silibinin (75 and 100 μg/ml) treatment, using TRI Reagent from Sigma (cat # T9424) following the manufacturer's protocol. Total RNA (25 μg) from each sample was electrophoresed on formaldehyde containing agarose gels followed by a transfer onto Hybond-N^+ membranes (Amersham Pharmacia Biotech). The transferred blots were hybridized using ^{32}P-labeled cDNA probes (Gilfix and Eckert, 1985). The probes for Northern hybridization analyses were made using the Rad-Prime DNA labeling system (Life Technologies). All probes were routinely obtained with specific activities of 2–4×10^8 dpm/μg. A 0.5 kb *Eco*R1–*Bam*H1 fragment from plasmid pGEM4-p21, a 0.7 kb *Hind*III–*Bam*H1 fragment from plasmid pSH-UP-GFP-UP-p27 and a 0.4 kb *Eco*R1 fragment from plasmid pCR 2.1-actin were used to make probes to Kip1/p27, Cip1/p21 and β-actin mRNAs, respectively. These plasmids were obtained from Dr James DeGregori and Dr Heide Ford of the UCHSC.

Determination of apoptosis by annexin V staining

To quantify silibinin-induced apoptotic death of human colon carcinoma HT-29 cells, annexin V/PI staining was performed followed by flow cytometry, as described recently (Singh *et al*., 2002b). Briefly, after treatment, both floating and attached cells were pooled and subjected to annexin V/PI staining using the Vybrant Apoptosis Assay Kit2 (Molecular Probes, Inc., Eugene, OR, USA) and following the step-by-step protocol provided by the manufacturer.

Caspase activity assay

Caspase 3 and 9 activities were assayed by the colorimetric protease assay ApoTarget kit (BioSource International, Inc., CA, USA) following the manufacturer's protocol. Briefly, at the end of treatment with silibinin (50–100 μg/ml dose) for 48 h, cells were collected and cell lysates prepared in cell lysis buffer (Tris-buffered saline containing detergent). A measure of 200 μg of protein lysate per sample was mixed with 2 × reaction buffer and 200 μM substrate (DEVD-*p*NA for caspase 3 and LEHD-*p*NA for caspase 9), and incubated at 37°C for 2 h in dark. These assay conditions are standardized, and products of the reaction remain in the linear range of detection. The developed color was measured at 405 nm in a microplate reader, and blank reading was subtracted from each sample reading before calculation.

Preparation of mitochondrial and cytosolic fractions for immunoblot analysis of cytochrome c

At the end of silibinin treatment, cells were washed with ice-cold PBS and collected by a brief trypsinization followed by two more washes with PBS. The cell pellet was resuspended in 200 μl of extraction buffer containing 210 mM manitol, 70 mM sucrose, 20 mM HEPES–KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM PMSF and protease inhibitors (Complete Cocktail; Roche Molecular Biochemicals, Indianapolis, IN, USA). After 20 min incuba-

tion on ice, cells were homogenized with a glass Dounce and pestle, and the resulting homogenates were left on ice for an additional 20 min. The homogenates were centrifuged at 500 *g* to remove the cell debris. The supernatants were further centrifuged at 18 000 *g* for 30 min at 4°C, to yield cytosolic extract (supernatant). Pellets representing mitochondrial fraction were washed with extraction buffer and dissolved in mitochondrial lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EGTA and complete protease inhibitors). A measuere of 70 μg protein per sample was used for SDS–PAGE and Western blotting. The membrane was probed with anticytochrome *c* antibody (BD PharMingen, San Diego, CA, USA) overnight at 4°C followed by 1 h incubation with HRP-conjugated secondary antibody. The protein was visualized by ECL detection system.

Study of bioavailability of silibinin in mice

To study the bioavailability of silibinin, SENCAR male mice (8 weeks old) were purchased from NCI and acclimatized for 1 week in our animal care facility. Mice were then fed with different doses of silibinin in saline by oral gavage for 16 days. Briefly, mice were divided into five groups ($n = 6$ mice/group): Group I, control (saline); Group II, 333 mg/kg silibinin; Group III, 666 mg/kg silibinin; Group IV, 1000 mg/kg silibinin; and Group V, 2000 mg/kg silibinin. The silibinin doses mentioned in each group were fed daily for 16 days, and body weight and diet consumption were monitored during the experiment. At the end of 16 days of treatment, all mice in each group were killed and plasma samples harvested from the blood and processed for the estimation of silibinin concentration by HPLC as described earlier (Singh *et al*., 2002a), using Cancer Pharmacology Core facility of the University of Colorado Cancer Center. Colons were also collected from all the groups of mice, and mucosa was scrapped at 4°C for silibinin analysis. Colon mucosa was thoroughly homogenized in three volumes of 50 mM Tris-HCl (pH 7.4) at 4°C using a Polytron PT-10 homogenizer (VWR Scientific, Painfield, NJ, USA). Tissue homogenates were further processed and analysed by HPLC system as described earlier (Singh *et al*., 2002a), using Cancer Pharmacology Core facility of the University of Colorado Cancer Center.

Statistical and densitometric analyses

The data were analysed using the Jandel Scientific SigmaStat 2.03 software. For all measurements, as needed, Student's *t*-test was employed to assess the statistical significance of difference between control and treated groups. A statistically significant difference was considered to be present at $P < 0.05$. Autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA). The mean density of each band was analysed by the Scion image program (NIH, Bathesda, MD, USA). In each case, blots were subjected to multiple exposures on the film to make sure that the band density is in the linear range. Densitometric data presented below the bands are fold change as compared to control for each treatment. All the cell culture data reported in the present study are representative of at least two to three independent experiments with similar observations.

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