Full paper

ERα down-regulation plays a key role in silibinin-induced autophagy and apoptosis in human breast cancer MCF-7 cells

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Abstract

The estrogen receptor alpha (ERα) has been proven to be one of the most important therapeutic targets in breast cancer over the last 30 years. Previous studies pointed out that a natural flavonoid, silibinin, induced apoptosis in human breast cancer MCF-7 cells. In the present study we report that exposure of MCF-7 cells to silibinin led to cell death through the down-regulation of ERα expression. Silibinin-induced apoptosis of MCF-7 cells through up-regulation of caspase 6 due to ERα signalling repression was further boosted by ERα antagonist. Moreover, up-regulation of autophagy induced by silibinin accounted for apoptotic exacerbation, being further enhanced by ERα inhibition. Upon ERα activation, series of downstream signalling pathways can be activated. We found that silibinin reduced the expressions of Akt/mTOR and extracellular-signal-related kinase (ERK), which respectively accounted for the induction of autophagy and apoptosis. These effects were further augmented by co-treatment with ERα inhibitor. We conclude that the treatment with silibinin of ERα-Positive MCF-7 cells down-regulates the expression of ERα, and subsequently mTOR and ERK signaling pathways, Erα downstream, finally resulting in induction of autophagy and apoptosis.

1. Introduction

In the modern world, breast cancer is one of the most common malignancies in women. It has been known more than a century (1) that breast cancer is associated with cell-surface estrogen receptor (ER). Human ER exists as two subtypes, ERα and ERβ, which regulate the transcription of various target genes upon binding to estrogen response elements (ERE) present within the regulatory region of the genes. In most ERα-positive breast cancers, especially in early stages, the expression level of ERα is considerably higher than that in normal breast epithelium (2). Therefore, targeting the ERα signalling pathway has already become a focal point in the development of breast cancer therapy.

Silibinin (Fig. 1A), a natural polyphenolic flavonoid, is a major bioactive component of silymarin isolated from the plant milk thistle Silybum marianum (L.) Gaertn. Silibinin has been extensively used for its hepatoprotective effectiveness in Asia and Europe (3,4). According to the reports, flavonoids have been shown to be cytotoxic to cancer cells (5,6). It has been reported that the estrogen receptor is required for flavonoid-induced cytotoxicity in breast cancer cell lines (7,8). Silibinin induces a loss of cell viability in MCF-7 cells. However, underlying mechanism has not been completely elucidated. It is not known if the estradiol-like effect of silibinin contributes to its anti-cancer potency. Therefore, we focused on ERα regulation in the cytotoxicity of silibinin in MCF-7 cells that inherently express high levels of ERα.

2. Materials and methods

2.1. Reagents

Silibinin with a purity of 99% was purchased from Jurong Best Medicine Material (Zhenjiang, Jiangsu, China). The reagent was dissolved in dimethylsulfoxide (DMSO) to make a stock solution.
Fig. 1. The cytotoxicity of silibinin in MCF-7 cells is correlated with the down-regulation of estrogen receptor α (ERα). (A) The chemical structure of silibinin. (B) The cells were treated with various doses of silibinin for 12, 24, 36 or 48 h. Viability was measured by MTT assay. (C) Western blot analysis of ERα levels after treatment with 100, 200 and 300 μM silibinin for 24 h β-actin was used as an equal loading control. (D) The cells were pre-treated with 10, 20 and 40 μM ICI 182,780 for 1 h prior to silibinin (200 μM) administration for 24 h, and the cell viability was measured by MTT assay, n = 3, mean ± S.E.M, *p < 0.05, compared with silibinin -treated group. (E) The different suppression of silibinin in ERα-high expressive MCF-7 and ERα-low expressing MDA-MB231 (MB231) human breast cancer cells. Compare between the two strains of human breast cancers at the specific concentration of silibinin, **p < 0.01. ERα levels were examined by western blot analysis. (F) Cells were cultured for 24 h and then incubated with different concentrations of MPP (a specific ERα antagonist MPP dihydrochloride) for 1 h, and then silibinin 200 μM was added and cultured for 24 h. The viability was determined by the MTT assay, n = 3, mean ± S.E.M, **p < 0.01, compared with silibinin-treated group. (G) The cells were transfected with control or ERα-targeting siRNA for 48 h, and then incubated with different concentrations of silibinin for 24 h. The viability was measured by MTT assay, n = 3, mean ± S.E.M, *p < 0.05, **p < 0.01, si-ERα compared with si-con group.
MEM and DMEM complete media were used for dilution and the final concentration of DMSO was kept below 0.1% in cell culture, which had no detectable effects on cells. Methyltryiazoyldiphényltetrazolium bromide (MTT), propidium iodide (PI), RNase A, monodansyl cadaverine (MDC), rhodamine 123, estrogen receptor (ER) antagonist Fulvestrant (ICI 182,780), estrogen receptor alpha (ERα)-specific antagonist MPP 1,3-bis-(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole-dihydr- chloride, and primary antibody against LC3 were purchased from Sigma Chemical (St. Louis, MO, USA). Polyclonal antibody against ERα primary antibody against LC3 were purchased from Proteintech Group (Chicago, IL, USA). Primary antibodies against p62, caspase-6, caspase-8, Bid, Akt1/2, p-Akt1/2, Bcl-2, Bax, cytochrome c, ERK1, p-ERK 1/2, GRB2, Raf, SOS, inhibitor of caspase-activated DNase (ICAD), poly-ADP-ribose polymerase (PARP) and β-actin, as well as horseradish peroxidase-conjugated secondary antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The SuperSignal West Pico Chemiluminescent Substrate used in conjunction with the horseradish peroxidase (HRP) enzyme was purchased from Thermo Scientific (Rockford, IL, USA).

2.2. Cells culture

Human breast cancer MCF-7, MDA-MB231 cells and human hepatoma G2 (HepG2) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). MCF-7 cells were cultured in Minimum Essential Medium (MEM, Gibco, Grand Island, NY, USA), while MDA-MB231 and HepG2 cells were in Dulbecco’s Modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA). All media were supplemented with 10% fetal bovine serum (FBS) (Beijing Yuanheng Shengma Research Institute of Biotechnology, Beijing, China), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were incubated at 37 °C with 5% CO2 in a humidified atmosphere. All experiments were performed on logarithmically growing cells.

2.3. Fluorescent microscopy of MDC staining

Autofluorescent compound MDC stains acidic lysosomes and thus it is widely used in studies of autophagy together with the measurement of other parameters (9). The cells were seeded into 24-well cell culture plates (Corning, NY, USA) at a density of 2 x 10^4 cells per well for 24 h. The cells in culture for another 24 h by the treatments with different reagents were examined. The cells treated were rinsed twice with ice-cold PBS, stained with 0.05 mM MDC solution in the dark at 37 °C for 30 min. They were observed with a fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Flow cytometry of MDC positive cells

Cells subjected to the indicated treatments were collected (5 x 10^5 cells per sample), incubated with 0.05 mM MDC solution in the dark at 37 °C for 30 min and analysed using a FACSscan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

2.5. Cytotoxicity assay

MCF-7 cells were seeded into 96-well cell culture plates (Corning, NY, USA) at a density of 5 x 10^3 cells/well and cultured for 24 h. Then the cells were subjected to the indicated treatments for 24 h. The cells were rinsed twice with ice-cold PBS and incubated with 100 μL of 0.5 mg/mL MTT solution at 37 °C for 3 h. Then the supernatant was discarded, and the residual cell layer was dissolved with 150 μL of DMSO. Thereafter, the optical absorbance (A value) was measured at 490 nm wavelength using a microplate reader (Thermo Scientific Multiskan MK3, Shanghai, China). Cell growth inhibitory ratio was calculated using the following equation:

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\text{Cell growth inhibitory ratio (\%)} = 100 \times \left( \frac{A_\text{control} - A_\text{test}}{A_\text{control}} \right) \\
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2.6. Flow cytometric analysis of dying cells

Propidium iodide (PI), a fluorescent dye that specifically binds to cellular DNA, was used to quantify DNA content. Cells subjected to the indicated treatments were collected (5 x 10^5 cells per sample), fixed with 70% (v/v) ethanol at 4 °C overnight, rinsed with ice-cold PBS twice and incubated with 1 mL of PI solution (PI 50 mg/L and RNase A1g/L) in the dark for 30 min. The cellular DNA content was next analyzed using a FACSscan flow cytometer. Cells with DNA content less than normal diploid (at subG0/G1 phase) are taken as dead cells.

2.7. Determination of mitochondrial membrane potential

The mitochondrial membrane potential was examined using the mitochondrial dye rhodamine 123. After incubation with indicated treatments, MCF-7 cells were collected and stained with 1 μg/mL rhodamine 123 at 37 °C for 30 min. The fluorescence intensity of the cells was analysed by FACSscan flow cytometry.

2.8. Transfection of siRNA

MCF-7 cells were transfected with negative control siRNA (NC-siRNA) or siRNA targeting ERα (ERα-siRNA) at a final concentration of 10 nM using siRNA-Mate (GenePharma, Shanghai, China) according to the manufacturer’s instructions. The sequences of the ERα-siRNA duplex were as follows: sense strand, 5'-GAGGGA-GAUGUUGAACAATT-3'; antisense strand, 5'-UGUUUCACAUUCUCCUCCTT-3'. The sequences of NC-siRNA duplex were: sense strand, 5'-UUUCUCCGAACGUACAGUTT-3'; antisense strand, 5'-ACGUGACAGUCGAGAGATT-3'. The transfected cells were maintained for another 48 h before subsequent experiments.

2.9. Western blot analysis

After the indicated treatments, both adherent and floating cells were collected at the predetermined time points and lysed with RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China) supplemented with PMSF (1 mM) for 30 min. After centrifugation at 12,000 x g for 10 min, the supernatant was collected and the protein concentration was determined with the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). The lysates that were adjusted with the total amount of proteins equal were separated on 10–13% SDS-PAGE. The separated protein bands were transferred onto Millipore Immobilon-P Transfer Membrane (Millipore Corporation, Billerica, MA, USA). After blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with primary antibodies at 4 °C overnight and then with corresponding HRP conjugated secondary antibodies at room temperature for 2 h. After that, the blots were visualized using the SuperSignal West Pico Chemiluminescent Substrate purchased from Thermo Scientific (Rockford, IL, USA).

2.10. Statistical analysis

All the data and results obtained by at least three independent experiments were expressed as mean ± S.D. Comparisons between groups were determined using Student’s t-test. One-tailed p-values are significant when p < 0.05.
Fig. 2. Treatment with a specific ERα antagonist MPP dihydrochloride (MPP) enhances the apoptosis of MCF-7 cells induced by silibinin. (A) Morphologic changes of apoptotic MCF-7 cells were observed at 24 h (a, 200 × magnification, bar = 20 μm). Con: control, Sili: silibinin. (B) Flow cytometric analysis of apoptotic cell ratios after PI staining (SubG1 cells). (a) The data are presented as the mean ± S.E.M. of the results for three independent experiments. (b) **p < 0.01 compared with silibinin-treated group. (C) The cells were incubated with 200 μM silibinin with/without 10 μM MPP for 24 h. The rhodamine-123 fluorescent intensity was analysed by flow cytometry. *p < 0.05 versus silibinin group. #p < 0.05 versus silibinin group.
Fig. 3. Inhibition of ERα enhances the MCF-7 cell death induced by silibinin through extrinsic and mitochondrial-apoptosis pathways. The cells were incubated with or without 10 μM MPP for 1 h. Then 200 μM silibinin was added to the cells which were cultured for 24 h. Cell lysates were separated by 11% SDS-PAGE, and the levels of (A) caspase 8, bid, (B) Bax (mitochondria), Bcl-2 (mitochondria), cytochrome c (cytoplasm), (D) caspase 6, PARP, ICAD were detected by Western blot analysis. Band density of the specific protein was analyzed with Quantity One image software and the results were expressed as relative density to β-actin. (C) The expression of caspase 3 was detected in the MCF-7 cells treated with silibinin and/or MPP. The expression in HepG2 cells was used as a positive control.
Fig. 4. Inhibition of ERα reinforces silibinin-induced MCF-7 cell autophagy. (A) The cells were incubated with or without 10 μM MPP for 1 h. Following the addition of 200 μM silibinin, the cells were cultured for 24 h. Changes of fluorescent intensity were observed by fluorescence microscopy with MDC staining (× 400 magnification, bar = 20 μm). (B) MCF-7 cells were cultured in the presence of 0, 100, 200 and 300 μM silibinin for 12 and 24 h. The MDC positive ratio was determined by flow cytometric analysis. (C) The positive ratio of MDC staining was detected by flow cytometric analysis, n = 3, mean ± S.E.M., **p < 0.01, compared with silibinin-treated group. (D) MCF-7 cells were lysed after the treatment with 100, 200 or 300 μM silibinin for 24 h, and the protein levels of p62 and LC3 were detected by western blot analysis. (E) The protein levels of p62 and LC3 were detected by western blot analysis. Band density of the specific protein was analysed with Quantity One image software. (n = 3, means ± SD; *p < 0.05 vs control group).
3. Results

3.1. ERα down-regulation was involved in silibinin-induced cytotoxicity of MCF-7 cells

To test the cytotoxicity of silibinin on MCF-7 cells, the cells were cultured with 100–300 μM silibinin for 12, 24, 36 and 48 h. Silibinin was found to decrease the proportion of viable cells in a time and dose-dependent manner with an IC50 (at 24 h) of 200 μM (Fig. 1B). As a flavonoid, silibinin might affect the estrogen receptor signalling pathways (5). Therefore, we investigated the levels of ERα. Western blot analysis showed that treatment with various concentrations of silibinin for 24 h reduced ERα expression in a concentration-dependent manner (Fig. 1C). Fulvestrant (ICI 182,780), an estrogen receptor antagonist which blocks both ERα and ERβ signalling, was applied with silibinin to examine the effect of estrogen receptor signalling pathway on MCF-7 cell growth. The results showed that ICI 182,780 augmented silibinin-induced MCF-7 cell growth inhibition (Fig. 1D). To further elucidate the role of ERα, we examined the cytotoxicity of silibinin on two different strains of human breast cancer cells: one is MCF-7 cells with an inherently high expression of ERα and the other is MDA-MB-231 cell with a rather low expression of ERα. MDA-MB-231 cells are often utilized as a negative control for the examination of ERα involvement (10). Western blot analysis of the ERα levels of MCF-7 and MDA-MB-231 indicated, in accordance with the previous reports, that the ERα expression in MCF-7 cells was high, but low in MDA-MB-231 cells (Fig. 1E (a)).

MTT assay showed that the growth inhibitory effect of silibinin on MCF-7 cells was higher than that on the MDA-MB-231 cells (Fig. 1E (b)), evidencing the positive correlation between ERα down-regulation and cytotoxicity. The specific ERα-antagonist, MPP (11), dose-dependently augmented silibinin-induced MCF-7 cell growth inhibition (Fig. 1F), even to a larger extent than ICI 182,780 (Fig. 1D). Since high dose of MPP (20 μM) alone also inhibited cell proliferation, we chose a lower dose (10 μM) of MPP in the following experiments. To further confirm the cytotoxicity of silibinin in combination with repression of ERα on MCF-7 cells, we transfected the siRNA targeting ERα (si-ERα) into cells (Fig. 1G (a)). We found that silencing of ERα markedly enhanced the cytotoxic effect of silibinin (Fig. 1G (b)). Therefore, silibinin exerted anticancer effects on MCF-7 cells through the inhibition of ERα pathways.

3.2. Treatment with MPP enhances silibinin-induced apoptosis of MCF-7 cells

To determine the features of MCF-7 cell growth inhibition, the morphologic changes were examined. Obvious changes of cell nuclei and cell number were observed in the cells treated with 200 μM silibinin plus 10 μM MPP for 24 h when compared with the cells treated with silibinin alone. (Fig. 2A). PI staining was applied to analysis of the changes of DNA contents: silibinin increased the ratio of cells at subG1 phase in cell cycle at 24 h, while co-treatment with MPP increased the ratio furthermore (Fig. 2B). These results suggested that apoptosis might be involved in this growth inhibition. It was well documented that many anticancer drugs induced apoptosis following mitochondrial dysfunctions (12). Therefore, we examined intactness of mitochondrial membranes by rhodamine 123 staining. Decrease in rhodamine-123 fluorescence intensity reflects the reduction in mitochondrial transmembrane potential (Δψm). Compared with silibinin-treated group, co-treatment with MPP resulted in a further reduction in Δψm (Fig. 2C), indicating that inhibition of ERα markedly enhances the cytotoxicity of silibinin on MCF-7 cells through mitochondrial dysfunction.

3.3. Treatment with MPP enhances the cytotoxicity of silibinin through both extrinsic and intrinsic-apoptosis pathways

Western blot assay was carried out to further determine the features of silibinin and MPP-induced MCF-7 cell death. The treatment with silibinin resulted in that extrinsic apoptosis-related protein pro-caspase-8 was cleaved into active caspase-8, as well as Bid was cleaved. MPP further augmented this effect (Fig. 3A). As for the intrinsic apoptosis pathway, translocation of the anti-apoptotic protein Bcl-2 to mitochondria was markedly decreased in the silibinin-treated group, while the pro-apoptotic translocation of Bax to mitochondria and the release of cytochrome c to cytoplasm were increased. MPP also augmented these processes furthermore (Fig. 3B). The results indicate that the intrinsic apoptosis pathway was also upregulated.

Caspase 3 is the downstream executor of apoptosis in many cells. However, MCF-7 cells were reported to be negative in the expression of caspase-3 (13,14). Accordingly, the expressions of pro-caspase 3 and activated caspase 3 were not detected in silibinin-treated MCF-7 cells (Fig. 3C). It was reported that silibinin

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Fig. 5. Autophagy promotes silibinin- and MPP-induced apoptosis. (A) The cells were treated with 10 μM MPP for 1 h, and then 200 μM silibinin was added and cultured for 24 h in the presence or absence of rapamycin (10 nM) or 3 MA (2 mM), and the cell viability was measured by MTT assay, n = 3, mean ± S.E.M, *p < 0.05, **p < 0.01, compared with silibinin and MPP-treated group. (B) The protein levels of caspase 6, PARP and ICAD were detected by western blot analysis.
activated caspase 3 in HepG2 cells (15). Therefore, silibinin-treated HepG2 cells were used as a positive control for the detection of caspase 3 (Fig. 3C). We examined the expression of another common downstream executor of apoptosis, caspase-6, and found that silibinin induced the activation of caspase 6. MPP treatment augmented the cleavage of pro-caspase-6 to caspase-6 furthermore. The substrates of caspase-6, ICAD and PARP, were cleaved accordingly (Fig. 3D). In conclusion, MPP pre-treatment enhanced
both intrinsic and extrinsic apoptotic pathways in silibinin-treated MCF-7 cells.

3.4. Inhibition of ERα augmented silibinin-induced MCF-7 cell autophagy

Autophagy is a fundamental cellular pathway by which cytoplasmic materials, including soluble macromolecules and organelles, are delivered into lysosomes for degradation (15). Autophagy plays an important role in the cellular responses towards stresses such as starvation, hypoxia and DNA damage. The morphologic changes were observed in the MDC-stained cells by fluorescence microscopy. Combined treatment with silibinin and MPP caused an obvious increase in the number of MDC-labelled autophagolysosomes compared with the silibinin alone group (Fig. 4A). The quantitative analysis of autophagy by MDC flow cytometric analysis showed the consistent results (Fig. 4B and C). Microtubule-associated protein light chain 3 (LC3) is now widely used to monitor autophagy. The levels of LC3 conversion from cytosolic LC3-I to membrane-bound lipidated LC3-II or the amounts of LC3-II were examined by immunoblotting analysis (16). An alternative method for detecting the autophagic flux is the measurement of p62 degradation, since p62 can bind LC3, thus serving as a selective substrate of autophagy (16). Dose-dependent up-regulation of the conversion from LC3-I to LC3-II and down-regulation of p62 caused by silibinin treatment were observed (Fig. 4D). Combined use of silibinin with MPP enhanced expressions of LC3-II and conversion from LC3-I to LC3-II significantly increased, while p62 amount was further reduced (Fig. 4E).

3.5. Autophagy promoted apoptosis in MCF-7 cells by the co-treatment with silibinin and MPP

To investigate the crosstalk of apoptosis and autophagy in silibinin and MPP-treated MCF-7 cells, 3 MA, a specific autophagic inhibitor, and rapamycin, an autophagic inducer, were employed. As shown in Fig. 5A, silibinin and MPP-induced cell growth inhibition was further augmented by rapamycin treatment but partially reversed by 3 MA. Western blot analysis showed that the activation of caspase-6 and the cleavage of caspase substrates, PARP and ICAD, caused by cotreatment with MPP and silibinin were reversed by 3 MA treatment (Fig. 5B). These findings indicated that the autophagy induced by silibinin and MPP co-treatment intensified the apoptotic process in MCF-7 cells.

3.6. Combination of silibinin and MPP treatments repressed the downstream signals of ERα

The pathways through Shc-Grb2-Sos (Src homology-growth factor receptor binding protein 2-son of sevenless) for activation of MAPK and are involved in the downstream of activated ERα (17). One of them is RAS/ERK pathway, which can induce oncogenic gene expression programs, being often activated in cancer cells. Another is PI3K/Akt pathway which is related with the mammalian target of rapamycin (mTOR). Hou X. and co-workers reported that ERα raised the phosphorylation levels of PI3K, Akt and mTOR in endometrial carcinoma cells (18). We examined the activation of these two ER downstream signaling pathways.

Western blot analysis showed that PI3K/Akt/mTOR pathway was down-regulated by either silibinin alone or silibinin and MPP co-treatment (Fig. 6A). As has been demonstrated in many studies, inhibition of mTOR promotes autophagy (19,20). Therefore, silibinin enhanced autophagy by down-regulating ERα downstream PI3K/Akt/mTOR pathway.

As shown in Fig. 6B, treatment with MPP obviously reduced the p-ERK expression compared with silibinin alone group. To examine the role of ERK, we pre-treated the cells with ERK inhibitor PD98059, and found that the cytotoxicity was obviously enhanced by ERK inhibition (Fig. 6C and D). Since p62 degradation and from LC3-I to LC3-II increased, we further confirmed significantly increased autophagy after PD98059 exposure (Fig. 6E). These findings indicated that apoptotic action of silibinin on MCF-7 cells was mediated through ERα downstream RAS/ERK pathway. EGFR signaling also leads to inhibition of ERK1/2. Crosstalk between EGFR and ER signaling has been established and proved to have significant impact on complex biological processes such as cell growth and cell survival of breast cancer cells. To test the relationship between down-regulation of ERα and EGFR, we examined the changes of expressed EGFR protein levels in MCF-7 cells treated with silibinin with/without MPP. We found that inhibition of ERα with silibinin or/and MPP, the expression levels of EGFR was down-regulated as shown in (Fig. 6F). Thus, the effect of silibinin on ERK is mediated by ERs that may have cross-talk with EGFR.

4. Discussion

Breast cancer is one of the most frequently diagnosed cancers and the leading cause of death by cancer among females worldwide. To develop breast cancer therapies, targeting of ERα, which is highly expressed in approximately 70% of all breast tumors (21), is taken as a targeting molecule.

ERα is a member of the steroid receptor superfamily that regulates processes such as growth and differentiation in various target cells by affecting transcription. ERα not only plays an important role in many human tissues and organs, such as mammary gland, genital tract, and central nervous system (22), but also it has a close correlation with gaining the peak bone mass and maintaining the bone mass (23). Women who are menopausal or postmenopausal usually produce less ERα, leading to bone loss (24). However, in the majority of breast cancers, ERα is greatly up-regulated than normal breast cells and its expression is a hallmark of hormone-dependent tumor growth (25). In addition, the presence of elevated levels of ERα in benign breast epithelium appears to indicate an increased risk of breast cancer, suggesting a role for ERα in breast cancer initiation, as well as progression (26).

Silibinin, which has been used as a liver protectant, induced apoptosis in human breast cancer MCF-7 cells (27). In this study, we report the evidence for the mechanism of silibinin-induced apoptosis in that downregulation of ERα expression in the MCF-7 cells exposed to silibinin underlies. Consistent with previous reports, sylimarin, which contains silibinin as a major component, has been used in the clinical treatment of hepatic diseases and pharmacologic studies have indicated that silymarin is not toxic even at high doses. Besides, pretreatment with silibinin 500 μM significantly inhibited UV-induced apoptosis in HaCaT cells after 9 h incubation (28). Therefore we believe that silibinin appears to indicate an increased risk of breast cancer, suggesting a role for ERα in breast cancer initiation, as well as progression (26).

The mechanism we propose here is supported by the finding that ERα-specific antagonist, MPP, combined with silibinin enhanced all the effects of silibinin on MCF-7 cells. The enhancement with MPP can be interpreted as inhibition of the activity of ERα remaining after downregulation with silibinin. Apoptosis is characterized by the sequential activation of caspase cascades. Caspase 8 is believed to be one of the initiator caspases that can
activate downstream caspase-3 (30). However, MCF-7 cells do not express caspase-3 (13,14). Instead, upregulation of the caspase-6 expression was observed for the MCF-7 cells treated with silybinin in accordance with the report by Kagawa S et al., showing that Bax-induced levels of caspase-6 activation could be used as an evaluation index of apoptosis when the cells did not express caspase-3 (31).

A number of studies showed a relationship between Erz expression and levels of apoptosis related proteins including Bcl-2 and Bax (32–34). The relationships between Erz and apoptosis pathways in silybinin-treated cells observed in the present study indicate that death rate of MCF-7 cells induced by silybinin involves both the extrinsic and intrinsic apoptosis pathways.

 Estradiol-bound Erz interacts with various signalling molecules such as PI3K (35) and MAP kinases (36). The PI3K/Akt and MEK/ERK signalling pathways are important downstream pathways of Erz. The observations in the present study that silybinin reduced the phosphorylation levels of both ER and Akt and that additional treatment with a specific inhibitor of ERK enhanced the cytotoxic effects on MCF-7 cells treated with silybinin and MPP are rationally interpreted, since they are downstream signaling pathways of Erz. Our results are in accordance with the results of the authors and their workers, who reported that MCF-7 breast carcinoma cells promoted survival through activation of PI3K/Akt cross-talk pathway (37). We therefore conclude that silybinin shows cytotoxic effect against MCF-7 cells through the down-regulation of Erz signalling pathway.

Physiological levels of autophagy are essential for normal cellular homeostasis. The absence of autophagy increases cell death during metabolic stress and on treatment with cytotoxic chemo-therapeutic agents. By contrast, excessive levels of autophagy promotes cell death (38). It is postulated that autophagic cell death induced by some anticancer agents underlines their potential as a new cancer therapy modality (39). MPP in combination with silybinin activates autophagy which enhances apoptosis in MCF-7 cells. The results are consistent with the notions in several studies in that autophagy is triggered in some cancers in response to various anticancer agents, including As2O3, tamoxifen and temozolomide (40).

In conclusion, the present study demonstrates that down-regulation of Erz expression by the treatment with silybinin of MCF-7 cells plays a key role in leading the cells to follow Erz-MEK/ERK and Erz-Akt/mTOR pathways to apoptosis. Erz inhibition with Erz-specific antagonist, MPP, augments apoptosis and autophagy by extrinsic-apoptosis and mitochondrial-apoptosis pathways. Autophagy plays a pro-apoptotic role with the detailed mechanism remaining to be cleared.

In the present study, we highlight just Erz signalling for the silybinin effect. However, it is not known whether the antitumor activity of Erz is involved. Estrogen receptors Erz and Erzβ share considerable sequence homology yet exert opposite effects on breast cancer cell proliferation. Bin et al. illustrated that modulation of Erz-specific antitumor activity can be considered as a molecular strategy for cancer therapy (41). It is suggested that the mechanisms of silybinin’s toxic effect on MCF-7 cells may also be associated with Erzβ. Up-regulation of Erzβ is another mechanism of toxic effect on cancer cells. Studies also have shown that Erzβ exerts antiproliferative effects in Erz expressing MCF7 cells, probably by initiating degradation of Erz. Meanwhile, Erzβ-selective agonist DPNI inhibited cell growth and induced apoptosis. In addition, there are reports which show that the proliferating MCF-7 cells express both Erz and Erzβ. In order to examine whether the cytotoxic effect of silybinin is related with Erz, we performed MTT experiments using MDA-MB231 cells that have low expression of Erz and positive Erzβ as a negative control. MTT assay showed that the growth inhibitory effect of silybinin on MCF-7 cells was higher than that on the MDA-MB-231 cells (Fig. 1E (b)), evidencing the positive correlation between Erz down-regulation and cytotoxicity. We assume that silybinin also dose-dependently induced cytotoxicity in MDA-MB231 cells and Erzβ expression in MDA-MB231 cells was increased.

In order to prove this hypothesis, further experiments are necessary. Thus, Erzβ could be another key mechanism of silybinin-induced apoptosis and autophagy in many cell lines as well as MCF-7 cell. Therefore, we are going to use Erzβ as a target object for future research.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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