Introduction

Silibinin, also known as silybin, is the major active constituent of silymarin, which is a mixture of flavonolignans extracted from milk thistle (Silybum marianum). Silibinin has hepatoprotective properties (1, 2) and anticancer effects against human prostate adenocarcinoma cells, estrogen-dependent and -independent breast carcinoma cells, ectocervical carcinoma cells, colon cancer cells, and both small and non-small human lung carcinoma cells (3 – 6). Our previous studies have shown that silibinin exhibited an anti-apoptotic effect on isoproterenol-treated rat cardiac myocytes by reducing the expression of inducible nitric oxide synthase (iNOS) (7) and by increasing superoxide dismutase (SOD) activity (8). Subsequently, silibinin was detected to promote sustained superoxide (O$_2^-$) production, which had a protective effect, as exogenous SOD markedly enhanced silibinin-induced apoptosis (9). In this study, silibinin displayed a cytotoxic effect on human fibroblast cells HT1080 through induction of reactive oxygen species (ROS) generation.

ROS, including hydroxyl radicals, superoxide anions, singlet oxygen, and hydrogen peroxide, are highly reactive owing to their possession of unpaired valence shell electrons. ROS is generated as a natural byproduct of cellular metabolism, primarily in the mitochondria. However, as environmental stress rises up, ROS levels increase dramatically, leading to significant impairment to cell structures, which cumulates into a state known as oxidative stress (10).

 Normally, autophagy is the cell’s internal course of catabolism, which is essential for cell growth, development, and homeostasis. With the involvement of the degradation of components through the lysosomal machinery, autophagy contributes to the balance between the synthesis, degradation, and subsequent recycling of cellular products. It has been illustrated that autophagy

Abstract. Hepatoprotectant silibinin has anticancer and chemo-preventive effects. In this study, silibinin showed significant inhibitory effect on human fibroblast HT 1080 cell growth cultured in media containing 10% fetal bovine serum or in serum free media, and in the latter case, silibinin exerted a more significant effect. Silibinin induced autophagy at 12 h, confirmed by monodansylcadaverine (MDC) staining, up-regulation of Beclin 1 (initiation factor for autophagosome formation), and conversion of LC3 I to LC3 II (autophagosome marker). It also induced apoptosis at 24 h, proved by observation of apoptotic body and activation of caspase-3. 3-Methyladenine (3-MA) inhibited silibinin-induced autophagy and promoted cell survival, suggesting that autophagy enhanced silibinin-induced apoptosis in HT1080 cells. Silibinin generated reactive oxygen species (ROS) in HT1080 cells, and the ROS scavenger N-acetylcysteine (NAC) reversed the cytotoxicity of silibinin, resulting in cell survival by inhibition of autophagic and apoptotic pathways. Application of specific antioxidants demonstrated that H$_2$O$_2$ was a major factor in silibinin-induced ROS since the H$_2$O$_2$ scavenger catalase reduced both autophagy and cell death. O$_2^-$ also contributed to silibinin-induced cell death.

Keywords: silibinin, H1080 cell, autophagy, apoptosis, reactive oxygen species
resulting in the total devastation of the cell is one of several types of programmed cell death (PCD) (11).

Apoptosis, another type of PCD, involves a trial of biochemical events in multicellular organisms, leading to a variety of cellular morphological changes and death. Morphological characteristics include blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (12).

Numerous investigations have shown silibinin’s apoptotic effect on various cancer cell lines (6, 9), while the study on its autophagic function is poor. In this study, the autophagic and apoptotic effects of silibinin were evaluated in condition of DMEM containing 10% fetal bovine serum or serum-free media (SFM). Cells grew fine in SFM at 48 h. Serum starvation can trigger autophagy to support cell survival. Media withdrawing serum provoked higher level of cell sensitivity to silibinin-induced autophagy and apoptosis. Since silibinin is a classic antioxidative traditional drug (13), involvement of ROS for induction of autophagy and apoptosis is indicated in this study.

Materials and Methods

Reagents

Silibinin was obtained from Beijing Institute of Biological Products (Beijing, China), and its purity was determined to be about 99% by HPLC measurement. Silibinin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution and diluted by DMEM (Gibco, Grand Island, NY, USA) before the experiments. DMSO concentration in all cell cultures was kept below 0.1%, which had no detectable effect on cell growth or death.

3-(4,5-Dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange (AO), propidium iodide (PI), 2′,7′-dichlorofluorescein diacetate (DCF-DA), monodansylcadervarine (MDC), 3-methyladenine (3-MA), N-acetylcysteine (NAC), catalase, SOD, and glutathione (GSH) were purchased from Sigma Chemical (St. Louis, MO, USA). Polyclonal antibodies against caspase-3 (sc-7148), Beclin 1 (sc-10086), LC3 (sc-134226), Actin (sc-7210), and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

HT1080, human fibroblast cells, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in DMEM medium supplemented with 10% heat inactivated (56°C, 30 min) fetal bovine serum (FBS) (Beijing Yuanheng Shengma Re-search Institution of Biotechnology, Beijing, China), 2 mM l-glutamine (Gibco, Grand Island, NY, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C in 5% CO2. The cells in the exponential phase of growth were used in the experiments.

Growth inhibition assay

The growth inhibitory effect of silibinin on HT1080 cells was measured by MTT assay. The cells were dispensed in 96-well, flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 1.5 × 104 cells per well. After 24-h incubation, they were treated with the tested agents for the indicated time periods. A 20-μl aliquot of MTT solution (5.0 mg/ml) was added to each well followed by 4-h incubation, and the optical density was measured using an ELISA reader (Tecan Spectra, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows:

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\text{Inhibitory ratio (\%)} = \left( \frac{A_{490, \text{control}} - A_{490, \text{sample}}}{A_{490, \text{control}} - A_{490, \text{blank}}} \right) \times 100
\]

Observation of morphologic changes

The HT1080 cells were divided into two groups and placed on culture plates for 24-h incubation. One group was treated with the control medium, another group was treated with silibinin, and the cellular morphology was observed using phase contrast microscopy (Olympus, Tokyo).

Nuclear damage observed by AO staining

The changes in nuclear morphology of apoptotic cells were examined by using cells labeled with the fluorescent, selective DNA and RNA-binding dye, AO. After incubation with silibinin for the indicated time periods, the cells were stained with 20 μg/ml AO at 37°C for 15 min, and then the morphology was observed under a fluorescence microscope (Olympus).

Flowcytometric analysis using PI, MDC, and DCF-DA

After treatment with drugs, HT1080 cells were harvested, rinsed with cold PBS, and then fixed in 70% ethanol at 4°C for at least 18 h. Then the cell pellets were stained with the fluorescent probe solution containing 50 μg/ml PI and 1 mg/ml DNase-free RNaseA in PBS on ice in the dark for 1 h. DNA fluorescence of PI-stained cells was evaluated by a FACSScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

The fluorescent compound MDC has been used as a tracer for autophagic vacuoles. HT1080 cells were harvested, rinsed with PBS, and then stained with 0.05 mM MDC at 37°C for 1 h. After incubation, the cells were washed once with PBS. The samples were analyzed by a FACSScan flow cytometer.
The treated HT1080 cells were incubated with 10 mM DCF-DA at 37°C for 30 min. The intracellular ROS mediated oxidation of DCF-DA to the fluorescent compound DCF. Then the cells were harvested and the pellets were suspended in 1 ml PBS. Samples were analyzed by a FACScan flow cytometer.

Western blot analysis
Both adherent and floating HT1080 cells were harvested, washed twice with ice-cold PBS, and then lysed in lysis buffer [50 mM Hepes (pH 7.4), 1% Triton-X 100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM edetic acid, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin] on ice for 1 h. After centrifugation of the cell suspension at 13,000 × g for 15 min, the protein content of supernatant was determined by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). The protein lysates were separated in 12% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Proteins were detected using polyclonal antibody and visualized using anti-rabbit, anti-mouse or anti-goat IgG conjugated with HRP and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the substrate of HRP.

Statistical analyses
All data represented at least three independent experiments and were expressed as the mean ± S.E.M. The data were analyzed by ANOVA using Statistics Package for Social Science (SPSS) software (version 13.0; SPSS, Chicago, IL, USA), and the LSD-post-hoc test was employed to assess the statistical significance of difference between the control and treated groups. *P*-values of less than 0.05 were considered statistically significant.

Results

Determination of the effect of silibinin to the HT1080 cells
The MTT cell-viability assay demonstrated that silibinin stimulated HT1080 cell proliferation at low dose (20 μM) and short-time (24 h) culture, while it inhibited cell proliferation at higher doses over 200 μM and longer-time (48 h) culture (Fig. 1). The growth inhibitory ratio of silibinin-treated HT1080 cells was increased by 10 times when serum was removed, suggesting that serum could protect HT1080 cells (Fig. 1).

Autophagy was induced in silibinin-treated HT1080 cells cultured with or without 10% FBS
MDC is used as a selective fluorescent marker for autophagic vacuoles (14). The silibinin-treated group showed higher fluorescent density and more MDC labeled particles in HT1080 cells compared with the control group at 12 h (Fig. 2A), indicating that silibinin increased MDC recruitment to autophagosomes in the cytoplasm of the cell. The flow cytometric analysis also indicated that the percentage of MDC-positive cells by silibinin treatment was increased compared to those in the control. Autophagy was more significant in silibinin-treated cells cultured in SFM for 12 h than in media containing 10% FBS (Fig. 2B). The MDC-positive cells were decreased when the specific autophagic inhibitor 3-MA (15) was applied (Fig. 2B). Beclin 1, associated with PI3K, has been identified to be responsible for autophagy (16). Once autophagy occurred, phosphatidylethanolamine is covalently linked to the cytosolic protein LC3 I to yield LC3 II, and this conversion is often regarded as a marker for autophagy (17). In order to further confirm the autophagy-augmenting effect of silibinin, Western blot analysis was performed to detect the expression of Beclin 1 and the conversion of LC3 proteins (Fig. 2C), indicating that silibinin induced autophagy in HT1080 cells.

Fig. 1. Effect of silibinin on HT1080 cell proliferation. The cells were cultured for 24 h and then incubated with different concentrations of silibinin for 12, 24, 36, and 48 h. A: The cells were cultured without serum. B: The cells were seeded in DMEM with 10% FBS. The viability was determined by the MTT assay. The data are presented as the mean ± S.E.M. of the results for three independent experiments.
Expression of Beclin 1 and conversion of LC3 were up-regulated at 50 and 100 μM of silibinin, but decreased at 200 μM of silibinin (Fig. 2C: a). In addition, the up-regulation of Beclin 1 and conversion of LC3 observed at 12 h was more obvious than those at 24 h (data not shown), suggesting that silibinin could trigger autophagy in HT1080 cells at specific dose and time. On the other hand, the much lower doses of silibinin up-regulated the expression of Beclin 1 and the conversion of LC3 in the cells cultured in SFM in a dose-dependent manner (Fig. 2C: b). High dose and long term application of silibinin might induce cell death through other death signal pathways, for instance, apoptosis or necrosis.

**Fig. 2.** Silibinin induced autophagy in HT1080 cells. A: Cellular fluorescent density was examined in the absence and in the presence of silibinin at 12 h (×400 magnifications); the cells were labeled with MDC. a and b: The cells were seeded in DMEM with 10% FBS. c and d: The cells were cultured without serum. B: Quantitative analysis detected a positive ratio of MDC staining by flow-cytometric analysis. The cells were treated with silibinin and the autophagy inhibitor 3-MA (1 mM) and harvested after 12 h. The data are presented as the mean ± S.E.M. of the results for three independent experiments. *P < 0.05, **P < 0.01. C: Expression of Beclin 1 and LC3 in silibinin-treated HT1080 cells at 12 h. a: The cells were seeded in DMEM with 10% FBS. b: The cells were cultured without serum. The density of each protein band was detected through Bandscan 5.0 software (Glyko, Novato, CA, USA).
Apoptosis was induced in silibinin-treated HT1080 cells cultured with or without 10% FBS

When HT1080 cells were cultured for 24 h with silibinin, marked morphologic changes were observed as compared with the control, and some of the silibinin-treated cells showed membrane blebbing, a hallmark of apoptosis (Fig. 3A: a–d). Meanwhile, silibinin-treated cells stained with AO showed remarkable chromatin condensation and nuclear fragmentation, as compared with the untreated control, which was stained homogeneously (Fig. 3A: e and f).

Caspase-3, a member of the family of aspartate-specific cysteinyl proteases, has been identified as a key mediator of apoptosis of mammalian cells by cleaving a variety of key cellular proteins, such as inhibitor of caspase-activated DNase (ICAD), poly (ADP-ribose) polymerase (PARP), and others (18). Both pro-caspase-3 and caspase-3 could be up-regulated by apoptotic stimuli (19). It was found that silibinin induced apoptosis by elevated expression of both inactive pro-caspase-3 and active caspase-3 in HT1080 cells either cultured in 10% FBS (Fig. 3B: a) or serum-free control media (Fig. 3B: b).

Inhibition of autophagy elevated survival of silibinin-treated HT1080 cells

Autophagy can function as a survival mechanism in starving cells. At the same time, extensive autophagy is commonly observed in dying cells, leading to its classification as an alternative form of PCD (20). MTT assay indicated that 3-MA decreased the inhibitory effect of silibinin on HT1080 cells in serum-free culture (Fig. 4). High dose (800 and 1,000 μM) of 3-MA slightly inhibited cell proliferation, in coordination with silibinin, and 600 μM of 3-MA showed the maximum protection to cells. Silibinin-induced autophagy led to the death of HT1080 cells, instead of survival (Fig. 4).

Silibinin induced apoptosis and autophagy by ROS generation in HT1080 cells

As an anti-oxidant traditional drug, silibinin was found
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to induce generation of ROS in HT1080 cells, which was markedly decreased by a ROS scavenger, NAC (21). Meanwhile, 3-MA also inhibited generation of ROS slightly (Fig. 5A), indicating that inhibition of autophagy reduced ROS in the cells. MTT assay was applied to examine the protective effect of NAC on silibinin-treated cells. Results showed that NAC could not only protect cells, but also, interestingly, convert the effect of silibinin from growth inhibition to stimulation (Fig. 5B). To determine if the protection by NAC is attributed to autophagy or apoptosis, MDC and PI staining were applied; NAC treatment lowered the MDC-positive ratio (Fig. 5C: a), and furthermore, it reduced the number of cells at the sub G0/G1 phase (Fig. 5C: b). Silibinin induced ROS generation in HT1080 cells and led cells to autophagy and apoptosis, and the ROS scavenger NAC could reverse both effects and rescue the cells.

$H_2O_2$ was the major ROS that participated in silibinin-induced cell death

Exogenous enzyme antioxidants (SOD, catalase) or non-enzyme antioxidants (GSH) were applied to determine the form of ROS in silibinin-treated cells. The generation of ROS was evaluated by DCF-DA staining. Catalase- or SOD-treatment reduced ROS generation; however, the catalase-treated group showed more significant ROS reduction than the SOD-treated group (Fig. 6A). The hydroxyl radical ($\cdot$OH) scavenger GSH (22) had no effect in this process. It is reported that SOD specifically catalyzes the reduction of superoxide anions ($O_2^-$) (23), while catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide ($H_2O_2$) into less reactive gaseous oxygen and water molecules (24). The result suggested that $H_2O_2$ is the major form of intracellular ROS generated by silibinin, and $O_2^-$ was involved as well in HT1080 cells. Autophagy of cells in media with different antioxidants plus silibinin was examined by MDC staining. Catalase decreased the positive ratio significantly; meanwhile, the SOD-treated group also showed lower MDC positive ratio (Fig. 6B). Silibinin-induced autophagy in cells was mainly mediated by $H_2O_2$, while $O_2^-$ played a secondary role. Contribution of antioxidants to proliferation of silibinin-treated cells was measured using the MTT assay. The result was parallel to the one of ROS and MDC staining that catalase demonstrated much more marked effect than SOD (Fig. 6C). It was concluded that both catalase and SOD diminished ROS generation, and autophagy proceeded and protected the cells. $H_2O_2$ was the predominant ROS induced by silibinin.

Discussion

Silibinin was reported to have anticancer properties. In this study, it was notable that silibinin moderately enhanced HT1080 cell growth at low dose and short-time culture, while silibinin suppressed cell growth at higher doses and longer-time culture, suggesting that there is some signal that switches silibinin’s function. Previous research demonstrated that dual efficacy of silibinin on ultraviolet B radiation–damaged HaCaT cells (25). It was reported that the protective effects of silibinin in HaCaT cells were lost at a higher dose of UVB and instead it further enhanced UVB-caused apoptosis (26). In this study, except for silibinin’s bi-directional regulation, more significant death ratio was observed under the starvation condition mimicked by serum-withdrawal. Silibinin might possess both survival and death effects. In the mild case, silibinin encourages cell proliferation. However, in the aggravated environment, inhibition ap-

Fig. 4. 3-MA inhibited the cytotoxicity of silibinin to HT1080 cells. Different doses of 3-MA were added to silibinin-treated cells. The cells were cultured in SFM for 24 h. The data are presented as the mean ± S.E.M. of the results for three independent experiments. *P < 0.05, **P < 0.01.
pears to be the major effect. Subsequent experiments demonstrated that silibinin induced marked autophagy at 12 h and apoptosis at 24 h in HT1080 cells. The functional relationship between autophagy and apoptosis is intricate. Autophagy acts as a stress adaptation that evades cell death and represses apoptosis in certain situations, whereas under other circumstances, it turns into an alternative cell death pathway. Autophagy and apoptosis may be evoked by common upstream signals, resulting in intermingled autophagy and apoptosis. In other proposed mechanisms, the cell switches between the two responses in a mutually exclusive manner (27). After inhibition of autophagy with 3-MA, the inhibitory ratio declined, suggesting autophagy induced by silibinin might enhance cell death.

On a molecular level, autophagic and apoptotic response machineries share some common pathways, for example, the PI3K/Akt signal pathway (28), MAPK/ERK1/2 signal pathway (29), and mitochondria pathway (30), which either link or polarize the cellular responses. Mitochondria are important regulators of both apoptosis and autophagy. One of the triggers for mitochondria-mediated apoptosis is ROS production. Mitochondria are both the source and target of ROS. Cytochrome c release from mitochondria, which triggers caspase activation, appears to be largely mediated by direct or indirect ROS action (31). Recently, several studies have demonstrated that ROS may be also involved in induction of autophagy. Recent investigations provided sound evidence for the participation of mitochondrially generated ROS production in the induction of autophagy, according to the formation of autophagosomes and autolysosomes (32).

ROS are molecules or ions that are formed by the incomplete one-electron reduction of oxygen, including superoxide anions \( \text{O}_2^{•−} \), hydrogen peroxide \( \text{H}_2\text{O}_2 \), hydroxyl radical \( \cdot\text{OH} \), and others. It has been found that silibinin is an active scavenger of various free radicals, including hydroxyl and peroxyl radicals, and the hypochlorite ion (33). Besides, silibinin augments the activity of the antioxidant enzymes SOD and GSH peroxidase.

![Graphs and diagrams](image)

**Fig. 5.** Silibinin induced apoptosis and autophagy through ROS in HT1080 cells. Silibinin: 20 µM. A: ROS generation was detected by a flow-cytometry staining with DCF-DA. B: The cells were incubated with silibinin or co-incubated with different concentrations of NAC, and the inhibitory ratio was measured by MTT assay. C: a: The cells were stained with PI and measured by a flow cytometry after collection. b: The MDC fluorescent intensity of treated cells was analyzed by flow cytometry. The data are presented as the mean ± S.E.M. of the results for three independent experiments. *\( P < 0.05 \), **\( P < 0.01 \).
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It is possible that the antioxidant activity of silibinin is on account of their ability to form inactive iron chelates, therefore reducing the formation of peroxides (33, 35). ROS was discussed in the initial investigation of silibinin’s function. Unexpectedly, our data showed that the antioxidant agent silibinin induced ROS generation in HT1080 cells, which was down-regulated by NAC, a ROS scavenger. NAC could not only help cells escape from the cytotoxicity of silibinin, but also switch silibinin’s effect from death to survival. Once all these species were cleared by NAC, silibinin then exerted its protective function, and the mechanism of this interesting promotive effect remains to be elucidated. Subsequently, the changes of MDC positive ratio and sub G0/G1 ratio showed that NAC reversed both autophagy and apoptosis induced by silibinin, indicating that NAC could affect both pathways to exert its protective effect.

In addition, ROS was reduced slightly by 3-MA, suggesting that the autophagic pathway initiated ROS generation. It was proposed that autophagy played an important role in the cell response to oxidative stress (36), and our result suggested that a positive feedback existed between autophagy and ROS.

Our previous study found that H2O2 is involved in autophagic PCD (37). Scherz-Shouval and his colleagues provided evidence for a mechanism by which H2O2 generated during starvation (deprivation of serum) serves as a signaling molecule that initiates autophagosome formation (38). Further research indicated that both H2O2 scavenger catalase and O2• scavenge SOD reduced ROS induced by silibinin, but the •OH scavenger GSH had no effect. Both Catalase and SOD inhibited autophagy and cell death induced by silibinin and catalase played a major role. According to these, silibinin triggered generation of ROS such as H2O2 and O2• and silibinin itself could not scavenge these ROS. Silibinin’s growth inhibition effect might mainly contribute to the induction of autophagy; and this induction might contribute to silibinin-induced H2O2, especially under the starvation condition.

References


