Full Paper

The Protective Effect of Silibinin Against Mitomycin C–Induced Intrinsic Apoptosis in Human Melanoma A375-S2 Cells

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Abstract. Silibinin is known for its hepatoprotective, anti-inflammatory, and anti-carcinogenic effects. We found that silibinin exhibited a protective effect against chemotherapeutic reagent mitomycin C–induced cell death in A375-S2 cells in a p53-dependent manner, which contradicted the findings of previous studies investigating the anti-neoplastic activity of silibinin and developing silibinin as a potential anti-neoplastic drug in clinical therapy. Mitomycin C administration triggered a time- and dose-dependent cell death in A375-S2 cells. Apoptotic morphology, DNA fragmentation, and caspase-3 activation demonstrated that the major cause of A375-S2 cell death by mitomycin C was apoptosis. This was associated with a marked increase of p53 level and changes in mitochondria associated proteins. However, preincubation with silibinin prior to mitomycin C treatment substantially suppressed cell apoptosis, attenuated the change of p53 and Bcl-2 expressions, blocked the translocation of Bax to mitochondrial outer membrane, and ameliorated the loss of mitochondrial membrane potential, but mitomycin C stimuli led to few changes in the protein levels of caspase 8, Fas ligand, and Fas-associated death domain protein, indicating that silibinin protected cells from mitomycin C–induced apoptosis mainly via suppressing the mitochondria-mediated intrinsic apoptosis pathway, but not in an extrinsic manner.

Keywords: silibinin, mitomycin C, mitochondria mediated apoptosis, p53

Introduction

Silymarin, a unique flavonoid derived from the milk thistle (*Silybum marianum* (L.) Gaertn) (1) (Fig. 1A), is known for its hepatoprotective (1–3), anti-inflammatory (4), and anti-carcinogenic effects (5, 6). It has already been found that silibinin is the main active structural component of silymarin (3). In the past few years, many researchers reported that silibinin had inhibitory effects on tumor cell invasion or growth. For instance, in non-small cell lung cancer cells and human osteosarcoma MG-63 cells, silibinin impeded tumor cell invasion (7, 8); in renal cell carcinoma, laryngeal squamous cell carcinoma, and prostate cancer cells, silibinin suppressed tumor cell growth (9–11).

Malignant melanoma is notorious for its insensitivity to DNA-damaging reagents, and other studies showed that combination of a DNA-damaging reagent (12) and silibinin exerted a synergistic pro-apoptotic effect in tumor cells (13). Therefore, A375-S2 cells, which belong to the malignant melanoma-type cells, were used to investigate whether silibinin also exerted a synergistic pro-apoptotic effect in cell death induced by mitomycin C, a DNA-damaging agent that is also used as a therapeutic drug. However, our study unexpectedly found that silibinin antagonized mitomycin C–induced cell death in A375-S2 cells and thus we further investigated the protective mechanisms of silibinin.

Apoptosis is an essential mechanism for cell death following many types of chemotherapy; It can be triggered by both intrinsic and extrinsic pathways. The intrinsic pathway is initiated in response to cellular signals resulting from DNA damage, loss of cell survival factors, or other types of severe cell stress. It is the

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Fig. 1. Chemical structure of silibinin (A) and the toxicity of mitomycin C in A375-S2 cells (B). B: The cells were treated with various doses of mitomycin C for 6, 12, or 24 h. Then the inhibitory ratio was measured by MTT assay. $n = 3$, mean $\pm$ S.D. All experiments were repeated more than three times.

equilibrium between Bax and Bcl-2 that dictates the fate of the cell, either death or survival, since an increased Bax/Bcl-2 ratio can lead to the increase of mitochondrial outer membrane permeabilization, allowing cytochrome *c* to diffuse from the intermembrane space to the cytosol, so that it can form an apoptotic complex with pro-caspase 9, thereby mediating intrinsic apoptosis (14, 15). On the other hand, the extrinsic pathway begins outside the cell through the activation of receptors on the cell surface by specific molecules known as pro-apoptotic ligands, including receptors DR4, DR5, and CD95L/Fas ligand (FasL) (receptor CD95/Fas) (16). Once activated, the ‘death domains’ of these receptors bind to the adaptor protein FADD (Fas-associated death domain protein), resulting in the assembly of the death inducing signaling complex, and recruitment and assembly of initiator caspase 8 and/or caspase 10. The two caspases are stimulated and processed, releasing active enzyme molecules into the cytosol, where they activate downstream caspases, thereby converging on the intrinsic pathway (17).

DNA damaging agents are an integral component in the treatment of a large variety of solid and hematological malignancies (18). DNA damage is a classical inducer of p53 activation, which orchestrates apoptosis or DNA repair depending on the cellular background (19) and during those processes, p53 functions as the

“guardian of genome integrity” since it elicits a block in the cell cycle, DNA repair, and eventually an apoptotic response after stress insults, compromising genomic integrity, oncogene activation, and hypoxia (20). More often than not, in executing the DNA lesion-induced cell death process, p53 functions as a crucial protein by switching on either intrinsic or extrinsic apoptosis or both. One important mechanism through which p53 mediates the biological response is transcriptional activation of proapoptotic target genes, including proapoptotic members of the Bcl-2 family proteins such as Bax, Bid, and death-related receptors such as FasR/DR5 (21); and this mechanism depends on p53 translocation from the cytoplasm to the nucleus. Besides, in the cytoplasm, p53 can directly interact with members of the anti- and pro-apoptotic Bcl-2 family members to either inhibit or activate them, finally leading to intrinsic/mitochondrial apoptosis or extrinsic apoptosis via activating caspase cascades (22).

It is worth noting that our study found the novel protective effect of silibinin against cell apoptosis in A375-S2 cells induced by the DNA-damaging reagent mitomycin C. This contradicted other studies that have well-established silibinin’s anti-tumor activities both in vivo and vitro. Considering the essential roles that p53 and mitochondria pathway-associated proteins play in a cell’s responses to DNA damage, we investigated the cyto-protective effect of silibinin, mainly focusing on its effects on the intrinsic apoptosis pathway.

Materials and Methods

Reagents

Silibinin was obtained from China Institute of Biological Products (Beijing, China). Silibinin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution and diluted by RPMI-1640 (Gibco, Grand Island, NY, USA). DMSO concentration in all cell cultures was kept below 0.1%, which had no detectable effect on cell growth or death. 5-Diphenyl tetrazolium bromide (MTT), 3,3-diaminobenzidine tetra hydrochloride (DAB), and rhodamine-123 were purchased from Sigma Chemical (St. Louis, MO, USA). Rabbit polyclonal antibodies against Bax, Bcl-2, SIRT-1, caspase 9, caspase 3, FasL, FADD, caspase 8, p53, mouse polyclonal antibody against cytochrome *c*, and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

A375-S2 cells, human melanoma cells, were obtained from American Type Culture Collection (ATCC, #CRL,

1872; Manassas, VA, USA) and were cultured in RPMI-1640 medium supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 mM L-glutamine (Gibco), 100 U/mL penicillin, and 100 mg/L streptomycin (Gibco) at 37°C in 5% CO₂. Cells in the exponential phase of growth were used in the experiments.

Cytotoxicity assay

A375-S2 cells were dispensed in 96-well, flat-bottomed microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells per well. After 24-h incubation, they were treated with or without silibinin at the given concentrations 1 h prior to the administration of mitomycin C for the indicated time periods and concentrations. The cytotoxic effect was measured using the MTT assay as described elsewhere (23) with a plate reader (Bio-Rad, Hercules, CA, USA). The percentage of cell growth inhibition was calculated as follows:

Cell growth inhibition (%)

$$= (A_{492, \text{control}} - A_{492, \text{sample}}) / (A_{492, \text{control}} - A_{492, \text{blank}}) \times 100$$

Observation of morphologic changes

The cells were divided into 4 groups and placed on culture plates for 24-h incubation. One group was treated with the control medium and incubated for 12 h. The other three groups were separately treated with or without silibinin for 1 h prior to mitomycin C administration and then co-incubated with mitomycin C for 12 h. Then the cellular morphology of each group was observed using phase contrast microscopy (Leica, Wetzlar, Germany).

Lactate dehydrogenase (LDH) activity-based cytotoxicity assay

LDH activity was assessed using a standardized kinetic determination kit (LDH kit; Zhongsheng, Beijing, China). LDH activity was measured in both floating dead cells and viable adherent cells (24). The floating cells were collected from culture medium by centrifugation ($240 \times g$) at 4°C for 5 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (extracellular LDH, or LDHe) was used as an index of necrotic death, and the LDH present in the adherent viable cells was taken as intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\begin{aligned} \text{Apoptosis\%} &= \text{LDHp} / (\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100 \\ \text{Necrosis\%} &= \text{LDHe} / (\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100 \end{aligned}$$

Flow cytometric propidium iodide (PI) staining analysis of DNA damage

The cells belong to the different tested groups were harvested at a density of 1×10^6 and washed once in cold PBS. Then cell pellets were fixed in 70% ethanol at 4°C overnight and washed in cold PBS. Then the pellets were suspended in 1 ml of PI solution containing 50 µg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) Triton X. Cell samples were incubated at 4°C in the dark for at least 15 min and analyzed by a flow cytometer with an excitation wavelength of 488 nm and an emission wavelength of 600 nm (25).

Western blot analysis

Tested groups were harvested (both adherent and floating cells), and then Western blot analysis was carried out as previously described (26) with some modifications. Briefly, cell pellets were resuspended in lysis buffer, including 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 (Sigma), 10 µg/mL aprotinin (Sigma), and 10 µg/mL leupeptin (Sigma), and lysed on ice for 60 min. After centrifugation of the cell suspension at $13,000 \times g$ for 15 min, the protein content of supernatant was determined by Bio-Rad protein assay reagent (Bio-Rad). The protein lysates were separated by 12% SDS-polyacrylamide gel electrophoresis and blotted onto a wet 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 horseradish peroxidase (HRP) and DAB as the HRP substrate.

Preparation of nuclear and cytosolic extract

The tested cell groups were collected by centrifugation at $200 \times g$ at 4°C for 5 min and then washed twice with ice-cold PBS. Cell pellets were resuspended in ice-cold homogenizing buffer, including 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 1 µg/mL aprotinin, and 1 µg/mL leupeptin. After homogenization (40 strokes), the homogenates were centrifuged at $5,000 \times g$ at 4°C for 20 min. The supernatant was used as the cytosol fraction, the pellet was resolved in lysis buffer and centrifuged at $5,000 \times g$ for 10 min and the supernatants were used as the nuclear extract (27).

Analysis of mitochondrial membrane potential by rhodamine 123

Rhodamine 123 as a cationic fluorescent dye can enter the mitochondrial matrix depending on mitochondrial

transmembrane potential. If the mitochondrial membrane potential is depleted, rhodamine 123 can be released from the mitochondria. The mean fluorescence intensity of rhodamine 123 was measured to determine the loss of mitochondrial membrane potential. The cells were pre-treated with or without silibinin for 1 h and then co-incubated with mitomycin C for 12 h. After incubation, the cells were collected and loaded with 5 μ M rhodamine 123 at 37°C for 30 min. The fluorescence intensity of the cells was observed under a fluorescence microscopy and analyzed by flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 525 nm (28). Since the decrease in rhodamine staining-positive cells stands for the loss of mitochondrial membrane potential (MMP), the relative loss of MMP compared to the control was calculated as follows: decrease in rhodamine retention% = $100 \times (\text{rhodamine positive cells\%} - \text{rhodamine positive cells\%})$.

Preparation of mitochondrial and cytosolic extracts

The tested cell groups were collected by centrifugation at $200 \times g$ at 4°C for 5 min and then washed twice with ice-cold PBS. Cell pellets were resuspended in ice-cold homogenizing buffer containing 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 1 μ g/mL aprotinin, and 1 μ g/mL leupeptin. After homogenization (40 strokes), the homogenates were centrifuged at $4,200 \times g$ at 4°C for 30 min. The supernatant was used as the cytosol fraction, and the pellet was resolved in lysis buffer as the membrane fraction (29).

Statistical analyses

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

Results

Mitomycin C administration induced apoptosis but not necrosis in a time- and dose-dependent manner

The cytotoxicity of mitomycin C was examined in the concentration range of 1–50 μ g/ml for 6, 12, and 24 h and then analyzed by MTT assay (Fig. 1B). Mitomycin C administration caused a time- and dose-dependent cell growth inhibition. The IC₅₀ value at 12 h is 18.6 μ g/ml; therefore, 20 μ g/ml was chosen as the

tested concentration in this study. The cells treated by mitomycin C showed marked apoptotic morphologic features, including cell membrane blebbing and typical apoptotic body formation observed by phase contrast microscopy (Fig. 2B: b).

Silibinin protected A375-S2 cells against mitomycin C-induced cell apoptosis

To evaluate the silibinin's ability to prevent mitomycin C-induced cell apoptosis, the cells were treated with silibinin at different concentrations for 1 h prior to mitomycin C incubation for 12 h and then analyzed by MTT assay. As shown in Fig. 2A, silibinin pre-treatment at each dose significantly increased the cell viability and groups treated with silibinin groups exhibited no obvious cell growth inhibition. Morphologic observation revealed that mitomycin C-induced marked apoptotic body formation was substantially reduced by pretreatment with silibinin for 1 h (Fig. 2B: b and c). To further investigate the role of silibinin in mitomycin C-induced A375-S2 cell death, the characteristics of cell death were analyzed by an LDH activity-based assay as shown in Fig. 2C. Results in Fig. 2C demonstrated that mitomycin C-induced A375-S2 cell death via apoptosis instead of necrosis, and this process was attenuated by silibinin pre-treatment.

The activation of caspase 9 and caspase 3, ICAD cleavage, and DNA fragmentation following mitomycin C administration were substantially alleviated by silibinin

In the mitochondria-mediated apoptotic process, procaspase 9 is cleaved into the active form caspase 9 and then activates its downstream caspase 3, which in turn cleaves caspase-activated DNase (CAD) inhibitor, ICAD, and promotes the dissociation of CAD from ICAD. Then CAD translocates into nucleus and degrades DNA into fragments, manifesting the apoptosis cascade (30). In terms of this process, the activation of caspase 3 and caspase 9 and ICAD cleavage were examined by Western blot analysis. The result revealed that caspase 3 and caspase 9 activation together with ICAD cleavage induced by mitomycin C administration for 12 h was significantly reduced by 150 μ M silibinin pretreatment (Fig. 3A). Cell death ratio was evaluated by flow cytometric assay of DNA fragmentation with PI staining. In the analytic diagrams of PI staining flow cytometry, the cursors M1, M2, M3, M4, and M5 define the sub-G0/G1 (stands for the DNA fragmentation), G0/G1, S, G2/M, and aneuploid phases of the cell cycle, respectively. Data in Fig. 3B showed that the percentage of Sub-G0/G1 phase was increased from 2.92% in the control group to 19.37% in the mitomycin C-treated group (Fig. 3B: a and b), demon-

Fig. 2. Silibinin protected cells from mitomycin C-induced death. A: Evaluation of cell viability by MTT assay. The cells were treated with or without silibinin at the indicated concentrations for 1 h, co-incubated with mitomycin C for 12 h, and then inhibitory ratio was analyzed by MTT assay. * $P<0.05$, ** $P<0.01$ vs. mitomycin C treatment alone group. B: Phase contrast micrographs of cell morphology. The cells were pre-incubated with or without silibinin for 1 h, co-incubated with mitomycin C for 12 h, and then the cells were observed by phase contrast microscopy. a: control group, b: mitomycin C treatment group, c: silibinin and mitomycin C co-incubation group, d: silibinin treatment alone group. Arrows indicate apoptotic cells. C: LDH analysis of cell death characteristic. The cells were treated with 100 or 150 μM silibinin for 1 h, incubated with mitomycin C for 12 h, and then the ratios of apoptosis and necrosis were examined by LDH analysis. $n=3$, mean $\pm$ S.D. ** $P<0.01$ vs. control, # $P<0.05$, ## $P<0.01$ vs. mitomycin C (alone)-treated group. All experiments were separately performed more than three times.

Fig. 3. The expression of apoptosis-associated proteins and DNA fragmentation induced by mitomycin C administration were suppressed after silibinin pretreatment. A: Western blot analysis of the activation of caspase 9 and caspase 3 and ICAD expression. The cells were pre-treated with or without silibinin for 1 h and then co-incubated with mitomycin C for 12 h. Then the cells were collected and lysed, and the protein levels of pro-caspase 3, pro-caspase 9, caspase 3, caspase 9, and ICAD were detected by Western blotting. B: Flow cytometric PI staining analyzed DNA fragmentation. The cells were treated in the same way as described in panel A and then collected and fixed with 70% ethanol; They were PI-stained for 1 h on ice before detection by FACS. a, control group; b, mitomycin C treatment group; c, silibinin and mitomycin C co-incubation group; d, silibinin treatment alone group. C: Data are the same as the data shown in panel B. $n=3$, mean $\pm$ S.D. ** $P<0.01$ vs. mitomycin C treatment group.

strating that notable DNA damage occurred in the cells with mitomycin C administration, and this increase was reduced to 3.62% in the silibinin and mitomycin C co-incubation group (Fig. 3B: c); the percentage of the Sub-G0/G1 phase was unaffected in the silibinin treatment alone group (Fig. 3B: d). The same result was also shown in the histogram of Fig. 3C. The PI-staining result represents the death ratio, while the result of the MTT assay represents growth inhibition that could be induced by both death and growth arrest. Therefore, the value of the cell death ratio analyzed by PI staining was lower than the value of growth inhibition analyzed by the MTT assay.

The marked increase in p53 expression and decrease in SIRT-1 expression after mitomycin C stimuli were substantially alleviated by silibinin

P53 often mediates apoptosis in cells suffering from serious DNA damage. SIRT-1, a deacetylase, can deacetylate p53 to reduce p53 stability and promote its degradation by mouse double minute-2 (MDM2) ubiquitin ligase (31). Therefore, the protein levels of p53 and SIRT-1 were examined by Western blot analysis: mitomycin C caused over-expression of p53, elevated translocation of p53 into the nucleus, and decreased SIRT-1 expression; and these effects were alleviated by silibinin to a large extent (Fig. 4).

There were no obvious changes in the protein expression of FasL, FADD, and pro-caspase 8 in mitomycin C-treated cells

To assess whether the death receptor Fas/CD 95 mediated cell apoptosis in mitomycin C treated A375-S2 cells, critical proteins associated with Fas-mediated signaling pathway were examined here. Results of Western blot analysis showed that protein expressions

of FasL, FADD, and pro-caspase 8 were not changed after mitomycin C treatment (Fig. 5), demonstrating that the Fas-mediated pathway was not involved in mitomycin C-induced cell death.

The loss of mitochondrial potential caused by mitomycin C treatment was reversed by silibinin pre-treatment

The importance of mitochondrial dysfunctions during apoptosis induced by anticancer drugs is well documented; in particular, mitochondrial permeability transition is considered a critical early event in the apoptotic process. Thus the change in MMP was examined here by rhodamine 123 staining and fluorescent microscopy (Fig. 6A) and the loss in rhodamine 123 retention was quantified by flow cytometry (Fig. 6B: a–d). Data in the the histogram of Fig. 6C showed that preincubation with silibinin ameliorated the loss of MMP by mitomycin C treatment.

The changes of Bcl-2 protein level, translocation of Bax, and cytochrome c by mitomycin C administration were suppressed by silibinin

Since accumulated evidences have been reported that apoptosis initiated by the loss of MMP depends on the lost dynamic equilibrium of Bcl-2 and Bax and mitomycin C-induced caspase 9 activation. Hereby, the protein levels of Bax, Bcl-2, and cytochrome c in mitochondria, cytosol, and the whole cells were examined. As shown in Fig. 7, the expression of Bcl-2 both in mitochondria and the whole cells decreased in response to mitomycin C stimuli, and this kind of decrease was attenuated by pretreatment with silibinin prior to mitomycin C administration. Bcl-2 expression in the cytosol was undetectable, possibly due to the low Bcl-2 levels in the cytoplasm (Fig. 7A). Furthermore, silibinin preincubation also reversed the conspicuous translocation of Bax from cytosol to mitochondria (Fig. 7B)

Fig. 4. Silibinin alleviated mitomycin C-induced p53 over-expression and SIRT-1 down-regulation. The cells were pre-treated with or without silibinin for 1 h, co-incubated with mitomycin C for 12 h, and then collected and lysed. Levels of SIRT-1 protein in the whole cell lysates and protein levels of p53 in whole lysates, nucleus, and cytosol lysates were detected by Western blot analysis. All experiments were carried out at least three times.

Fig. 5. There were no changes in the protein levels of Fas receptor-associated proteins in mitomycin C-induced cell death. The cells were treated in the same way as depicted in Fig. 3A. Then each group of cells was collected and lysed. The expression of FADD, FasL, and pro-caspase 8 were detected by Western blot analysis. Experiments were repeated more than three times with similar results.

Fig. 6. Silibinin reversed the loss of MMP caused by mitomycin C stimuli. The cells were pre-treated with or without silibinin for 1 h and co-incubated with mitomycin C for 12 h. Then the cells were collected and loaded with the membrane-sensitive probe rhodamine 123 (1 μ g/ml) at 37°C for 30 min, washed, and observed by fluorescence microscopy or flow cytometry. The fluorescent micrographs of treated cells are shown in A: a, control group; b, mitomycin C treatment group; c, silibinin and mitomycin C co-incubation group; d, silibinin (alone) treatment group. The FACS histograms of treated cells are presented in B: a, control group; b, mitomycin C treatment group; c, silibinin and mitomycin C co-incubation group; d, silibinin (alone) treatment group; the histogram in C showed the relative loss of rhodamine 123 retention in each treatment group compared to the control group. $n = 3$, mean $\pm$ S.D. ** $P < 0.01$ vs. mitomycin C (alone) treatment group. All experiments were performed more than three times with similar results.

Fig. 7. The effect of silibinin on the expression and translocation of critical proteins in the mitochondrial pathway. The cells were pre-treated with or without silibinin for 1 h and then cultured with mitomycin C for a further 12 h. Then the cells were collected and lysed. The protein levels of Bcl-2 (A), Bax (B), and cytochrome *c* (C) in the whole cell lysates and mitochondria lysates were separately detected by Western blot analysis. All experiments were repeated more than three times.

and cytochrome *c* releasing from the mitochondrial intermembrane space to the cytosol (Fig. 7C).

Discussion

Until now, the development of silibinin as a chemotherapeutic assistant drug has been anticipated. Several in vitro studies by others and us have shown that silibinin inhibited growth in many tumor cells mainly via modulating multiple signaling pathways that related to cell growth, apoptosis, and cell cycle arrest (5, 6, 32–34). In some studies, it was found that silibinin increased IGFBP-3 (insulin-like growth factor-binding protein 3) expression, thus inhibiting IGF-1R signaling, and also inhibited nuclear factor κ B and epithelial growth factor receptor signaling (35). Silibinin itself also showed a moderate pro-apoptotic effect both in vitro and vivo. It has been observed that silibinin activated the ataxia telangiectasia mutated-check point signaling kinase-2 pathway, leading to G2-M arrest as well as apoptosis in colon carcinoma cells (33, 34).

To our surprise, in our study, silibinin's cyto-protection in A375-S2 cells was associated with the inhibition of p53 expression, and this protection was not found in HeLa cells and Hep-G2 cells (data not shown). The discrepancies in different cells might lie in the various characteristics of different cell types, which need to be further investigated. Among these cells, p53 is constitutively highly expressed in melanoma cells and Bcl-2 plays an essential role in melanoma resistance to chemotherapeutic drugs (36, 37). Therefore, this protective mechanism might be attributed to the co-operation between reduced p53 expression and augmented anti-apoptotic Bcl-2 expression in melanoma and more attention should be paid to the development of silibinin as a potential drug in cancer therapy or prevention.

Having a short half-life, p53 is normally maintained at low levels both in unstressed normal mammalian cells and p53 wild-type transformed cells by continuous ubiquitylation and subsequent degradation by the proteasome (38, 39). Whereas, when cells face stress such as DNA damage, oxidative stress, or hypoxia, p53 degradation is suppressed and it starts to accumulate in the cells, where it is activated and stabilized by undergoing multiple covalent modifications including phosphorylation and acetylation (40).

Phosphorylation of p53 often occurs at the serine residues in the N-terminal activation domains, sometimes occurring in the C-terminal domains. Acetylation of p53 has been shown to augment p53 DNA binding and to regulate the stability of p53 by inhibiting its ubiquitination by MDM2 ubiquitin ligase. In vivo, acetylation of p53 at Lys320, 373, and 382 is induced by many genotoxic agents, including UV-radiation, ionizing radiation, hypoxia, and oxidative stress (40). P53 can also be deacetylated by histone deacetylases such as SIRT-1.

SIRT-1 is a NAD-dependent deacetylase critically involved in the stress response, cellular metabolism, and possibly, ageing (41). The tumor suppressor p53 represents the first non-histone substrate of SIRT-1, functionally regulated by acetylation and deacetylation; and it has been reported that SIRT-1 promotes cell survival by deacetylating p53 and that hyperacetylation of p53 increases radiation-induced apoptosis in SIRT-1-deficient cells (42, 43).

In the present study, mitomycin C stimuli led to apparent DNA fragmentation and apoptosis in A375-S2 cells, which were accompanied with the over-expression of p53 protein. Silibinin pre-treatment promoted cell survival substantially and attenuated p53 over-expression, demonstrating that silibinin protected cells from mitomycin C-induced apoptosis possibly via suppressing the pro-apoptotic effect of p53. In addition, silibinin

treatment alone escalated SIRT-1 expression and even when co-incubated with mitomycin C, silibinin reversed mitomycin C-induced SIRT-1 down-regulation, suggesting that the elevated SIRT-1 expression might be involved in silibinin's protective effects; these observations suggest that silibinin inhibits p53-mediated cell death through deacetylation of p53 to decrease the stability of p53. Although mitomycin C caused an increased expression of p53, there were no changes in p53 levels in the nucleus, implicating that translocation of p53 did not participate in p53-mediated apoptosis.

In contrast to no changes in the critical proteins levels in Fas receptor-mediated signaling, mitochondrial pathway-associated proteins played an essential role in starting the apoptosis program in mitomycin C-induced cell death in A375-S2 cells. Mitomycin C treatment led to a marked Bax localization into mitochondrial outer membrane and cytochrome *c* release into the cytosol. Meanwhile, the levels of anti-apoptotic Bcl-2 protein also decreased. However, when the cells were pre-exposed to silibinin and then treated with mitomycin C, the above phenomena were reversed. Many researchers showed that the increase in p53 expression was sometimes involved in the mitochondrial apoptosis pathway, via promoting Bax translocation or interacting with Bcl-2 protein and neutralizing their inhibitory effects on pro-apoptotic Bax (44, 45). The same phenomena observed in this study indicated that p53 induced apoptosis by promoting Bax translocation and enhancing Bcl-2 degradation, resulting in increased releases of MMP and cytochrome *c* into the cytosol, finally leading to cell apoptosis.

To summarize, our present study showed that p53 plays a role in mitomycin C-induced intrinsic apoptosis in A375-S2 cells, and this is associated with the decrease in Bcl-2 levels and elevated Bax translocation from the cytosol to mitochondrial outer membrane; silibinin antagonized mitomycin C-induced intrinsic apoptosis in A375-S2 cells probably through increasing the expression of SIRT-1 protein and inhibiting p53 expression.

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