Silibinin induces G1 arrest, apoptosis and JNK/SAPK upregulation in SW1990 human pancreatic cancer cells

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Abstract. The aim of the present study was to investigate the inhibitory effect of silibinin on SW1990 pancreatic cancer cells. An MTT assay following silibinin treatment demonstrated an inhibitory effect on AsPC-1 and SW1990 cells in a dose- and time-dependent manner. Propidium iodide staining analysis identified the cell cycle arrest of G1 phase and western blotting analysis demonstrated that the expression levels of cyclin D1, cyclin E2, cyclin A and cyclin B1 were decreased. The expression of G1-associated cell cycle-dependent kinases, cyclin-dependent kinase (CDK)4 and CDK6, were also decreased, whereas the expression of p15 (p15^{INK4B}) was increased. In addition, after SW1990 cells were incubated with various concentrations of silibinin, early and late apoptotic cells were detected using flow cytometry. Silibinin increased the activities of caspase-9 and caspase-3, and subsequent cleavage of poly (ADP-ribose) polymerase (PARP) was also observed. The expression levels of B-cell lymphoma (Bcl)-2, Bcl-2-like 1 and myeloid cell leukemia 1 were decreased, whereas the expression of Bcl-like protein 4 did not alter and the expression levels of Bcl-2-like 1 small and Bcl-2-like protein 11 were increased. The expression levels of c-Jun N-terminal kinase (JNK) and phospho-JNK were also increased. In conclusion, silibinin inhibited cell proliferation, induced cell cycle G1 arrest via upregulating p15^{INK4B} and induced mitochondrial apoptosis via upregulating JNK/stress-activated protein kinase (SAPK) signaling pathway in human pancreatic cancer SW1990 cells.

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Introduction

Pancreatic cancer is one of the most frequently malignant gastrointestinal cancer types and has a high mortality rate for the majority of patients in advanced stages, with a median survival of 3-6 months and a 5-year survival rate of less than 5% (1). Pancreatic cancer ranks as the tenth highest incidence, and the sixth most frequent cause of mortality in China (2). In addition, the incidence and mortality rate of pancreatic cancer has been increasing yearly (3,4). The high mortality incidence is due to the tumors being frequently metastatic at the time of initial diagnosis, and despite an aggressive clinical approach systemic therapies fail to treat pancreatic cancer (5). There are no specific clinical symptoms in the early stages of the disease, and only mild symptoms will appear in stage I pancreatic cancer, including epigastric discomfort or indigestion. Surgery remains the primary treatment for pancreatic cancer; however, only 5-25% of patients with clinically diagnosed pancreatic cancer are eligible to receive surgical treatment. Following complete resection of tumors and adjuvant administration of gemcitabine, the median disease-free survival for patients with pancreatic cancer is 13.4 months. The 5-year survival rate following pancreatic cancer resection is <10%, and 50% of patients who have survived for 5 years succumb to recurrence of disease within the subsequent 5 years (6,7). With respect to the development of surgical treatments for pancreatic cancer, novel therapy options remain challenging.

Patients who are not recommended for surgical resection require adjuvant therapy, including chemotherapy and radiotherapy, to extend their survival. Currently, the chemotherapy drugs of choice for patients with pancreatic cancer are gemcitabine, capecitabine, fluorouracil, mitomycin, adriamycin and arsenic trioxide, and these are used in combination (8). However, as pancreatic cancer is frequently insensitive to chemotherapy, the curative effect of chemotherapy for this cancer type is poor (9). Therefore, it is important to identify chemotherapy drugs to which patients with pancreatic cancer

are sensitive, or to identify alternative methods to improve the sensitivity of patients to chemotherapeutic agents. It is also important to reduce the side effects of radiation and chemo-therapy to improve the therapeutic effects and the prognosis of patients with pancreatic cancer. There have been numerous previous studies that have focused on natural anti-tumor drugs, and there is increasing consideration of the anti-tumor effects of natural products. A group of anti-cancer drugs from natural products, including camptothecin and paclitaxel, have been established in clinical practice and have demonstrated a curative effect for certain types of cancer (10).

Silibinin is a flavonoid that is extracted from milk thistle plants and has traditionally been used for detoxification and in the treatment of liver diseases, as it is a natural drug that protects the liver (11). Silibinin has potential clinical value and has exhibited positive effects in the treatment of liver diseases, diabetes, mushroom poisoning, neurodegenerative diseases and numerous types of cancer (12,13).

In vitro and *in vivo* studies, silibinin has been established to have inhibitory effects in a number of cancer types, including cutaneous carcinoma, breast, cervical, prostate, colorectal, gastric carcinoma, bladder, lung, ovarian, kidney, tongue, hepatocellular carcinoma and leukemia (14). However, there have only been a small number of studies on the inhibition effects of silibinin on pancreatic cancer cells (15,16). Therefore, the present study used pancreatic cancer SW1990 cells as a model to investigate the inhibitory effects of silibinin on cell proliferation in pancreatic cancer.

Materials and methods

Cell lines and reagents. The LO2 human hepatic cell line and the AsPC-1 and SW1990 human pancreatic cancer cell lines were obtained from the Department of Biochemistry, Medical College of Jinan University (Guangzhou, China). All cells were maintained in RPMI-1640 supplemented with 5% fetal bovine serum at 37°C in 95% humidified air containing 5% CO_2. Silibinin (Fig. 1A) was purchased from Sichuan Weikeqi Biological Technology Co., Ltd. (Chengdu, China).

Fetal bovine serum, RPMI-1640, trypsin and EDTA were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MTT, dimethyl sulfoxide (DMSO), Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The antibody against cleaved caspase-3 was purchased from Epitomics (Burlingame, CA, USA). Antibodies against cyclin D1 (cat. no. 2978; dilution 1:1,000), cyclin E2 (cat. no. 4132; dilution 1:1,000), cyclin A (cat. no. 4656; dilution 1:1,000), cyclin B2 (cat. no. 4135; dilu-tion 1:1,000), cyclin-dependent kinase (CDK)4 (cat. no. 12790; dilution 1:1,000), CDK6 (cat. no. 13331; dilution 1:1,000), p15 (cat. no. 8762; dilution 1:1,000), p21 (cat. no. 2947; dilution 1:1,000), B-cell lymphoma 2 associated protein X (Bax; cat. no. 5023; dilution 1:1,000), B-cell lymphoma 2 (Bcl-2; cat. no. 15071; dilution 1:1,000), Bcl-2-like 1 (Bcl-xl; cat. no. 2764; dilution 1:1,000), myeloid cell leukemia 1(Mcl-1; cat. no. 94296; dilution 1:1,000), Bcl-2-like 1 small (Bcl-xs; cat. no. 2764; dilution 1:1,000), Bcl-2-like 11 (Bim; cat. no. 2933; dilution 1:1,000), Jun N-terminal kinase (JNK; cat. no. 9252; dilution 1:1,000), phosphorylated JNK (p-JNK;

cat. no. 4668; dilution 1:1,000), caspase-9 (cat. no. 9508; dilution 1:1,000), caspase-3 (cat. no. 9665; dilution 1:1,000), poly (ADP-ribose) polymerase (PARP; cat. no. 9532; dilution 1:1,000), GAPDH (cat. no. 5174; dilution 1:1,000) and horse-radish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. 7054; dilution, 1:5,000), and horseradish peroxidase-conjugated goat anti-mouse immu-noglobulin G secondary antibody (cat. no. 7056; dilution, 1:5,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell growth and viability assay. Cell growth and viability were measured using an MTT assay. The normal liver cell LO2 and human pancreatic cancer AsPC-1 and SW1990 cell lines in logarithmic growth phase were seeded into 96-well plates at a density of $5x10^3$ cells/well and incubated at 37°C overnight. Subsequently, the cells were treated with varying concentrations of silibinin (50, 100, 200, 300, 400 and 500 μM) and DMSO (<0.1%) was used as the vehicle control (5 repeats were performed in each group). Cells were incubated for 24 to 72 h in standard culture conditions (37°C containing 5% CO_2). Subsequently, 20 μl MTT (5 mg/ml) reagent was added to each well followed by an additional incubation of 4 h at 37°C. The supernatants were discarded and 150 μl of DMSO was added, and the cells were agitated to dissolve the purple precipitate. A spectrophotometer was used to measure the absorbance at a wavelength of 570 nm. The cell inhibitory rate was calculated using the following equa-tion: Cell inhibitory rate=$(1-A_{treatment}/A_{blank})$ x100%, where A was the absorbance.

Flow cytometry analysis for cell cycle distribution. SW1990 cells in the logarithmic growth phase were seeded at a density of $3x10^5$ cells/well into 6-well tissue culture plates. The cells were treated with various concentrations of silibinin (50, 100 and 200 μM) and DMSO (<0.1%) was used as the vehicle control. Following 48 h of incubation at 37°C, cells were harvested using trypsin. The cells were washed twice with ice-cold PBS and fixed in 70% ethanol overnight at 4°C. Subsequently, the cell pellets were washed three times with PBS and incubated with PI (500 g/l) and RNase (20 U/ml) for 30 min at room temperature in the dark. The DNA content was determined by flow cytometry by ModFit 2.0 software (FCM; Beckman Coulter, Inc., Brea, CA, USA).

Annexin V-FITC/PI staining and flow cytometric analysis to detect apoptosis. SW1990 cells in logarithmic growth phase were seeded at a density of $3x10^5$ per well into 6-well tissue culture plates. Subsequently, cells were treated with various concentrations of silibinin (50, 100 and 200 μM), and DMSO (<0.1%) was used as the vehicle control. Following 48 h of incubation at 37°C, the cells were harvested using trypsin. The cells were washed twice with ice-cold PBS, re-suspended in 200 μl of binding buffer (HEPES, NaCl and $CaCl_2$) and incubated with 10 μl Annexin V-FITC and 5 μl of PI for 15 min at room temperature in the dark. A flow cytometer was used to analyze the percentage of early and late apoptotic cells. A total of 10,000 cells were analyzed by WinMDI version 2.9 software (The Scripps Research Institute, San Diego, CA, USA).
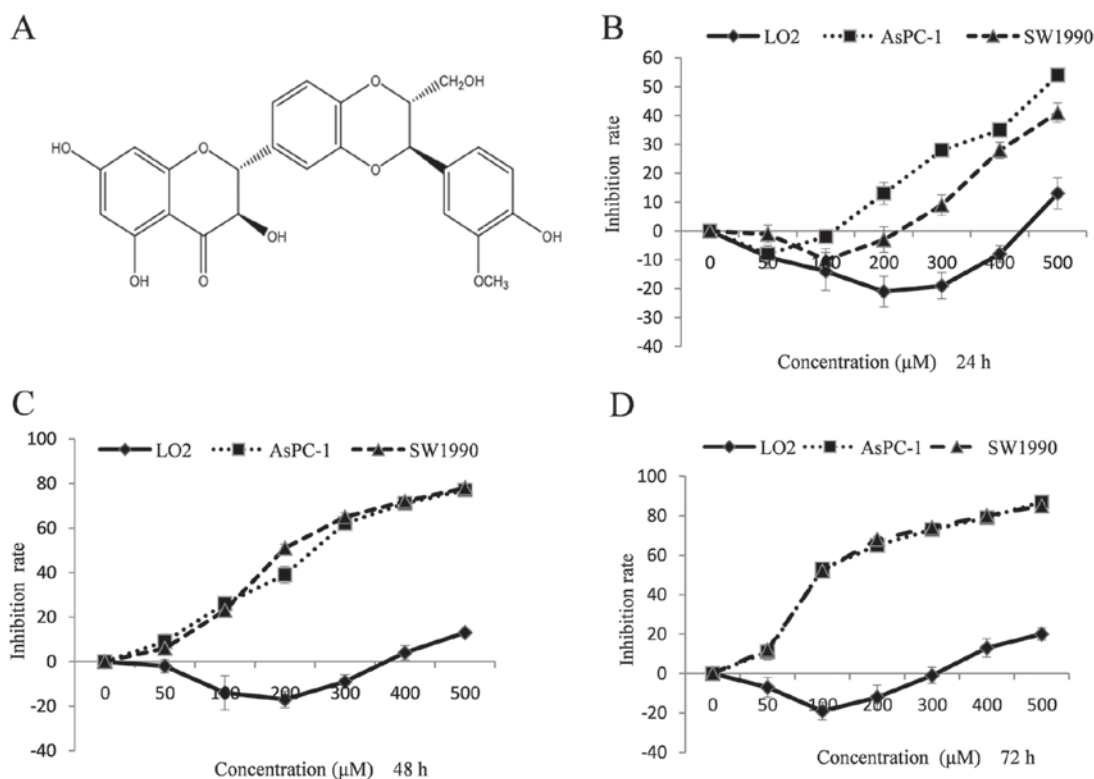
Figure 1. Inhibition of proliferation of LO2, AsPC-1 and SW1990 cells by silibinin. (A) Structure of silibinin. LO2, AsPC-1 and SW1990 cells were incubated with various concentrations of silibinin for (B) 24, (C) 48 and (D) 72 h. The cell inhibitory rates were determined using an MTT assay and the half maximal inhibitory concentration values were calculated as described. Each value represents the mean ± standard deviation of 3 independent experiments.

Western blotting analysis. SW1990 cells were seeded at a density of 3×10^5 per well into 6-well tissue culture plates. The cells were incubated at 37°C with various concentrations of silibinin (50, 100, 150 and 200 μM) for 48 h, and DMSO (<0.1%) was used as the vehicle control. Subsequently, the cells were harvested and washed twice with ice-cold PBS. The cells were lysed in buffer (Biospec, Inc., Bartlesville, OK, USA) on ice for 30 min and centrifuged at 12,000 x g for 15 min at 4°C. The cell proteins were extracted and the concentrations were determined using a bicinchoninic acid assay. A total of 30 μg of protein was denatured by the addition of 5X reducing sample buffer (Biospec, Inc.), incubated for 5 min at 100°C and separated by SDS-PAGE (10% or 15%). The proteins were transferred to polyvinylidene difluoride membranes. Following blocking with 5% dried skimmed milk, the membranes were incubated overnight at 4°C with the appropriate primary antibodies. Subsequently, the membranes were washed 3 times in TBST (10 mM Tris, 100 mM NaCl and 0.1% Tween 20) for 5 min each and incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Following washing in TBST, the bound antibody complex was detected using an electrochemiluminescence reagent (HRP Substrate Luminol Reagent and HRP Substrate Peroxide Solution; Biospec, Inc.) and XAR film (Kodak, Rochester, NY, USA) as described previously (ImageJ v1.46d; National Institutes of Health, Bethesda, MD, USA) (17). The data were quantitated from at least three separate experiments.

Statistical analysis. The results are expressed as the mean ± standard error from 3 independent experiments using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Student's t-tests were used to determine the significance between the control and test groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Silibinin inhibits the proliferation of LO2, AsPC-1 and SW1990 cells. To investigate the effects of silibinin on cell proliferation, an MTT assay was used to analyze the proliferation of LO2, AsPC-1 and SW1990 cells. The results demonstrated that varying concentrations of silibinin had little effect on LO2 cells. Notably, silibinin enhanced LO2 cell proliferation at concentrations <300 μM (Fig. 1B-D). The proliferation of AsPC-1 and SW1990 cells was significantly inhibited with silibinin treatment in a dose- and time-dependent manner (Fig. 1B-D). The half maximal inhibitory concentration (IC$_{50}$) values of silibinin on AsPC-1 at 48 and 72 h were 224.20 and 87.25 μM, respectively. The IC$_{50}$ values on SW1990 cells at 48 and 72 h were and 218.41 and 86.91 μM, respectively.

Effects of silibinin on cell cycle distribution. As the decrease in cell growth was observed following silibinin treatment, which may be due to the induction of cell cycle arrest, the effect of silibinin on cell cycle distribution was examined in SW1990 cells. Flow cytometry following PI staining assay was used to analyze the effects of silibinin on cell cycle distribution. As presented in Fig. 2A, the population of cells was arrested in G1 phase (48.6-77.0% compared with 46.8% in
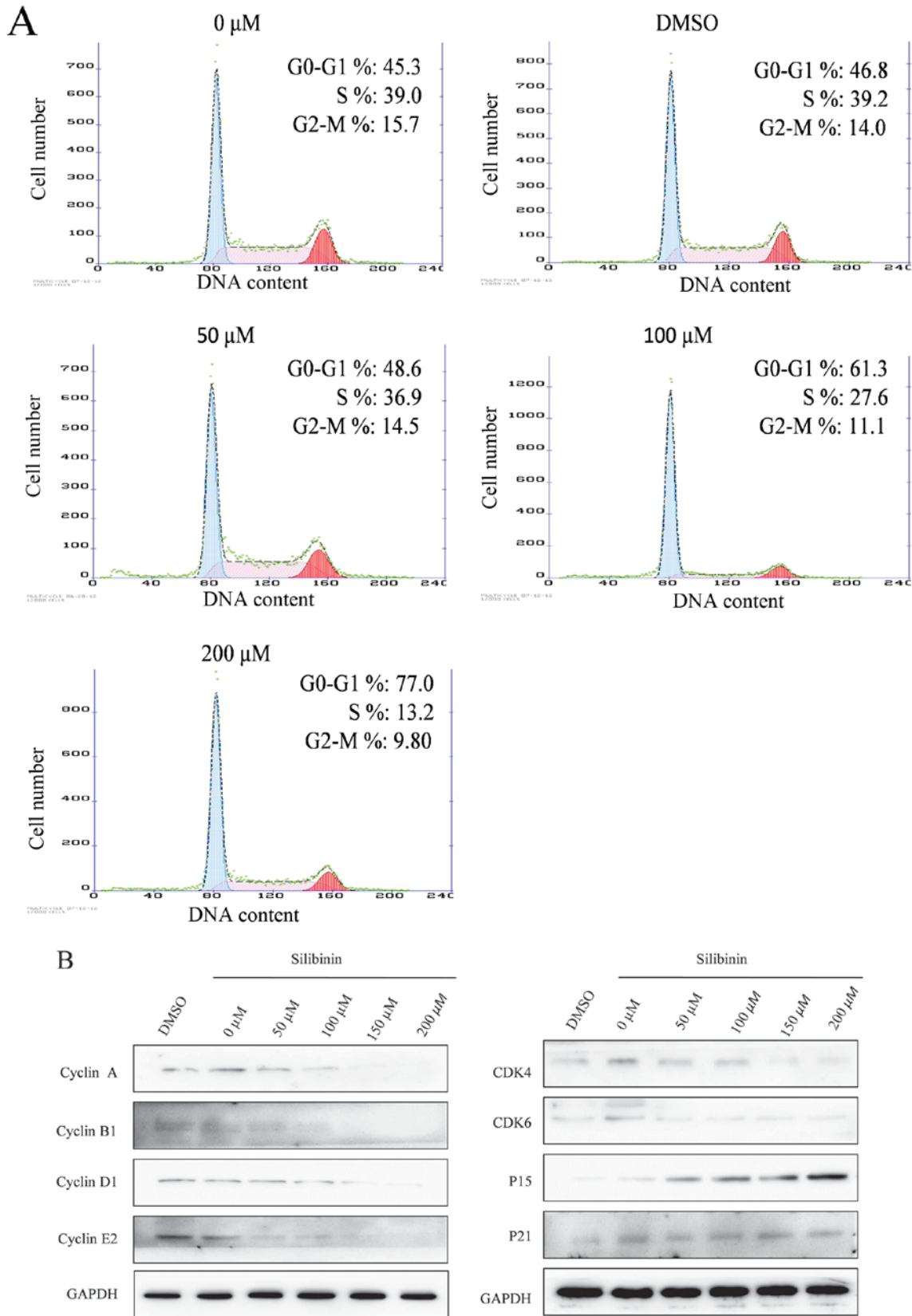
Figure 2. Effect of silibinin on cell cycle distribution and on the expression of cell cycle-associated proteins in SW1990 cells. SW1990 cells were incubated with a number of silibinin concentrations for 48 h. (A) Flow cytometry to detect cell cycle distributions, (B) Western blotting assay to detect the expression of cell cycle-associated proteins. The data represent one of three experiments that yielded similar results. DMSO, dimethyl sulfoxide; CDK, cyclin-dependent kinase.

DMSO-treated controls). The populations of cells in S phase and G2-M phase were reduced with increasing concentrations of silibinin. These results suggest that cell cycle progression was arrested in G1 phase.

Silibinin modulates the expression of cell cycle regulatory proteins. As significant cell cycle arrest was observed following silibinin treatment, the expressions of cell cycle regulatory proteins as Cyclins, CDKs and CDK inhibitors were analyzed using western blotting analysis. The results demonstrated that the protein expression levels of cyclin D1, cyclin E, cyclin A, cyclin B1 and G1 phase-associated CDK4 and CDK6 were significantly reduced following treatment with silibinin for 48 h. However, the expression level of p15 increased, whereas the level of p21 was not altered (Fig. 2B).

Silibinin induces apoptosis in human pancreatic cancer SW1990 cells. To determine whether silibinin induces apoptosis, the SW1990 cells were treated with silibinin at 0, 50, 100 or 200 μM or 0.1% DMSO for 48 h. Cells were double stained with Annexin V-FITC and PI. Annexin V assay identified that the populations of apoptotic cells increased with higher silibinin concentrations. The apoptosis rate was 27.69% when the SW1990 cells were treated with silibinin at a concentration of 200 μM for 48 h, while in the control group, the apoptosis rate was 8.84% (Fig. 3A).

To investigate the underlying mechanism by which silibinin induced apoptosis, western blotting analysis was conducted with antibodies against caspase-3, caspase-9 or PARP. Treatment with silibinin for 48 h promoted the activation of a number of apoptotic proteins, including caspase-9, caspase-3 and its substrate, PARP, via protein cleavage (Fig. 3B). Increased silibinin concentrations resulted in a reduction of the intact proteins and an increase in proteolytic cleavage bands in a concentration-dependent manner.

Detection of the protein expression levels of Bcl-2 family proteins and JNK using western blotting analysis. The mitochondrial death pathway is controlled by members of the Bcl-2 family. The Bcl-2 family serves a central regulatory role in deciding the fate of cells via the interaction between pro- and anti-apoptotic members (18). Therefore, whether the mitochondrial-mediated apoptosis in SW1990 cells induced by silibinin treatment was modulated by Bcl-2 family members was investigated.

Silibinin suppressed the expression levels of Bcl-2, Mcl-1 and Bcl-xl, but did not alter the expression of Bax. The expression of pro-apoptotic protein (Bcl-xs) and the BH3-only protein, Bim, increased (Fig. 4A). As a result of these effects, the ratios of Bcl-2/Bax, Mcl-1/Bax and Bcl-xl/Bax were reduced during apoptosis. The protein expression levels of JNK and p-JNK increased with higher concentrations of silibinin (Fig. 4B). These results suggest that silibinin induces apoptosis in SW1990 cells through the activation of JNK and altering the expression levels of the Bcl-2 anti-apoptotic proteins and BH3-only proteins triggering the mitochondrial apoptosis signaling pathway.

Discussion

Certain anti-cancer agents have been extracted and purified from plants, including taxol and camptothecin. These drugs have been established in the clinic as effective chemotherapy agents and have demonstrated beneficial effects in certain types of carcinoma. In addition, numerous plant extracts including, tea polyphenol, resveratrol, ginger extract and soy isoflavones, have demonstrated potential anti-tumor effects, which may provide a novel direction for the study of potential anti-cancer drugs. Pancreatic cancer has poor outcomes with regards to surgical resection and chemotherapy (19,20). In China, the incidence of pancreatic cancer has continued to increase yearly (21). The search for an effective chemotherapeutic drug with low toxicity is an important focus of studying pancreatic cancer treatment. Silibinin serves a role in anti-oxidation, anti-fibrosis, anti-inflammation, immunoregulation and in the promotion of hepatocyte regeneration (22). It has been used in the clinic for >20 years for the treatment of hepatitis, fatty liver, cirrhosis and other diseases (23). Apart from its hepatoprotective effects, silibinin has received increased interest due to its anti-tumor efficacy. It was identified that silymarin and silibinin exhibit anti-tumor effects and may inhibit the growth of prostate, skin, bladder, lung, colon, breast, ovarian, renal, liver and tongue cancer (24-29). Notably, silibinin has been used in phase I clinical trial for the treatment of prostate cancer. Silymarin and silibinin exhibit low toxicity, and consequently their median lethal dosage values are unable to be measured in animal experiments. Additionally, silibinin is used as an antioxidant food additive in the United States and Europe (30,31).

The present study investigated the inhibitory effects of silibinin on the proliferation of SW1990 pancreatic cancer cells and the potential underlying mechanisms of action. An MTT assay was used to detect the inhibitory effect of varying concentrations of silibinin on the proliferation of LO2 normal human liver cell line and on AsPC-1 and SW1990 pancreatic cancer cell lines. The results demonstrated that there was no marked inhibitory effect of silibinin on the proliferation of LO2 cells, whereas the inhibition of proliferation of AsPC-1 and SW1990 cells was significantly altered and occurred in a time- and dose-dependent manner.

The effects of silibinin treatment on cell cycle distribution differ between cell types. For example, silibinin promotes a G2 phase cell cycle arrest in human gastric carcinoma SGC7901 cells (32); however, it also triggers G2/M and G1 arrest in the TCC-SUP and T-24 human bladder transitional cell carcinoma cell lines, respectively (33). Silibinin promotes AsPC-1 human pancreatic cancer cells to arrest in G1 phase but does not markedly affect BxPC-3 and Panc-1 cells (17). Kaur *et al* (34) indicated that silibinin treatment notably inhibits the growth of Lovo cells and induces increased levels of the apoptosis-associated cleaved caspases (caspases 3 and 9) and cleaved PARP, and therefore promotes cell cycle arrest at G1 phase.

Using flow cytometry assay, a significant G1 phase arrest in the silibinin group was identified and western blotting analysis demonstrated that silibinin increased the expression of p15[INK4B] and did not alter the expression of p21[Cip1] in SW1990 cells. p15, which is upstream of CDK4/CDK6, inhibits CDK4/CDK6 activity, as well as inhibits the activity of the CDK4/cyclin D and CDK6/cyclin D complexes. In the current study, a decrease in the expression of the G1 phase-associated cell cycle proteins cyclin D1 and the cyclin-dependent kinases CDK4 and CDK6 was revealed using western blotting analysis. As the majority of cells cannot proceed to the next phase, the expression of late G1 phase-, S phase- and G2 phase-associated cell cycle
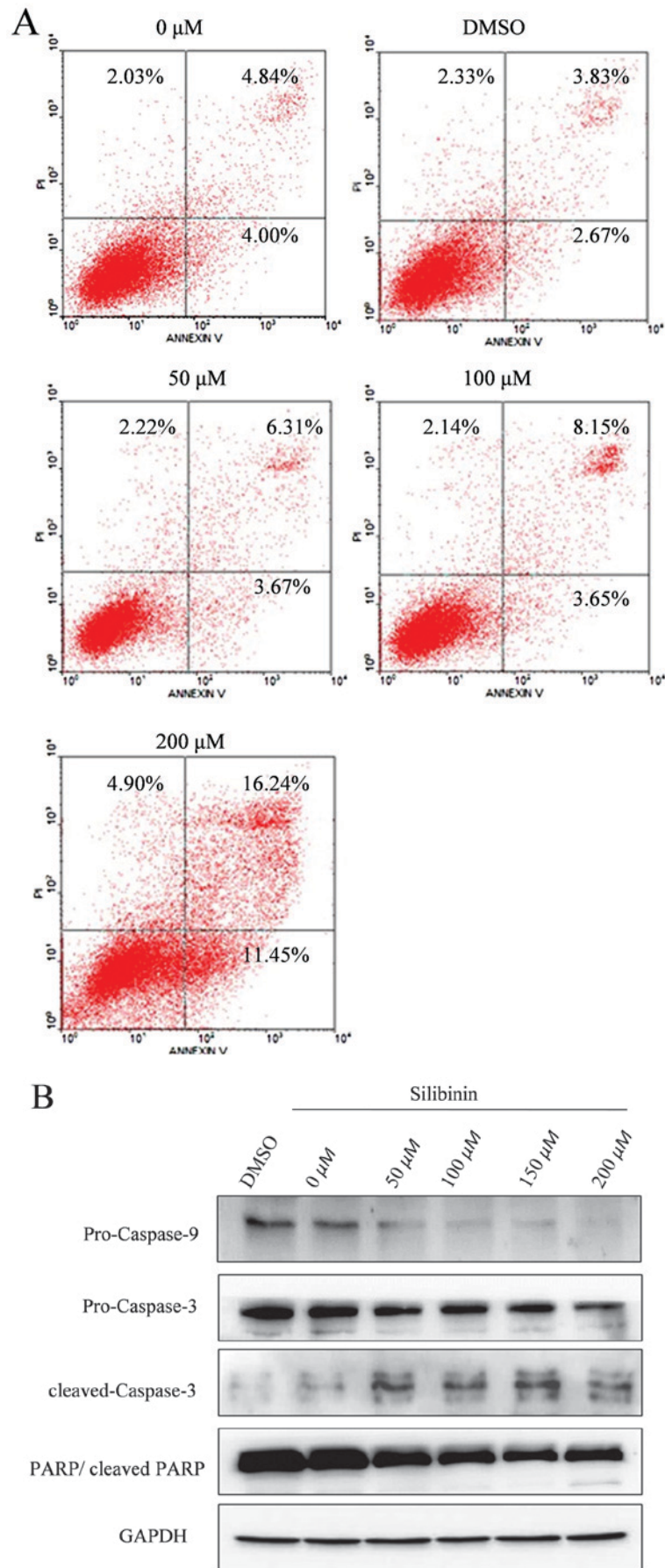
Figure 3. Effect of silibinin on apoptosis in SW1990 cells. SW1990 cells were incubated with different concentrations of silibinin for 48 h. (A) Flow cytometry assay to detect the apoptotic cells using Annexin V-fluorescein isothiocyanate and PI staining. (B) Western blotting assay to detect the expression of apoptosis-associated proteins caspase-3, caspase-9 and PARP. The data represent one of three experiments that yielded similar results. PARP, poly(ADP-ribose) polymerase; DMSO, dimethyl sulfoxide; PI, propidium iodide.
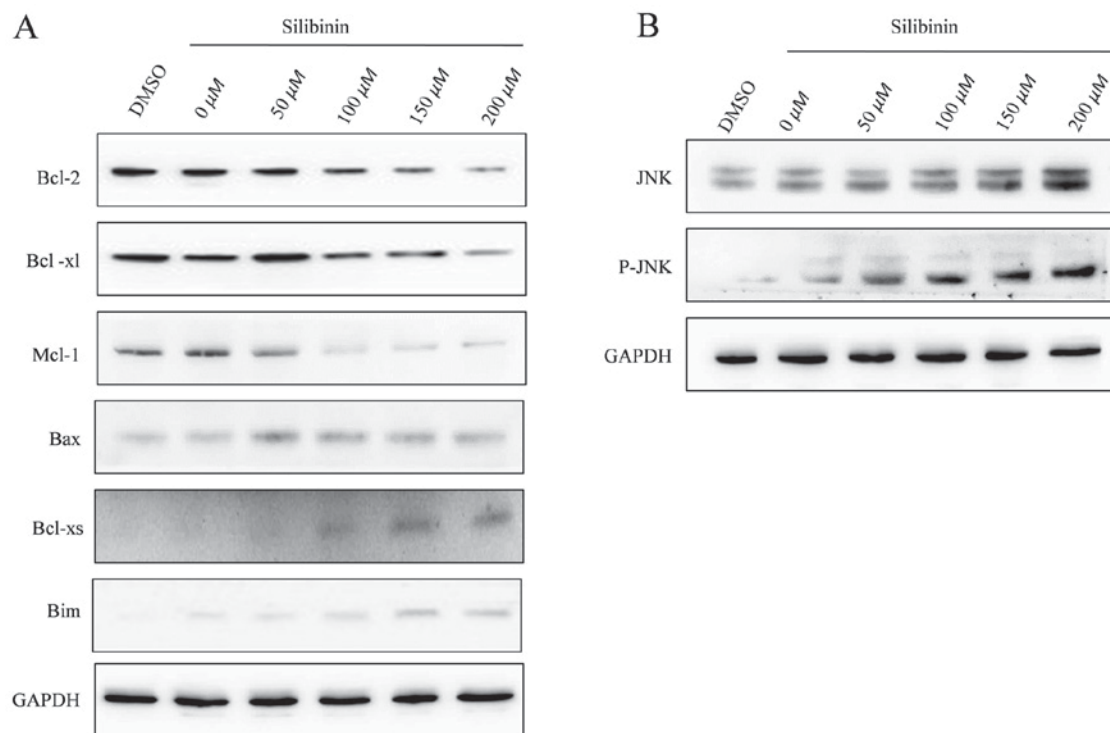
Figure 4. Expression of apoptosis-associated proteins analyzed using western blotting analysis. SW1990 cells were incubated with different concentration of silibinin for 48 h. (A) The expression of Bcl-2 family proteins and (B) the expression of JNK and P-JNK were detected using western blotting assay. The data represent one of three experiments that yielded similar results. DMSO, dimethyl sulfoxide; Bcl, B-cell lymphoma; Bcl-xl, Bcl-2-like-1; Mcl-1, myeloid cell leukemia 1; Bax, Bcl-2 associated protein X; Bcl-xs, Bcl-2-like 1 small; Bim, Bcl-2-like 11; JNK, Jun N-terminal kinase; p-JNK, phosphorylated JNK.

proteins, including cyclin E2, cyclin A and cyclin B1, was also reduced in the cells that were treated with silibinin. These results are consistent with G1 phase arrest, as well as with the results of the flow cytometry experiments.

Previous studies have demonstrated that apoptosis may occur via three signaling pathways, including the death receptor-mediated pathway, the mitochondrial- mediated pathway and the endoplasmic reticulum-associated pathway (35,36). Caspase-9 is linked to the mitochondrial death pathway and once the mitochondrial pathway is initiated, pro-caspase-9 is cleaved into its active form. Activated caspase-9 cleaves its downstream signaling protein pro-caspase-3, which promotes caspase-3 activation. PARP, an enzyme that is involved in the recognition of genomic integrity and DNA repair, is inactivated by caspase cleavage and is therefore regarded as a molecular marker of apoptosis (29,37-39).

In the present study, SW1990 cells were stained with Annexin V-FITC and PI and the cells in the silibinin-treated groups were apoptotic; the percentage of apoptotic cells increased with higher concentrations of silibinin. These results also demonstrated that SW1990 cells treated with silibinin have activated pro-caspase-9 and pro-caspase-3, and PARP was also cleaved into its active form. These data suggest that silibinin induces apoptosis via the mitochondrial-mediated signaling pathway in SW1990 cells and this was consistent with the results of flow cytometry analysis.

The mitochondrial-mediated apoptotic signaling pathway is associated with the expression and activation of the Bcl-2 protein family. Bcl-2 proteins are grouped into three classes: Those that inhibit apoptosis (Bcl-2, Bcl-xl, Bcl-2-like 2, Mcl-1, Bcl-10, Boo/Diva, NR-13 and Bcl-2-related protein A1);

those that promote apoptosis (Bak, Bax, Bcl-xs, Bcl2-related ovarian killer); and the pro-apoptotic BH3-only proteins (Bad, Bid, Bcl2-interacting killer, Bim, B lymphocyte kinase, Bcl2-modifiying factor (Bmf), harakiki Bcl2-interacting factor, Bcl2-interacting protein 23, NIP3-like protein X and Noxa) that bind and regulate the anti-apoptotic Bcl-2 proteins (40,41). In the present study, western blotting analysis demonstrated that with increasing concentrations of silibinin, the expression of the anti-apoptotic Bcl-2 family of proteins Bcl-2, Bcl-xl and Mcl-1 was decreased, whereas the expression of the BH3-only protein Bim was increased in a concentration-dependent manner. The expression of the pro-apoptotic protein Bcl-xs was increased, whereas the expression of another pro-apoptotic protein Bax was unaltered. These results suggest that Bim may form a heterodimer with Bcl-2 or Mcl-1 and promote dissociation of Bax/Bcl-2 or Bax/Mcl-1 heterodimers. This event promotes Bax oligomerization and cytochrome-c release from the mitochondria, which initiates the mitochondrial-mediated apoptotic signaling pathway. The expression of the pro-apoptotic protein Bcl-xs was increased, and therefore Bcl-xs may be able to activate Bak or Bax through inhibiting the function of voltage-dependent anion channel 2 and Bcl-xl (42). Although the protein levels of anti-apoptotic proteins, including Bcl-2, Mcl-1 and Bcl-xl, were decreased, the ratios of Bax/Bcl-2, Bax/Mcl-1 and Bax/Bcl-xl were increased, resulting in an increased quantity of unbound Bax and initiation of the mitochondrial-mediated apoptotic signaling pathway.

JNK serves an important role in the death receptor-initiated extrinsic apoptotic signaling pathway and the mitochondrial intrinsic apoptotic signaling pathway. JNK promotes apoptosis

through two actions. Firstly, activated JNK locates to the nucleus and activates transcription factors, including c-Jun. This activation of c-Jun/AP1 or p53/p73, leads to the increased expression of pro-apoptotic genes. The second action requires JNK to locate into the mitochondria and phosphorylate BH3-only Bcl-2 proteins, including Bad Ser128, which inhibits 14-3-3 protein interactions and therefore reducing the anti-apoptotic activity of Bcl-2 and Bcl-xl. Phosphorylation of 14-3-3ξ at Ser184 by JNK does not allow 14-3-3ξ to relocate. JNK is able to induce the cleavage of Bid, independently of caspase-8, in HeLa cells during JNK-induced apoptosis. The 21 kDa Bid fragment promotes the release of the second mitochondria-derived activator of caspase, which is a pro-apoptotic protein (43). Apoptosis may also be initiated by JNK activation of Bax or Bak via the phosphorylation of Bim and Bmf, which has been demonstrated in HEK293T cells during UV-induced apoptosis (44). Finally, JNK phosphorylates Bcl-2 at Ser70 and causes the dissociation of the Bcl-2/Bax heterodimer and promotes cell apoptosis (45). The present study identified that following treatment with silibinin, the expression levels of JNK and p-JNK were increased in SW1990 cells, which was consistent with the results of Duan *et al* (46). The results of the present study indicated that the pro-apoptotic effects on SW1990 cells were mediated via the JNK signaling pathway, and the activation of this signaling pathway may activate the downstream mitochondrial-associated apoptotic signaling pathway to induce cell apoptosis.

In conclusion, silibinin suppresses the proliferation of pancreatic cancer cells by the induction of G1 phase cell cycle arrest and through an increase in the expression of p15^{INK4B}. In addition, silibinin induced JNK activation and initiated the mitochondrial-associated apoptotic signaling pathway, which promoted the pancreatic cancer SW1990 cells to undergo apoptosis. The signaling pathway by which silibinin upregulates p15^{INK4B} in pancreatic cancer SW1990 cells remains to be elucidated. Additionally, whether the activation of JNK by silibinin promotes the dissociation of Bcl-2/Bax or Bcl-xl/Bax and the oligomerization of Bax, and whether silibinin suppresses the growth of pancreatic cancer in SW1990 xenograft mice are yet to be determined.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

JJ and MC contributed to the conception of the study, and conceived and designed the experiments. XZ performed the experiments and data analyses and wrote the manuscript. JL, PZ and LD helped perform the analysis with constructive discussions, and contributed significantly to analysis and manuscript preparation. ZW and LW contributed reagents, materials and analysis tools and helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Quan JL, Feng Y, Chen J and Fu DL: Current status and progress of pancreatic cancer in China. World J Gastroenterol 21: 7988-8003, 2015.
2. Chen WQ, Li H, Sun KX, Zheng RS, Zhang SW, Zeng HM, Zou XN, Gu XY and He J: Report of cancer incidence and mortality in different areas of China, 2014. Zhonghua Zhong Liu Za Zhi 40: 5-13, 2018 (In Chinese).
3. Kongkam P, Benjasupattananun P, Taytawat P, Navicharoen P, Sriuranpong V, Vajragupta L, Klaikaew N, Ridtitid W, Treeprasertsuk S, Rerknimitr R and Kullavanijaya P: Pancreatic cancer in an Asian population. Endosc Ultrasound 4: 56-62, 2015.
4. Gall TMH, Tsakok M, Wasan H and Jiao LR: Pancreatic cancer: Current management and treatment strategies. Postgrad Med J 91: 601-607, 2015.
5. Pliarchopoulou K and Pectasides D: Pancreatic cancer: Current and future treatment strategies. Cancer Treat Rev 35: 431-436, 2009.
6. Luo G, Guo M, Liu Z, Xiao Z, Jin K, Long J, Liu L, Liu C, Xu J, Ni Q and Yu X: Blood neutrophil-lymphocyte ratio predicts survival in patients with advanced pancreatic cancer treated with chemotherapy. Ann Surg Oncol 22: 670-676, 2015.
7. Foley K, Kim V, Jaffee E and Zheng L: Current progress in immunotherapy for pancreatic cancer, Cancer Lett 381: 244-251, 2016.
8. Shi S, Yao W, Xu J, Long J, Liu C and Yu X: Combinational therapy: New hope for pancreatic cancer? Cancer Lett 317: 127-135, 2012.
9. Seicean A, Petrusel L and Seicean R: New targeted therapies in pancreatic cancer. World J Gastroenterol 21: 6127-6145, 2015.
10. Rodeiro I, Magarino Y, Ocejo O, Garrido G and Delgado R: Use of natural products in anti-cancer alternative therapy: Risk of interactions with conventional anti-cancer drugs. Bol. Latinoam Caribe Plant Med Aromaticas 7: 332-344, 2008.
11. Sozen H, Celik OI, Cetin ES, Yilmaz N, Aksozek A, Topal Y, Cigerci IH and Beydilli H: Evaluation of the protective effect of silibinin in rats with liver damage caused by itraconazole. Cell Biochem Biophys 71: 1215-1223, 2015.
12. Cheung CW, Gibbons N, Johnson DW and Nicol DL: Silibinin-a promising new treatment for cancer. Anti Cancer Agents Med Chem 10: 186-195, 2010.
13. Gazák R, Walterová D and Kren V: New and emerging applications in medicine. Curr Med Chem 14: 315-338, 2007.

14. Tiwari P and Kaushala PM: Silibinin in cancer therapy: A promising prospect. Cancer Res Front 1: 303-318, 2015.
15. Ge Y, Zhang Y, Chen Y, Li Q, Chen J, Dong Y and Shi W: Silibinin causes apoptosis and cell cycle arrest in some human pancreatic cancer cells. Int J Mol Sci 12: 4861-4871, 2011.
16. Nambiar D, Prajapati V, Agarwal R and Singh RP: In vitro and in vivo anticancer efficacy of silibinin against human pancreatic cancer BxPC-3 and PANC-1 cells. Cancer Lett 334: 109-117, 2013.
17. Deng R, Jun T, Xia LP, Li DD, Zhou WJ, Wang LL, Feng GK, Zeng YX, Gao YH and Zhu XF: ExcisaninA, a diterpenoid compound purified from Isodon MacrocalyxinD, induces tumor cells apoptosis and suppresses tumor growth through inhibition of PKB/AKT kinase activity and blockade of its signal pathway. Mol Cancer Ther 8: 873-882, 2009.
18. Brunelle JK and Letai A: Control of mitochondrial apoptosis by the Bcl-2 family. J Cell Sci 122: 437-441, 2009.
19. Conroy T, Bachet JB, Ayav A, Huguet F, Lambert A, Caramella C, Maréchal R, Van Laethem JL and Ducreux M: Current standards and new innovative approaches for treatment of pancreatic cancer. Eur J Cancer 57: 10-22, 2016.
20. Yang X, Hao J, Zhu CH, Niu YY, Ding XL, Liu C and Wu XZ: Survival benefits of western and traditional Chinese medicine treatment for patients with pancreatic cancer. Medicine (Baltimore) 94: e1008, 2015.
21. Sultana A, Tudur Smith C, Cunningham D, Starling N, Tait D, Neoptolemos JP and Ghaneh P: Systematic review, including meta-analyses, on the management of locally advanced pancreatic cancer using radiation/combined modality therapy. Brit J Cancer 96: 1183-1190, 2007.
22. Pradhan S and Girish C: Hepatoprotective herbal drug, silymarin from experimental pharmacology to clinical medicine. Indian J Med Res 124: 491-504, 2006.
23. Magliulo E, Gagliardi B and Fiori GP: Results of a double blind study on the effect of silymarin in the treatment of acute viral hepatitis, carried out at two medical centres (author's transl). Med Klin 73: 1060-1065, 1978 (In German).
24. Deep G and Agarwal R: Antimetastatic efficacy of silibinin: Molecular mechanisms and therapeutic potential against cancer. Cancer Metastasis Rev 29: 447-463, 2010.
25. Zheng D, Wang Y, Zhang D, Liu Z, Duan C, Jia L, Wang F, Liu Y, Liu G, Hao L and Zhang Q: In vitro antitumor activity of silybin nanosuspension in PC-3 cells. Cancer Lett 307: 158-164, 2011.
26. Wu KJ, Zeng J, Zhu GD, Zhang LL, Zhang D, Li L, Fan JH, Wang XY and He DL: Silibinin inhibits prostate cancer invasion, motility and migration by suppressing vimentin and MMP-2 expression. Acta Pharmacol Sin 30: 1162-1168, 2009.
27. Hogan FS, Krishnegowda NK, Mikhailova M and Kahlenberg MS: Flavonoid, silibinin inhibits proliferation and promotes cell-cycle arrest of human colon cancer. J Surg Res 143: 58-65, 2007.
28. Zhang Y, Li Q, Ge Y, Chen Y, Chen J, Dong Y and Shi W: Silibinin triggers apoptosis and cell-cycle arrest of SGC7901 cells. Phytother Res 27: 397-403, 2013.
29. Lah JJ, Cui W and Hu KQ: Effects and mechanisms of silibinin on human hepatoma cell lines. World J Gastroenterol 13: 5299-5305, 2007.
30. Wu K, Zeng J, Li L, Fan J, Zhang D, Xue Y, Zhu G, Yang L, Wang X and He D: Silibinin reverses epithelial-to-mesenchymal transition in metastatic prostate cancer cells by targeting transcription factors. Oncol Rep 23: 1545-1552, 2010.
31. Tyagi A, Bhatia N, Condon MS, Bosland MC, Agarwal C and Agarwal R: Antiproliferative and apoptotic effects of silibinin in rat prostate cancer cells. Prostate 53: 211-217, 2002.
32. Zhang Y, Ge Y, Chen Y, Li Q, Chen J, Dong Y and Shi W: Cellular and molecular mechanisms of silibinin induces cell-cycle arrest and apoptosis on HeLa cells. Cell Biochem Funct 30: 243-248, 2012.
33. Tyagi A, Agarwal C, Harrison G, Glode LM and Agarwal R: Silibinin causes cell cycle arrest and apoptosis in human bladder transitional cell carcinoma cells by regulating CDKI- CDK-cyclin cascade, and caspase 3 and PARP cleavages. Carcinogenesis 25: 1711-1720, 2004.
34. Kaur M, Velmurugan B, Tyagi A, Deep G, Katiyar S, Agarwal C and Agarwal R: Silibinin suppresses growth and induces apoptotic death of human colorectal carcinoma LoVo cells in culture and tumor xenograft. Mol Cancer Ther 8: 2366-2374, 2009.
35. Indran IR, Tufo G, Pervaiz S and Brenner C: Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochem Biophys Acta 1807: 735-745, 2011.
36. Gogvadze V, Orrenius S and Zhivotovsky B: Mitochondria as targets for cancer chemotherapy. Semin Cancer Biol 19: 57-66, 2009.
37. Costantini P, Bruey JM, Castedo M, Métivier D, Loeffler M, Susin SA, Ravagnan L, Zamzami N, Garrido C and Kroemer G: Pre-processed caspase-9 contained in mitochondria participates in apoptosis. Cell Death Differ 9: 82-88, 2002.
38. Bialik S, Zalckvar E, Ber Y, Rubinstein AD and Kimchi A: Systems biology analysis of programmed cell death. Trends Biochem Sci 35: 556-564, 2010.
39. Lüthi AU and Martin SJ: The CASBAH: A searchable database of caspase substrates. Cell Death Differ 14: 641-650, 2007.
40. Kang MH and Reynolds CP: Bcl-2 inhibitors: Targeting mitochondrial apoptotic pathways in cancer therapy. Clin Cancer Res 15: 1126-1132, 2009.
41. Ola MS, Nawaz M and Ahsan H: Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol Cell Biochem 351: 41-58, 2011.
42. Plötz M, Gillissen B, Hossini AM, Daniel PT and Eberle J: Disruption of the VDAC2-Bak interaction by Bcl-x(S) mediates efficient induction of apoptosis in melanoma cells. Cell Death Differ 19: 1928-1938, 2012.
43. Deng Y, Ren X, Yang L, Lin Y and Wu X: A JNK-dependent pathway is required for TNFalpha-induced apoptosis. Cell 115: 61-70, 2003.
44. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S and Korsmeyer SJ: Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2: 183-192, 2002.
45. Wei Y, Sinha S and Lebive B: Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. Autophagy 4: 949-951, 2008.
46. Duan WJ, Li QS, Xia MY, Tashiro S, Onodera S and Ikejima T: Silibinin activated p53 and induced autophagic death in human fibrosarcoma HT1080 cells via reactive oxygen species-p38 and c-Jun N-terminal kinase pathways. Biol Pharm Bull 34: 47-53, 2011.