

Silibinin Efficacy against Human Hepatocellular Carcinoma

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Abstract Purpose: Hepatocellular carcinoma (HCC) is one of the most common recurrent malignancies, for which, currently, there is no effective therapy. Considering the antihepatotoxic activity of silibinin, a widely used drug and supplement for various liver disorders, together with its strong preventive and anticancer efficacy against various epithelial cancers, we investigated the efficacy of silibinin against human HCC cells.

Experimental Design: Silibinin effects were examined on growth, cytotoxicity, apoptosis, and cell cycle progression in two different HCC cell lines, HepG2 (hepatitis B virus negative; *p53* intact) and Hep3B (hepatitis B virus positive; *p53* mutated). At molecular level, cell cycle effects of silibinin were assessed by immunoblotting and in-bead kinase assays.

Results: Silibinin strongly inhibited growth of both HepG2 and Hep3B cells with a relatively stronger cytotoxicity in Hep3B cells, which was associated with apoptosis induction. Silibinin also caused G₁ arrest in HepG2 and both G₁ and G₂-M arrests in Hep3B cells. Mechanistic studies revealed that silibinin induces Kip1/p27 but decreases cyclin D1, cyclin D3, cyclin E, cyclin-dependent kinase (CDK)-2, and CDK4 levels in both cell lines. In Hep3B cells, silibinin also reduced the protein levels of G₂-M regulators. Furthermore, silibinin strongly inhibited CDK2, CDK4, and CDC2 kinase activity in these HCC cells.

Conclusion: Together, these results for the first time identify the biological efficacy of silibinin against HCC cells, suggesting the importance of conducting further investigations in preclinical HCC models, especially on *in vivo* efficacy, to support the clinical usefulness of silibinin against hepatocellular carcinoma in addition to its known clinical efficacy as an antihepatotoxic agent.

Hepatocellular carcinoma (HCC) is the fifth most common cancer with more than 1 million fatalities occurring annually worldwide (1). It has been estimated that 17,550 new cases of liver cancer and 15,420 associated deaths will occur in the year 2005 in the United States alone (2). Many factors play a major role in the etiology of HCC; most important of them include hepatitis B and C viruses, alcohol, and aflatoxin exposure (1, 3). Most HCCs, unlike their normal counterparts, are quite resistant to death receptor-mediated apoptosis because cell-surface death receptors are cross-linked with either agonistic antibodies or soluble death ligand proteins (4, 5). HCCs also display high resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated cell death (6, 7), which, together with other apoptosis resistance mechanisms, suggests that alternative approaches are needed to control HCC growth and metastasis.

It is now well established that a variety of cell cycle proteins play an important role in cancer etiology including liver carcinogenesis (8). Amplification of the chromosome 11q13 region occurs in HCC, and cyclin D1 gene maps to this region, suggesting that increased cyclin D1 expression might play an important role in the development of a subset of human HCC by affecting normal cell cycle progression (9, 10). In addition, cyclin E and CDKs are also generally activated or overexpressed in HCC (11–13). Based on these observations, although several clinically used chemotherapeutic drugs are preferably aiming at the cell cycle machinery in HCC (14–16), their outcomes have not been promising, including drug resistance and systemic toxicity specifically at the advanced stage of this deadly malignancy (17–20). Together, these caveats suggest that additional agents that are nontoxic in nature should be evaluated for their effects in modulating cell cycle regulators in HCC cells, causing cell growth inhibition and/or apoptotic death as a resultant biological response for the prevention and/or intervention of human HCC growth. In this regard, natural products are a rich source of pharmacologically active compounds with least toxicity and certain traditionally used herbal medicines and phytochemicals have been shown to inhibit human HCC growth by affecting the cell cycle progression (21, 22).

Extract from the seeds of milk thistle [*Silybum marianum* (L), Gaertn.] is a widely used traditional herbal/dietary supplement around the world including the United States for its strong antihepatotoxic activity against almost any kind of human liver damage/toxicity (23, 24); for the same reason, it is also used clinically at least in Europe and Asia (23). The crude form of

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milk thistle extract, silymarin, and the major pure pharmacologically active flavonoid, silibinin (25), have been shown to be *in vivo* immune-response modifiers (26). In terms of their efficacy against cancer, over the last 15 years, several studies by us and others have shown that both silymarin and silibinin are highly effective in the prevention and intervention of various cancers in both rodent and cell culture models and that their mechanisms of efficacy involve cell cycle arrest and/or apoptosis (27–34). The cancer sites showing silymarin and silibinin efficacy include prostate (27–29), skin (30), breast (31), colon (32), lung, and bladder (33, 34); however, to our knowledge, no study has reported anticancer effects of these agents against HCC. Considering the exceptionally strong antihepatotoxic activity of silibinin both in a clinical setup and as a dietary supplement worldwide and its efficacy against various epithelial cancers, but not HCC, here for the first time we investigated silibinin efficacy against HCC, employing HepG2 and Hep3B cells as model systems. Our results show that silibinin strongly inhibits growth and causes cell cycle arrest in human HCC cells by modulating CDK inhibitor-CDK-cyclin circuitry.

Materials and Methods

Cell lines and reagents. Human HCC cell lines HepG2 and Hep3B were purchased from American Type Culture Collection (Manassas, VA) and cultured in MEM with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin under standard culture conditions (37°C, 95% humidified air and 5% CO₂). Silibinin used in the present study was from Sigma-Aldrich Chemical Co. (St Louis, MO) and analyzed by high-performance liquid chromatography as a pure compound (35). The primary anti-Kip1/p27 antibody was from NeoMarkers (Fremont, CA). Antibodies to CDK2, CDK4, CDK6, cyclin D1, cyclin D3, cyclin E, and Rb-glutathione S-transferase fusion protein were from Santa Cruz Biotechnology (Santa Cruz, CA) and secondary antibodies were from Cell Signaling Technology (Beverly, MA). Antibody for β-actin was from Sigma, histone H1 from Boehringer Mannheim Corp. (Indianapolis, IN), and [γ -³²P]ATP (specific activity, 3,000 Ci/mmol) and enhanced chemiluminescence detection system from Amersham Corp. (Arlington Heights, CA). All other reagents used were of analytic grade.

Cell growth, cytotoxicity, and apoptosis assays. HepG2 and Hep3B cells were plated at 5,000/cm² in 60-mm dishes and, after 24 hours, were treated with different concentrations of silibinin (100, 200, and 300 µmol/L) in DMSO; controls received only DMSO and the final DMSO concentration in all treatments did not exceed 0.15% (v/v). After 12, 24, 48, and 72 hours of these treatments, cells were collected following brief trypsinization, washed twice with ice-cold PBS, and finally counted using a hemocytometer. Trypan blue dye exclusion method was used to determine cell viability. For apoptosis assay, following similar treatments, cells were collected and stained with Annexin V/propidium iodide (Molecular Probes, Inc., Eugene, OR) following the step-by-step protocol of the vendor. The early (Annexin V-stained) and late (both Annexin V- and propidium iodide-stained) apoptotic cell populations were then analyzed immediately by flow cytometry in the fluorescence-activated cell sorting analysis core facility of the University of Colorado Cancer Center (Denver, CO) as published earlier (32).

Cell cycle phase distribution by flow cytometry. HepG2 and Hep3B cells were plated and treated with either DMSO alone or various doses of silibinin, similar to those for cell growth and cytotoxicity studies. After 6, 12, 24, 48, and 72 hours of treatment, cells were collected by trypsinization and $\sim 0.5 \times 10^6$ cells in 0.5 mL of saponin/propidium iodide solution (0.3% saponin, 25 µg/mL propidium

iodide, 0.1 mmol/L EDTA, and 10 µg/mL RNase) were incubated overnight at 4°C in the dark. Cell cycle distribution was then analyzed by flow cytometry (32).

Western immunoblotting and kinase activity assay. HepG2 and Hep3B cells were cultured and treated with DMSO alone or with various doses of silibinin (100, 200, and 300 µmol/L) in DMSO. After 6, 12, 24, and 48 hours of treatment, cell lysates were prepared in nondenaturing lysis buffer as recently published (32). Protein concentration was determined using Bio-Rad detergent-compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA), and as needed, 50 to 70 µg of protein from each lysate sample were denatured in 2× SDS-PAGE sample buffer and subjected to SDS-PAGE on 12% Tris-glycine gel. Separated proteins were transferred onto nitrocellulose membrane followed by blocking with 5% nonfat milk in Tris-buffered saline for 1 hour at room temperature. Membranes were then probed for phosphorylated or total protein levels of desired molecules using specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibody, and visualized by enhanced chemiluminescence detection system. CDK2-, CDK4- and CDC2 (CDK1)-associated kinase activity was determined as described in detail recently (32).

Statistical analysis. Statistical significance of differences between control and treated samples was calculated by Student's *t* test (SigmaStat 2.03, Jandel Scientific, San Rafael, CA). *P* < 0.05 was considered significant. Autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA) and the mean density of each band was analyzed by the Scion Image program (NIH, Bethesda, MD). In each case, blots were subjected to multiple exposures on the film to make sure that the band density is in the linear range. Densitometry data presented below the bands are "fold change" as compared with control for silibinin treatment in each case. Unless otherwise mentioned, all the data shown in the study for cell growth inhibition, cytotoxicity, cell cycle phase distribution, immunoblotting, and kinase activities are representative of two or three independent experiments.

Results

Growth inhibitory, cytotoxic, and apoptotic effects of silibinin in human hepatocellular carcinoma HepG2 and Hep3B cells. As this is the first attempt to assess silibinin efficacy against human HCC, we conducted dose-response and time-response studies to determine the effect of silibinin on HepG2 and Hep3B cell growth inhibition and cytotoxicity. As shown in Fig. 1A and B, silibinin treatment inhibited the growth of both HepG2 and Hep3B cells in a dose- and time-dependent manner in which 100, 200, and 300 µmol/L doses of this agent resulted in 30% to 65% (*P* < 0.001) and 43% to 77% (*P* < 0.001) growth inhibition in HepG2 cells (Fig. 1A) and 45% to 69% (*P* < 0.001) and 40% to 75% (*P* < 0.001) growth inhibition in Hep3B cells (Fig. 1B) after 48 and 72 hours of treatment, respectively. The highest growth inhibitory effect of silibinin was observed at 300 µmol/L dose and maximum treatment time of 72 hours whereas cell growth was minimally affected by the lowest dose (100 µmol/L) in both cell lines. In terms of the cytotoxic effects of silibinin, the highest dose (300 µmol/L) used in the study significantly reduced (*P* ≤ 0.001) the viability of human HCC Hep3B cells even after 12 hours of treatment; however, Hep2G cells were relatively resistant to silibinin as estimated by the trypan blue dye exclusion assay. As shown in Fig. 1C, treatment of HepG2 cells with different doses (100–300 µmol/L) of silibinin for 12 to 72 hours resulted in a cell death comparable to that of DMSO-treated vehicle controls, except treatment for 72 hours at 200 to 300 µmol/L doses that caused 5% to 7% cell death compared with 3% in controls. In

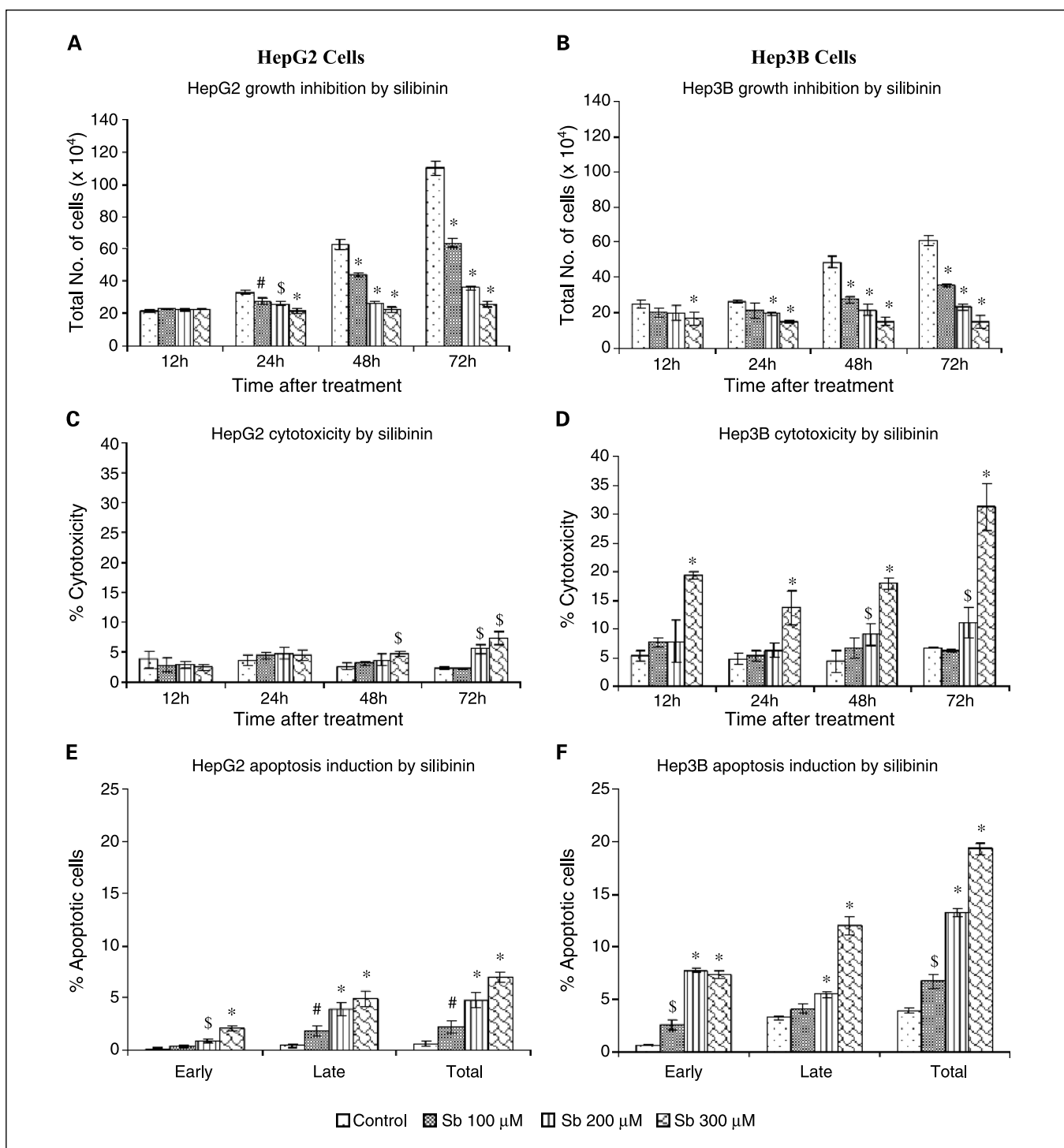


Fig. 1. Growth inhibitory, cytotoxic, and apoptotic effects of silibinin on human hepatocellular carcinoma cells. HepG2 (A) and Hep3B (B) cells were treated with DMSO (control) or different concentrations of silibinin, and after 12, 24, 48, or 72 hours, cells were counted as detailed in Materials and Methods. C and D, trypan blue dye exclusion method was used to evaluate cytotoxicity. E and F, apoptotic effect of silibinin was examined by Annexin V and propidium iodide staining as described in Materials and Methods. Only Annexin V – stained cells were accounted for early apoptotic cells and both Annexin V – and propidium iodide – stained cells were accounted for late apoptotic cells. Columns, mean of three separate plates; bars, SD. Results were reproducible in two additional independent experiments. #, $P \leq 0.05$; \$, $P \leq 0.01$; *, $P \leq 0.001$. The P value is determined by comparing with control group. Sb, silibinin.

case of Hep3B cells, however, similar silibinin treatment showed relatively higher cell death, specifically at 300 μmol/L dose that resulted in 14% to 31% cell death following 12 to 72 hours of treatment (Fig. 1D). The lower silibinin dose

(100 μmol/L), however, also did not show a significant cytotoxic effect in Hep3B cells at all the time points examined in the study. In other studies assessing whether cytotoxicity caused by silibinin in these HCC cells is associated with

apoptotic death, as shown in Fig. 1E and F, silibinin treatment of these cells at 100 to 300 $\mu\text{mol/L}$ doses for 24 to 72 hours resulted in moderate and strong apoptotic deaths (both early and late) of HepG2 and Hep3B cells, respectively, both in a dose- and time-dependent manner (data are shown only for 72-hour treatments). Together, these observations suggest that silibinin causes growth inhibition of both HepG2 and Hep3B cells but is more effective in inducing cytotoxicity and associated apoptotic death in Hep3B cells.

Silibinin induces cell cycle arrest in human hepatocellular carcinoma HepG2 and Hep3B cells. As silibinin treatment showed a strong growth inhibitory affect in both HepG2 and Hep3B cell lines, we next assessed whether this is associated with an arrest in the cell cycle progression of these human HCC cells. Compared with vehicle-treated controls, silibinin treat-

ment induced an accumulation of both HCC cell lines in the G_1 phase of cell cycle (Fig. 2). In the time-response study, the G_1 arrest by silibinin was evidenced in HepG2 cells as early as 12 hours of treatment and remained statistically significant through both 24 and 48 hours of treatment (Fig. 2A-C). The observed G_1 arrest in HepG2 cells by silibinin was also dose dependent, except in a few cases where the highest dose (300 $\mu\text{mol/L}$) also caused a marginal increase in G_2 -M population following 12 hours of treatment, which further increased at 24 and 48 hours (Fig. 2A-C). Overall, the observed cell cycle arrest by silibinin in HepG2 cells was accompanied by a decrease in the S-phase cell population (Fig. 2A-C). Unlike the results in HepG2 cells, in case of Hep3B cells, silibinin treatment resulted in both G_1 and G_2 -M arrests where 200 and 300 $\mu\text{mol/L}$ doses showed considerable effect following

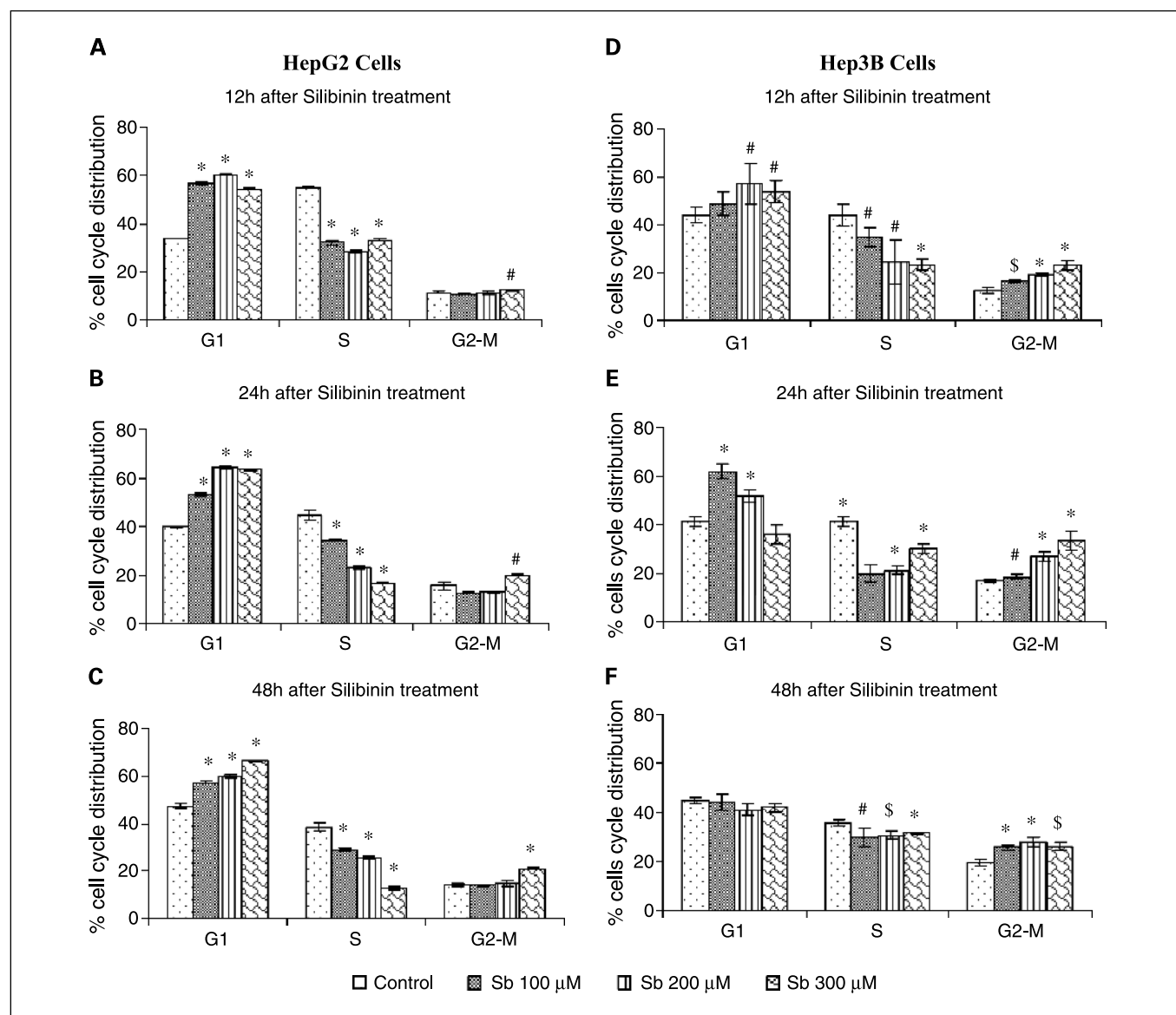
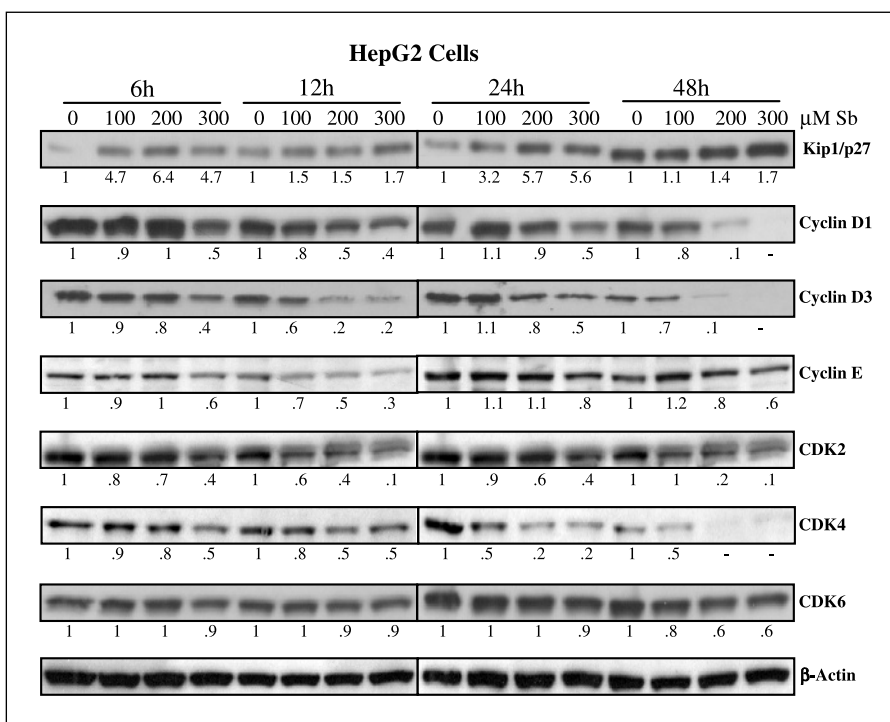


Fig. 2. Cell cycle inhibitory effect of silibinin on human hepatocellular carcinoma cells. The cells were cultured as described in Materials and Methods and treated with either DMSO alone (control) or varying concentrations of silibinin for 12, 24, 48, and 72 hours. Cells were then collected and incubated overnight with saponin/propidium iodide solution and subjected to fluorescence-activated cell sorting analysis. Silibinin induces G_1 phase arrest in HepG2 cells (A-C) and G_1 as well as G_2 -M arrest in Hep3B cells (D-F). Columns, mean of three independent plates; bars, SD; the results were reproducible in two additional independent experiments. , $P \leq 0.05$; \$, $P \leq 0.01$; *, $P \leq 0.001$. The P value is determined by comparing with control group.

Fig. 3. Silibinin induces Kip1/p27 protein level and modulates the expression of G₁ phase cyclins and CDKs in human HCC HepG2 cell line. Cells were cultured as described in Materials and Methods and treated with either DMSO (control) or varying concentrations of silibinin. Lanes 0, DMSO alone. Total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting. Membranes were probed with antibodies to Kip1/p27, CDK2, CDK4, CDK6, cyclin D1, cyclin D3, cyclin E, and β -actin, followed by peroxidase-conjugated appropriate secondary antibodies, and visualized by enhanced chemiluminescence detection system. The densitometric data (arbitrary) shown under the immunoblots are presented as fold changes as compared with their respective control.



12 hours of treatment; 24 hours of silibinin treatment caused strong G₁ arrest at 100 and 200 μ mol/L doses together with a stronger G₂-M arrest at 200 and 300 μ mol/L doses (Fig. 2D-F). When cells were treated for longer time (48 hours) with these silibinin doses, there was no difference in the cell cycle distribution of control and treated Hep3B cells with respect to G₁ phase; however, all doses showed a strong and statistically significant increase in G₂-M cell population (Fig. 2D-F).

Silibinin induces cyclin-dependent kinase inhibitor Kip1/p27 but decreases protein levels of G₁ regulatory cyclin-dependent kinases and cyclins in human hepatocellular carcinoma HepG2 and Hep3B cells. Based on the above findings showing that silibinin causes cell cycle arrests in both HepG2 and Hep3B cells, we first studied mechanisms of these effects by examining the effect of silibinin on cell cycle regulatory molecules involved in the G₁ phase of the cell cycle progression. As shown in Figs. 3 and 4, silibinin treatment of HepG2 and Hep3B cells resulted in a strong increase in the protein levels of Kip1/p27 in a dose-dependent manner as quantified by the densitometry of the immunoblots. Interestingly, the effect of silibinin on Kip1/p27 induction in HepG2 cells seems biphasic, causing stronger effect at 6 and 24 hours as compared with that at 12 and 48 hours (Fig. 3). In case of Hep3B cells, however, silibinin showed a dose-response effect at 6 hours that reduced to a second or third level at 12 and 24 hours, but surprisingly, 48 hours of treatment caused 6- to 10-fold increase in Kip1/p27 levels (Fig. 4). The observed induction in Kip1/p27 protein levels by silibinin was not due to a change in protein loading as confirmed by probing of the membrane with anti- β -actin antibody.

In other studies assessing the effect of silibinin on the protein levels of G₁ cyclins and CDKs, it strongly decreased their levels in both HepG2 and Hep3B cells in a dose-dependent manner and, in some cases, in a time-dependent manner (Figs. 3 and 4). In HepG2 cells, silibinin treatment at the 200 or 300 μ mol/L dose

resulted in a strong decrease in the levels of cyclin D1, cyclin D3, and cyclin E at almost all the time points starting at 6 hours to 48 hours (Fig. 3). Identical silibinin treatment in Hep3B cells also caused a strong down-regulation in the protein levels of all these cyclins with a better magnitude of effect in this cell line compared with HepG2 (Fig. 4); even lower silibinin dose (100 μ mol/L) was effective in reducing the levels of these cyclins in Hep3B cells (Fig. 4). In terms of its effect on CDK levels, silibinin also caused a strong decrease in CDK2 and CDK4 levels in both the cell lines in a dose- and/or time-dependent manner; however, it showed marginal effect on the reduction of CDK6 level only in HepG2 cells following 48 hours of treatment (Figs. 3 and 4).

Silibinin decreases protein levels of G₂-M regulators in human hepatocellular carcinoma Hep3B cells. Because treatment of Hep3B cells with silibinin resulted in an increase in G₂-M phase cell population, we next assessed the effect of silibinin on the molecules involved in the regulation of G₂-M phase of the cell cycle. These studies were done only in Hep3B cells as we observed a strong G₂-M arrest only in these cells by silibinin. Complex of CDC2 with cyclin B is known to perform an important function in controlling the G₂-M phase where the dephosphorylated form of CDC2 is active and the Ser¹⁵ phosphorylated form is inactive, thereby regulating the entry of cells into M phase, and the CDC25C phosphatase and Wee1 kinases are responsible for the dephosphorylation and phosphorylation of CDC2, respectively (36). As shown in Fig. 5, silibinin treatment of Hep3B cells resulted in a decrease in pCDC25C (Ser²¹⁶), CDC25C, pCDC2 (Tyr¹⁵), CDC2, and cyclin B1 protein levels without any noticeable change in Wee1 levels. Membranes were stripped and reprobed for β -actin as loading control (Fig. 5).

Silibinin inhibits cyclin-dependent kinase activity in human hepatocellular carcinoma HepG2 and Hep3B cells. To further

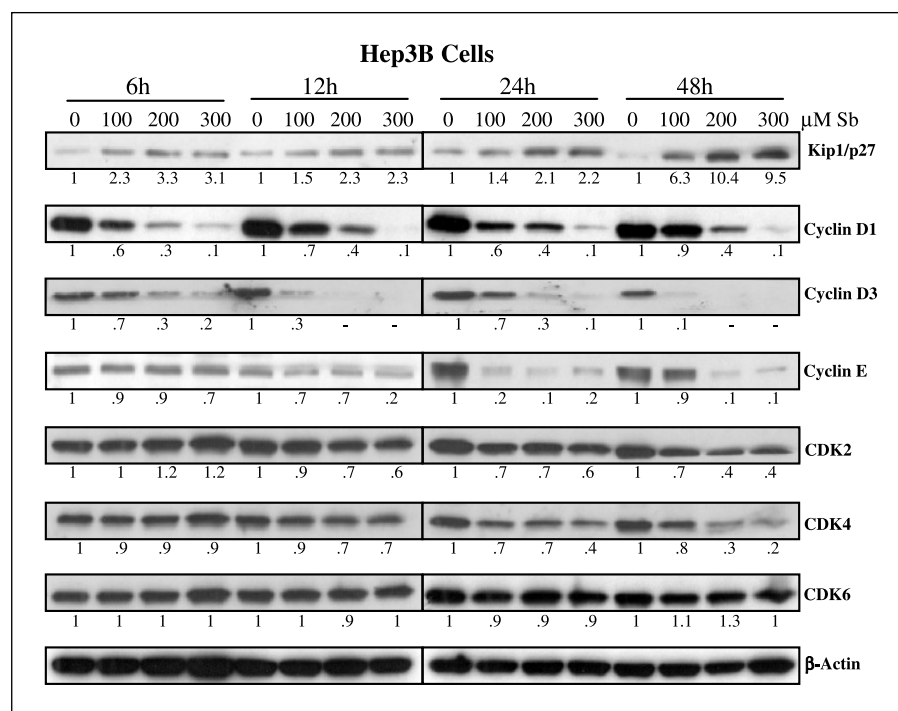


Fig. 4. Silibinin induces Kip1/p27 protein level and modulates the expression of G₁ phase cyclins and CDKs in human HCC Hep3B cell line. Cells were cultured as described in Materials and Methods and treated with either DMSO (control) or varying concentrations of silibinin. Different treatments are as labeled in the figure; lanes 0, DMSO alone. Total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting. Membranes were probed with antibodies to Kip1/p27, CDK2, CDK4, CDK6, cyclin D1, cyclin D3, cyclin E, and β-actin, followed by peroxidase-conjugated appropriate secondary antibodies, and visualized by enhanced chemiluminescence detection system. The densitometric data (arbitrary) shown under the immunoblots are presented as fold changes as compared with their respective control.

study the functional significance of the observed effects of silibinin on Kip1/p27 induction together with a decrease in cyclins and CDKs involved in G₁ and G₂-M phases of the cell cycle, we finally assessed the effect of silibinin on the kinase activity of the CDKs. As shown in Fig. 6A and B, compared with DMSO-treated vehicle controls, silibinin treatment of both HepG2 and Hep3B cells resulted in a strong inhibition of CDK2 and CDK4 kinase activity as evidenced by a reduction in phosphorylation of histone H1 and Rb-glutathione S-transferase fusion protein used as substrates, respectively. In addition, as shown in Fig. 6C, the kinase activity of CDC2 was also strongly inhibited following silibinin treat-

ment of Hep3B cells. Taken together, these results suggest that alterations in the levels of cell cycle regulators by silibinin play a major role in its effect on human HCC cells in terms of cell cycle arrest and cell growth inhibition together with possible apoptosis induction.

Discussion

The results of the present study show that silibinin has a strong anticancer activity against human HCC cells, which is associated with alteration in cell cycle machinery, leading to cell cycle arrests at G₁ phase in HepG2 cells and at G₁ as well as

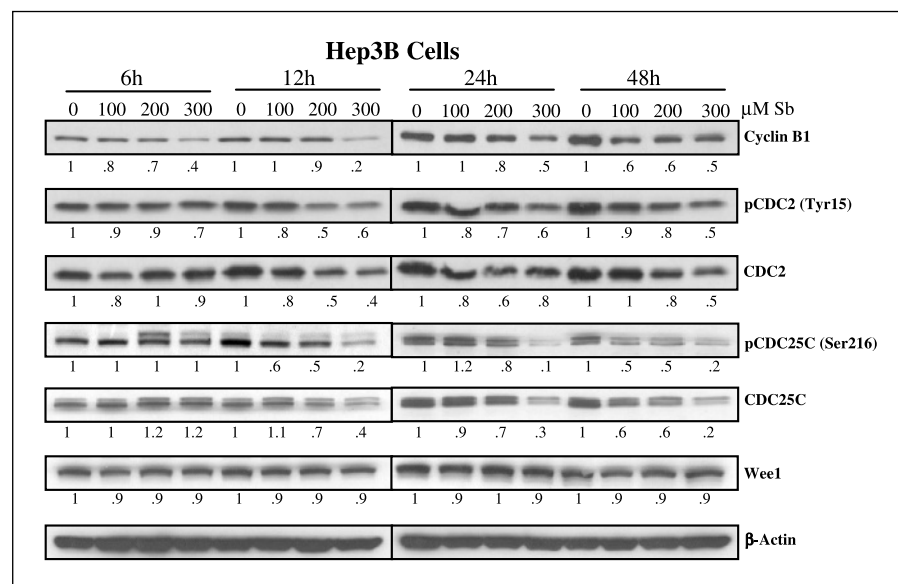


Fig. 5. Silibinin modulates the protein expression of G₂-M cell cycle regulators in Hep3B cells. Cells were cultured and treated as described in Fig. 4. Total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting. Membranes were probed with antibodies to pCDC25C (Ser²¹⁶), CDC25C, pCDC2 (Tyr¹⁵), CDC2, cyclin B1, and β-actin, followed by peroxidase-conjugated appropriate secondary antibodies, and visualized by enhanced chemiluminescence detection system. The densitometric data (arbitrary) shown under the immunoblots are fold changes as compared with their respective control.

G₂-M phases in Hep3B cells. Hep3B cell line is positive for hepatitis B viral genome and *p53*, one of the most important tumor suppressor genes, is deleted in it (37, 38). In HepG2 cells, however, there is no integration of hepatitis B viral genome and the *p53* gene is intact. It is interesting in this regard that among other etiologic factors, hepatitis B viral infection ranks first in Asia and Africa, accounting for ~80% in the causation of HCC (39). Retinoblastoma (pRb), the phosphorylation status of which regulates the restriction point in late G₁ and decides whether the cell has to divide or not, is intact in both the cell lines at genetic level and carries a defect in transcription in Hep3B cells, causing a lack of both mRNA and protein expression (37).

As it was evidenced by the fluorescence-activated cell sorting analysis that silibinin treatment caused G₁ arrest in HCC cell lines, we conducted a detailed analysis of the molecular mechanism involved in cell cycle regulation. The D-type cyclins and CDK4/CDK6 complex that drive cells through the G₁ restriction point play an important role in G₁-S transition by phosphorylating pRb (40). When it is phosphorylated by cyclin-CDK complex, E2F, the transcription factor essential for genes that are required for S phase transition, is released from the Rb-E2F complex (41). This facilitates the transcription of the desired genes, one of them being the gene for cyclin E, and the cell cycle proceeds to S phase (42). Silibinin treatment was effective in down-regulating the level of the cell cycle proteins, such as cyclin D1, cyclin D3, and CDK4, which are involved in G₁-S progression. Further downstream, silibinin effectively inhibited pRb phosphorylation as estimated by the *in vitro* kinase activity of CDK4 for Rb-glutathione S-transferase phosphorylation. Cyclin E is expressed later in G₁ and forms an active complex with CDK2 and is required for the G₁-S transition and initiation of DNA synthesis (40, 42). In the present study, we observed that silibinin treatment reduced the levels of cyclin E and CDK2 as well as the kinase activity associated with CDK2 in HCC cells, which might have resulted in the accumulation of cells in G₁ phase rather than to progression to S phase. These effects of silibinin in HCC cells were comparable to those reported earlier in prostate (28, 29), breast (31), colon (32), and bladder (34) cancer cells.

In addition to the CDKs regulation by phosphorylation, their activity is also negatively controlled by the binding of inhibitory proteins called CDK inhibitors to CDK/cyclin complexes, in which the Waf/Kip family of CDK inhibitors (e.g., Kip1/p27) plays a pivotal role (43, 44). Inactivation of Kip1/p27 is believed to be one of the fundamental steps in carcinogenesis, and a decreased Kip1/p27 expression was correlated with advanced tumor stages and poorer disease-free survival of patients with HCC at a variety of evolutionary stages (45, 46). Furthermore, HCC patients with higher expression levels of the Kip1/p27 protein experience longer disease-free survival and acquired Kip1/p27 expression is considered to be a favorable independent prognostic variable for HCC (47–49). Consistent with these facts, our study also revealed that the induction of Kip1/p27 by silibinin would have positively contributed to the G₁ accumulation because silibinin treatment of HCC cell lines, HepG2 and Hep3B, showed a strong G₁ arrest and a consistent increased expression of Kip1/p27 protein. These effects of silibinin were also in accord with its previously reported mechanisms of action in other cancer cells (28–34).

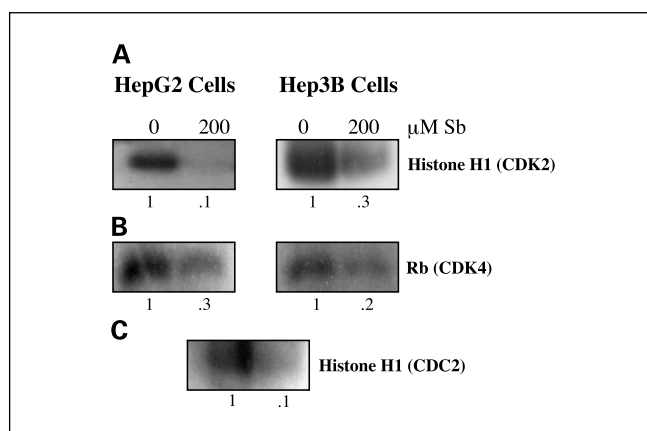


Fig. 6. Silibinin inhibits CDK2, CDK4, and CDC2 kinase activity in human HCC cells. Cells were cultured and treated with either DMSO or 200 μmol/L silibinin for 24 hours, and at the end of treatments, total cell lysates were prepared. Lanes 0, DMSO alone. For kinase activity, CDK2, CDK4, and CDC2 were immunoprecipitated from total cell lysates by using specific antibodies followed by kinase assay employing histone H1 (for CDK2 and CDC2) or Rb-glutathione S-transferase (for CDK4) substrate as described in Materials and Methods. *A* and *B*, CDK2 and CDK4 kinase activity, respectively, in HepG2 and Hep3B cells; *C*, CDC2 kinase activity in Hep3B cells. The densitometric data (arbitrary) shown under the autoradiograms are fold changes as compared with their respective control.

The G₂-M transition requires activity of CDK1 (CDC2), which is positively regulated by the association with cyclin B1 (50). The phosphorylation (by Wee1) and dephosphorylation (by CDC25C) of Thr¹⁴ and Tyr¹⁵ are also crucial in regulating CDC2-associated kinase activity (50, 51). In cell cycle analysis, we observed that silibinin causes a prominent increase in G₂-M cell population in Hep3B cells as compared with HepG2 cells. To find out the molecular mechanisms that play a role in silibinin-induced G₂-M arrest in Hep3B cells, we analyzed the expression and phosphorylation levels of these G₂-M proteins. Silibinin treatment reduced cyclin B1 as well as phospho- and total levels of both CDC2 and CDC25C without any effect on Wee1 level. Kinase activity of CDC2 was reduced by silibinin treatment. Although these results partially explain how Hep3B cells arrest in G₂-M phase following silibinin treatment, more studies are needed to further identify a G₂-phase arrest versus an M-phase arrest and the role of CDC25C and CDC2 activation and inactivation in this process. There are reports showing that G₂-M arrest is mostly followed by an enhanced cytotoxicity (52). Consistent with such reports, silibinin showed increased cytotoxicity in Hep3B cells which was associated with an induction of apoptosis. Because Hep3B cells are mutated in *p53*, it could be likely that silibinin has greater G₂-M arrest (as well as apoptosis)-inducing effect in such cells as compared with those having intact *p53*, such as HepG2 cells. However, more studies are needed to examine the role of *p53* in silibinin-induced cell cycle arrest and apoptosis in HCC cells.

In conclusion, silibinin exerts strong anticancer activity against human HCC cells by modulating cell cycle and associated proteins causing growth inhibition as well as apoptotic death, as it does for many other human cancer cells. These encouraging results suggest the importance of conducting further investigations in preclinical HCC models, especially on *in vivo* efficacy, to support the clinical usefulness of silibinin against hepatocellular carcinoma in addition to its known clinical efficacy as an antihepatotoxic agent.

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