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 antihapatotoxic 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 antihapatotoxic agent.
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 antiproliferative 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 0.5 × 106 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
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 μmol/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 treatment induced an accumulation of both HCC cell lines in the G1 phase of cell cycle (Fig. 2). In the time-response study, the G1 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 G1 arrest in HepG2 cells by silibinin was also dose dependent, except in a few cases where the highest dose (300 μmol/L) also caused a marginal increase in G2-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 G1 and G2-M arrests where 200 and 300 μmol/L doses showed considerable effect following a 12-hour treatment.

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 G1 phase arrest in HepG2 cells (A-C) and G1 as well as G2-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 ≤ 0.01; ** P ≤ 0.001. The P value is determined by comparing with control group.
12 hours of treatment; 24 hours of silibinin treatment caused strong G1 arrest at 100 and 200 μmol/L doses together with a stronger G2-M arrest at 200 and 300 μmol/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 arrest 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 μmol/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 μmol/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 Weel 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 (Ser116), CDC25C, pCDC2 (Tyr15), CDC2, and cyclin B1 protein levels without any noticeable change in Weel 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
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...
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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 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).

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 phase population in Hep3B cells 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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