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ORIGINAL ARTICLE

Inhibitory effects of Silibinin combined with doxorubicin in hepatocellular carcinoma; an *in vivo* study

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Summary

Purpose: *Hepatocellular carcinoma (HCC) is the one of the most common cancers and the third leading cause of cancer related mortality in the world. Unacceptable side effect and development of treatment resistance are the major concerns with the conventional chemotherapeutic agents. Combination therapy using phytotherapeutic agents is attracting the attention of investigators in view of the current needs.*

Methods: *In the present study we have evaluated the synergistic effect of silibinin, a nontoxic phytotherapeutic agent in combination with doxorubicin, in advanced HCC using HEPG2 cells and an orthotopic rat model of HCC.*

Results: *The results showed that silibinin strongly synergized with doxorubicin-induced growth inhibition, G2-M arrest, and apoptosis of HEPG2 cells. Silibinin-doxorubicin combination also inhibited cdc2/p34 kinase activity when histone H1 was used as substrate. The combination regimen also moderately increased the expression of cdc25C-cyclin B1-cdc2/p34 associated upstream kinases (Chk1). Simultaneous treatment with silibinin-doxorubicin combination showed a 41% increase in the apoptotic cell death (p=0.01), which was 3-fold higher than what was observed with silibinin or doxorubicin individually. In the orthotopic rat model treatment with silibinin-doxorubicin reduced tumor growth by close to 30% at nearly twice lower dose of individual drugs in the combination group.*

Conclusions: *Our study suggests that combination therapy using silibinin-doxorubicin may show a better therapeutic efficacy in patients with HCC. These findings need to be further validated in human clinical trials.*

Key words: *doxorubicin, hepatocellular carcinoma, liver cancer, phytotherapeutics, silibinin*

Introduction

HCC is one of the most common cancers and the third leading cause of cancer related mortality in the world [1,2]. Despite advancements in early diagnosis, its prognosis remains poor. There are about 35 million new cases diagnosed each year and over 600,000 patients die as a result of liver cancer annually [3]. Current therapeutic options for HCC include chemotherapy, radiotherapy, and surgery. However, with conventional chemotherapy there is a major risk of developing resistance to treatment, leading to failure and death [4]. Doxorubicin is an agent widely used for the treatment of patients with solid tumors [5,6]. However, the effectiveness of doxorubicin in HCC patients is limited, because its sub effective doses due to systemic toxicity [5]. Owing to these limitations, there has been an increased scientific interest in the use of other approaches, possibly active in HCC. Combination therapy is one such approach which aims to decrease the side effects of chemotherapeutic agents and at the same time maintaining the effectiveness of the drug [7,8]. In this regard, phytotherapeutic agents possessing anticancer efficacy, limited toxicity and synergistic effect with known chemotherapeutic agents are ideal candidates and attracted much interest of the scientific community in recent years [9-12].

Silibinin, an antioxidant flavonoid, extract-

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ed from *Silybum marianum* L. (milk thistle plant) has been reported to show therapeutic efficacy in cancers of epithelial origin, such as liver cancer [12-18]. In recent times therapeutic efficacy of oral silibinin on a solid tumor xenograft in athymic nude mice has also been reported and found to be free of adverse events in humans [19-21]; there is no known LD_{50} for silibinin in laboratory animals [22,23].

In the present study we have evaluated the synergistic effect of a nontoxic phytotherapeutic agent (silibinin) with doxorubicin in advanced HCC using HEPG2 cells and an orthotopic rat model of HCC. The results obtained clearly show that silibinin strongly synergizes with doxorubicin to induce apoptosis, G2-M arrest and doxorubicin-induced growth inhibition. These results further support the hypothesis that lower doses of doxorubicin in combination with silibinin increases the efficacy of the former in patients with advanced HCC.

Methods

Cell lines and reagents

The human liver cancer cell lines HepG2 and Morris hepatoma (MH)-3924A cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cells were maintained in DMEM medium (Gibco, USA), supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), 10% fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel), and 5% CO_2 at 37°C. Silibinin was purchased from Sigma Chemical Co. (St. Louis, MO), and its purity was 100%. Doxorubicin was obtained from Cetus Oncology (Emeryville, CA). Antibodies to Chk1, cdc25C, cdc2/p34, and cyclin B1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was from Sigma Chemical Co. (St. Louis, MO).

Cell growth assay

Combination index (CI)/isobologram method [24] was used to analyze the combined growth-inhibitory effect. Under the standard culture conditions, cells were plated at 5000 cells/cm^2 in 60-mm dishes as described above. One day after the initial seeding, cells were treated with either DMSO alone (0.1% v/v, control), different doses of silibinin (10-100 µM) dissolved in DMSO or doxorubicin (10-100 nM) alone, or both agents in different combinations. Each treatment and time point had three plates. After 48 hrs of these treatments, both attached and floating cells were collected by trypsinization and counted using a hemocytometer. On the basis of the results of these experiments, combinations of 100 µM silibinin with 25 nM doxorubicin were further studied, where cells were treated with silibinin or doxorubicin either alone or first with silibinin and 24 hrs later with doxorubicin, or vice versa, or both agents together for a total of 48 hrs, and cell growth and viability were determined using cell proliferation assay kit (Abcam, Cambridge, UK).

Cell cycle analysis

HepG2 cells at 60% confluency were treated with silibinin and doxorubicin either alone or in combination for 48 hrs. At the end of treatment, cells were processed and incubated in saponin/PI solution [0.3% saponin (w/v), 25 µg/ml PI (w/v), 0.1 mM EDTA, and 10 µg/ml RNase in PBS] at 4°C for 24 hrs in the dark. Cell cycle distribution was then analyzed by flow cytometry.

Western blotting

For cell cycle regulatory molecules, at the end of treatment, attached cells were washed twice with PBS, and cell lysates were prepared in non-denaturing lysis buffer as described earlier [17]. For immunoblot analysis, 70 µg of protein lysates/sample were denatured in 2X SDS-PAGE sample buffer and subjected to SDS-PAGE on 12% Tris-glycine gel. The separated proteins were transferred on to nitrocellulose membrane followed by blocking with 5% nonfat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) for 1 hr at room temperature or overnight at 4°C. Membranes were probed for the protein levels of Chk1, cdc25C, cdc2/p34, and cyclin B1 using specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibody, and visualized by the ECL detection system.

Cdc2/p34 kinase assay

Cdc2/p34-associated H1 histone kinase activity was determined as described recently by our team [17] with some modifications. Briefly, 200 µg of protein lysates from each sample were precleared with Protein A/G-plus agarose beads, and cdc2/p34 protein was immunoprecipitated using anti-cdc2/p34 antibody (2 µg) and Protein A/G-plus agarose beads. Beads were washed three times with lysis buffer and finally once with kinase assay buffer [50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 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 [-32P] ATP (specific activity, 3000 µCi/mmol), 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 samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography.

Apoptotic cell death assay

To quantify apoptosis, HepG2 cells were stained with annexin V and propidium iodide (PI) using Vy-

brant Apoptosis Assay Kit2 from Molecular Probes, Inc. (Eugene, OR) following the step-by-step protocol as provided by the manufacturer and analyzed by flow cytometry. Briefly, at the end of treatment with 100 μM silibinin and 25 nM doxorubicin either alone or in combination for 48 hrs, both floating and attached cells were collected, washed twice with PBS, and subjected to annexin V (conjugated with fluorophores and Alexa fluro 488 dye) and PI staining. Flow cytometry was performed within 30 min with a 488-nm line of an argon-ion laser for excitation.

Animal experiments

All the experimental procedures involving animals were conducted under a protocol reviewed and approved by the Ethics Committee of Zhumadian Center Hospital, Yicheng, China. All efforts were made to minimize the number of animals used and to ameliorate their suffering. An orthotopic rat model of HCC was used, in which subcutaneously injected Morris hepatoma (MH)-3924A cells ($5x10^6$) in syngeneic ACI rats (Harlan, USA) led to formation of a subcutaneous tumor within 14 days. Subsequently, the livers of 12 to 18 weeks old male ACI rats were surgically implanted with portion of the tumor as described by Vives et al. [25]. On day 5 after tumor implantation, the rats were randomized into three groups of 6 animals each receiving placebo (normal saline), doxorubicin (2mg/kg), or the combination of Silibinin (1.9mg/kg) and doxorubicin. The agents were administered by intraperitoneal injection once a week for five weeks. Rats were euthanized on day 40 after the first injection. Serum, plasma, tumors and livers were harvested. Tumor volume was calculated ex vivo.

Statistical and densitometric analyses

The data were analyzed using SigmaStat 2.03 software. Statistical significance of differences was assessed by Student's t test and it was set at $p<0.05$. Autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA) and adjusted for brightness and contrast for minimum background. The mean density of each band was analyzed by the Scanimage program (NIH, Bethesda, MD). In densitometric data presented below the bands are fold change as compared with control.

Results

Synergistic effect of silibinin-doxorubicin combination on HEPG2 cell growth inhibition

To investigate the effect of silibinin and doxorubicin combination on HEPG2 cell growth, cells in the exponential growth phase were treated with different doses of silibinin (10, 50, and 100 μM) and doxorubicin (10, 25, 50, and 100 nM) for 48 hrs. At the end of treatment, a dose dependent inhibition of cell growth was observed, accounting for 21-43% and 39-81% inhibition, respectively (Figure 1). On the basis of these results, we used 50 and 100 μM doses of silibinin (for 48 hrs) in combination with 25 nM dose of doxorubicin (added for the last 24 hrs), which showed a strong synergistic growth inhibition of HepG2 cells (CI= 0.589, 0.173), where CI<1 means synergism, CI=1 means additive effects, and CI>1 means antagonism. Based on this study a dose of 100 μM silib-

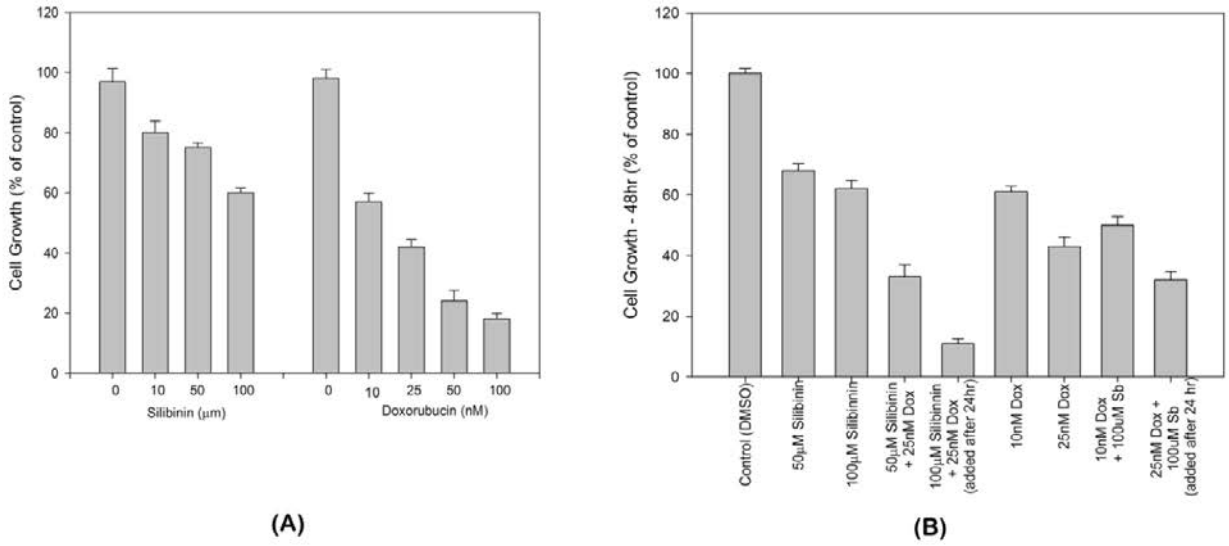

Figure 1. Effect of silibinin and doxorubicin alone and in combination on HEPG2 cell growth. **A:** Cells were treated with 0 (DMSO control), 10, 50, and 100 μM silibinin or 10, 25, 50, and 100 nM doxorubicin alone for 48 hrs. **B:** control (DMSO), 50 and 100 μM silibinin alone, and in combination with 25 nM doxorubicin, 10, and 25 nM doxorubicin alone and in combination with 100 μM/silibinin, which was added for the last 24 hrs. Data are the means of triplicate samples for each treatment. Error bar represents SD (p = 0.040).

inin and 25 nM doxorubicin was used for further experiments.

Synergistic effect of silibinin-doxorubicin on selective G2-M arrest in HepG2 cells

To further study the synergistic effect of silibinin-doxorubicin combination we investigated the effect of 100 μM silibinin and 25 nM doxorubicin, either alone or in combination on cell cycle progression in HEPG2 cells. Treatment with silibinin showed a G1 arrest of 56% compared to 45% in the control; treatment with doxorubicin showed 41% G2-M arrest compared to 20% in the control after 48 hrs of treatment. There was a decrease in the S-phase cells on treatment with silibinin, while treatment with doxorubicin showed decrease in G1 population (Figure 2). Interestingly, addition of doxorubicin after treatment with silibinin increased silibinin-induced G1 arrest to a predominantly G2-M arrest (61 vs 21% in control). Similar pattern was observed when silibinin treated cells were further exposed to doxorubicin which showed increase in G2-M arrest to 61% compared to 42% in doxorubicin alone. On simultaneous addition of silibinin and doxorubicin to HEPG2 cells predominant G2-M arrest (85%) was observed. Therefore, our results showed that that combination of silibinin and doxorubicin causes a significant G2-M arrest compared to cell cycle arrest observed individually. Therefore, the cell growth inhibition of HEPG2 could be attributed to the cell cycle arrest at the G2-M check point.

Effect of silibinin-doxorubicin combination regimen on the G2-M cell cycle regulators

On observing strong G2-M arrest with silibinin-doxorubicin combination we further investigated the molecular events leading to this modulation. Immunoblot analysis showed that treatment with 100 μM silibinin and 25 nM doxorubicin for 48 hrs showed a prominent increase in cdc25C, cdc2/ p34, and cyclin B1 protein levels compared to what was observed with these compounds individually (Figure 3). Silibinin-doxorubicin combination also inhibited cdc2/p34 kinase activity when histone H1 was used as substrate. The combination regimen also moderately increased the expression of cdc25C-cyclin B1-cdc2/ p34 associated upstream kinases (Chk1). Based on these observations we postulated that down-regulation of the Chk1/2-cdc25C-cyclin B1-cdc2/p34 pathway could be a possible mechanism associated with the synergistic effect of silibinin-doxoru-

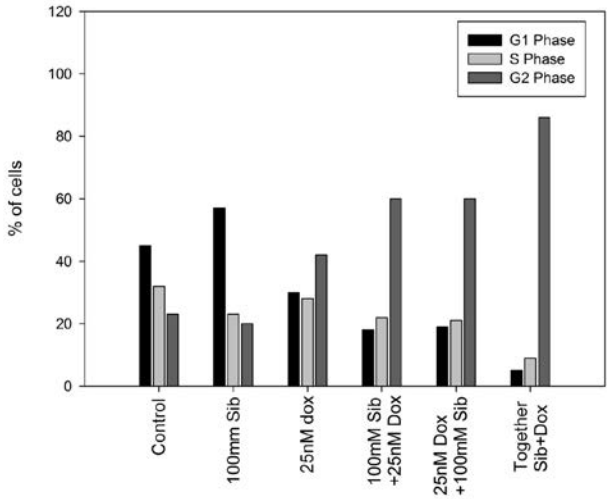

Figure 2. Effect of silibinin and doxorubicin alone and in combination on the cell cycle progression of HEPG2 cells. Cultured cells were treated with either DMSO alone (control); 100 μM silibinin (Sib); 25 nM doxorubicin (dox), 100 μM Sib and 24 hrs later 25 nM dox; 25 nM Dox and 24 hrs later 100μM Slb, or both 100 μM Sib and and 25 nM Dox together for a total of 48 hrs. At the end of treatments, cells were harvested and stained with saponin and PI. The percent of cells in G1 phase was calculated (p = 0.038).

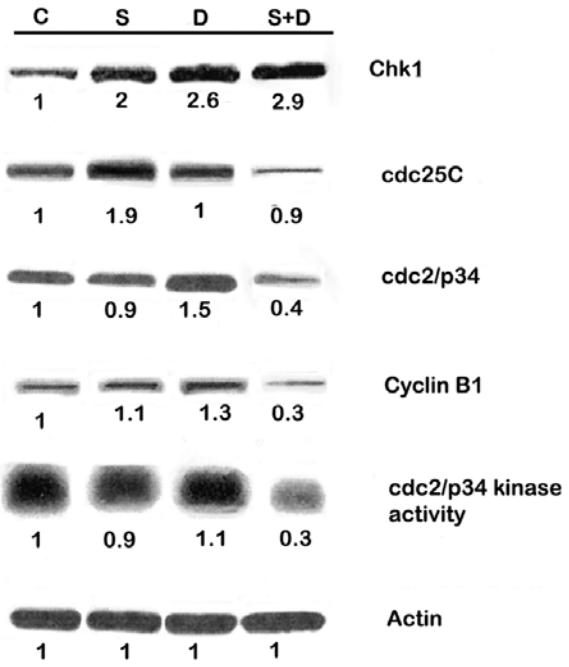

Figure 3. Effect of silibinin and doxorubicin alone and in combination on the expression of G2-M cell cycle regulators in HEPG2 cells. Cultured cells were given the following treatment: DMSO alone in the control (C) group, 100μM silibinin (S), 25 nM doxorubicin (D) and combination of both (100μM S and 25 nM D) for 48 hrs. At the end of treatment total protein was subjected to SDS-PAGE and Western immunoblotting and probed with anti Chk1, cdc25C, cdc2/p34, Cyclin B1, cdc2/p34 kinase activity and actin antibodies (p= 0.029).

bicin combination on G2-M arrest and apoptotic cell death.

Effect of silibinin-doxorubicin combination on the apoptotic death of HEPG2 cells

Using quantitative apoptotic cell death assay we investigated whether increased G2-M cell cycle arrest also resulted in an increased apoptotic cell death. There was an increase in the apoptotic cell death from 9% in the control to 13% in the silibinin treated cells and 11% in the doxorubicin treated cells, after 48 hrs. Addition of silibinin to the doxorubicin treated cells showed a significant increase of 22% in the apoptotic cell death ($p<0.05$) (Figure 4). However, when doxorubicin was added to silibinin treated cells the increase in apoptotic cell death was not significant ($p=0.11$). Furthermore, when silibinin-doxorubicin combination was simultaneously added to HEPG2 cells for 48 hrs there was a 41% increase in the apoptotic cell death ($p=0.01$), which was 3-fold higher than what was observed with silibinin or doxorubicin individually. In view of the observed findings, we further postulate that the biological activity of silibinin-doxorubicin combination in HEPG2 cells could due to apoptosis.

In vivo evaluation

The *in vivo* effect of treatment with silibinin plus doxorubicin combination was analyzed in an orthotopic rat model of HCC. Upon 5-week treatment adverse effects such as weight loss or hyper or hypo-activity was observed. In the HCC rat model, administration of doxorubicin was found to inhibit 10% of tumor growth (Figure 5). Treatment with silibinin-doxorubicin reduced tumor growth by close to 30%. Statistical analysis showed that the result for the group treated with the combination of silibinin-doxorubicin was statistically significant ($p=0.019$), whereas for the group treated with doxorubicin alone, it was close to being statistically significant ($p=0.053$). Thus, the combination of silibinin with doxorubicin slowed the progression of HCC in rats more significantly than doxorubicin alone, nearly twice lower dose of individual drugs in the combination group.

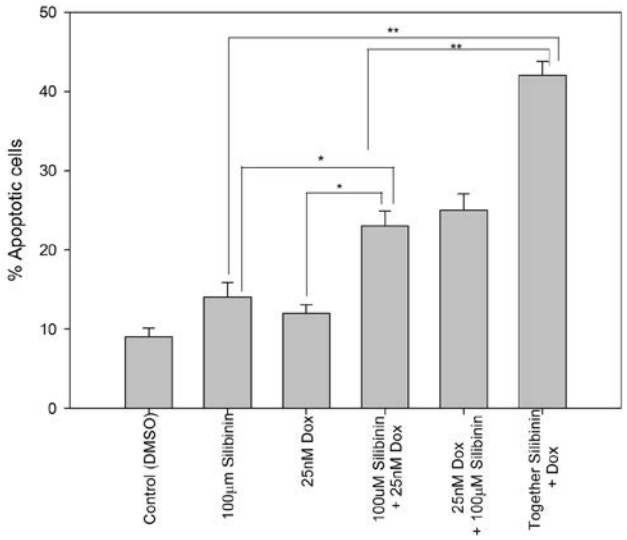

Figure 4. Effect of silibinin and doxorubicin alone and in combination on apoptotic death in HEPG2 cells; *p= 0.029; **p = 0.01.

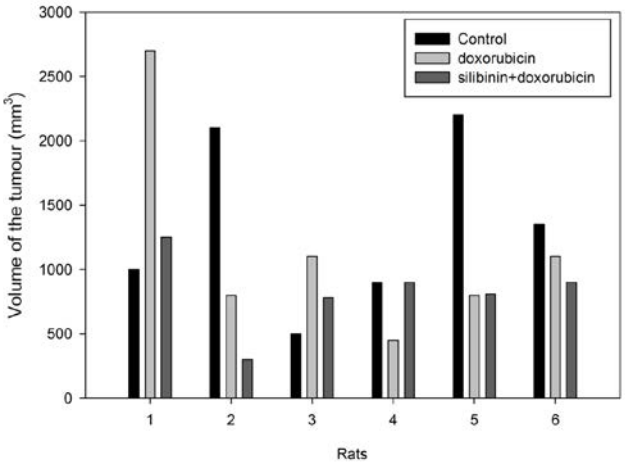

Figure 5. Effect of doxorubicin and silibinin+ doxorubicin combination on tumor growth in each rat. Rats were administered intraperitoneal injection of either normal saline (control) or doxorubicin (2 mg/kg), or combination of silibinin and doxorubicin (1.9 mg/kg). The size of tumor was evaluated using vernier calliper at the end of 5 weeks. Silibinin plus doxorubicin group demonstrated significant reduction in tumor size ($p=0.019$).

Discussion

Silibinin has been shown to inhibit various types of cancer cells, such as lung, colorectal, breast, prostate, brain, ovarian and renal carcinoma [26-28]. The anti-tumor effect of silibinin involves signalling pathways such as NF-kB signalling [27], vascular endothelial growth factor (VEGF) receptor signaling [26], extracellular signal-regulated kinase signaling (ERK) [29], protein kinase B signaling (Akt) [29], and signal transducers and activators of transcription (STATs) signaling [30]. However, the mechanism responsible for the anti-tumor effects of silibinin on HCC and its synergistic effect with doxorubicin has not been fully understood. In the present study, silibinin treatment resulted in a dose- and time-dependent inhibition of cell viability as well as increased ef-

ficacy with doxorubicin in HCC.

The take home message of the present study is that silibinin strongly synergizes the therapeutic effect of doxorubicin in human HCC, HEPG2 cells, via induction of G2-M arrest and apoptotic cell death. This is also supported by results from orthotopic rat model of HCC. Doxorubicin and other cytotoxic drugs are associated with high systemic toxicity including immunosuppression and cardiomyopathy, which is also associated with a risk of developing primary or secondary drug resistance to standard chemotherapy [5,6].

Combination chemotherapy has received much attention in recent times with an aim of identifying compounds with a known mechanism of action that can enhance the therapeutic index of clinical anticancer drugs, decreasing at the same time the unacceptable side effects [31]. Since dietary supplements generally have beneficial effect on overall health they support a logical rationale for being used as combination agents with standard chemotherapy. Apart from their own biological efficacy, dietary supplements with anticancer activity are the first choice in combination chemotherapy. Our study shows that silibinin is an excellent agent to be used in a combination regimen for the treatment of solid tumors such as HCC. We also hypothesized that combining silibinin with doxorubicin could produce synergistic efficacy.

We have observed that the in vitro therapeutic effect of doxorubicin on cell growth inhibition at concentration of 100 nM dose could be achieved at its one-fourth concentration (25 nM dose used in the present study) when combined with the 100 μM dose of silibinin in HEPG2 cells. In studies exploring the mechanism of observed effect we found that silibinin and doxorubicin combination caused strong synergistic effect on the G2-M arrest in cell cycle progression.

G2-M transition is an effective checkpoint in the cell cycle progression which is regulated through cyclin complexes and cdc-family proteins (tyrosine kinases and phosphatases) [32]. It is known that cdc25C functions as a mitotic activator by dephosphorylating cdc2/p34 that forms a complex with cyclin B1 and drives the cell from G2 to M phase. The activation of cdc25C is further controlled through its inhibitory phosphorylation by the upstream kinase Chk1/2, in case of DNA damage, and in turn inhibits mitosis [33]. Therefore, targeting regulation of G2-M transition can be a logical approach in targeting cell proliferation and apoptotic death [34-36]. With this background we used silibinin in combination with doxorubicin, and observed inhibition of cdc2/p34 kinase activity and down-regulation of cdc25C and cyclin B1 in HepG2 cells.

Strong evidence supports that G2-M arrest drives cells to death through the apoptotic pathway [31,37-39]. Consistent with earlier reports, our data shows that silibinin-doxorubicin combination induces 3-fold higher apoptotic cell death than each agent used alone [40,41]. Evidence also suggests that many HCC patients use medicines of herbal origin which does not have an established mechanism of action, due to the side effects of conventional chemotherapeutic agents. Therefore, our study suggests that combination therapy using silibinin-doxorubicin may show a better therapeutic efficacy in patients with HCC. These findings need to be further validated in human clinical trials.

Conflict of interests

The authors declare no confict of interests.

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