Inhibition of Insulin-like Growth Factor 1 Receptor Signalizing Enhanced Silibinin-Induced Activation of Death Receptor and Mitochondrial Apoptotic Pathways in Human Breast Cancer MCF-7 Cells

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Abstract. Silibinin, which had been used as a hepatoprotectant, was shown to have anticancer activity. In this study we investigated the mechanisms of silibinin-induced apoptosis in human breast cancer MCF-7 cells. Expressions of Fas ligand (FasL), Fas-associated death domain protein (FADD), and Bax were significantly up-regulated in silibinin-treated cells, whilst silibinin induced a conspicuous translocation of Bax to mitochondria and release of cytochrome c to the cytosol. Therefore, both the extrinsic Fas death receptor and intrinsic mitochondrial death pathways played essential roles in silibinin-induced apoptosis. It was also found that silibinin markedly decreased protein expression of SIRT1, a mammalian homologue of yeast Sir2, which was proved to have a role in sequestering Bax away from mitochondria. Insulin-like growth factor 1 receptor (IGF-1R), a receptor tyrosine kinase with a crucial role in malignancy development, is expressed in most human primary breast carcinomas. Our results showed that silibinin-induced apoptosis was significantly reinforced by blocking IGF-1R signaling with tyrphostin AG1024, a specific inhibitor of IGF-1R auto phosphorylation. Up-regulation of FADD, down-regulation of SIRT1 expression, and activation of the mitochondrial death pathway were apparently enhanced by AG1024 in the silibinin-treated MCF-7 cells.

Keywords: silibinin, insulin-like growth factor 1 receptor (IGF-1R), MCF-7 cell, apoptosis

Introduction

Silibinin, a naturally occurring polyphenolic flavonoid (Fig. 1), constitutes a major biologically active portion of the plant extract from milk thistle (Silybum marianum) that is widely consumed as a dietary supplement. Silibinin and its crude form silymarin have been clinically used to treat certain liver complications (1). In recent years, accumulating evidence showed that silibinin had anticancer and chemopreventive effects, as well as cardioprotective, neuroactive, and neuroprotective activities (2–5).

Apoptosis is defined as genetically programmed autonomous cell death, and its deregulation is a hallmark of malignancy. Several molecular strategies designed to activate tumor cell apoptosis are under clinical investigation (6). There are two principal pathways leading to apoptotic cell death, the “extrinsic” or death receptor-initiated pathway and the “intrinsic” or mitochondrial pathway. The extrinsic pathway originates with binding of death-promoting ligands (e.g., FasL, TNFα) to their

Fig. 1. Chemical structure of silibinin.
Increase of Bax/silibinin was measured by HPLC and determined to be about 99%. Silibinin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The concentration of DMSO was kept below 0.1% in all the cell cultures and did not exert any detectable effect on cell growth or cell death.

$3-(4,5$-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), Acridine Orange (AO), L(+)-sodium lactate, iodonitrotetrazolium chloride (INT), phenazine methosulfate (PMS), NAD$^+$, and 3,3-diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit polyclonal antibodies against Bax, Bel-2, cytochrome c, FasL, FADD, caspase-8, SIRT1, and horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against IGF-1Rβ (C-20) and phosphorylated IGF-1R (Tyr1131) were purchased from Chemicon (Temecula, CA, USA). Tyroshostin AG1024 (3-bromo-5-tert-butyl-4-hydroxy-benzylidenonaminate) and genistein were obtained from Sigma Chemical Co.

**Cell culture**

Human breast cancer MCF-7 (#HTB-22) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Dalian Biological Reagent Factory, Dalian, China), L-glutamine (2 mM, Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO$_2$. The cells in the exponential phase of growth were used in the experiments.

**Growth inhibition assay**

The growth inhibitory effect of silibinin on MCF-7 cells was measured by MTT assay. The cells were dispensed in 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) at a density of $1.5 \times 10^4$ cells/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 resulting crystals were dissolved in DMSO. Absorbance (A) was measured with an ELISA reader (Tecan Spectra, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows:

$$\text{Inhibitory ratio (\%)} = \frac{A_{490,\text{control}} - A_{490,\text{experiment}}}{A_{490,\text{control}} - A_{490,\text{blank}}} \times 100$$

The combination index (CI) assay by Chou (15) was used to analyze the effect of the combination of silibinin and AG1024. CI value was calculated by CalcuSyn software (version 2.0; Biosoft Inc., Ferguson, MO, USA). CI>1, CI = 1, and CI<1 indicate antagonism, additive...
effect, or synergy, respectively.

**LDH activity-based cytotoxicity assay**

Lactate dehydrogenase (LDH) activity assay was performed as previously described (16). LDH activity was measured in both floating dead cells and viable adherent cells. MCF-7 cells were incubated in 96-well plates at a density of $1.5 \times 10^4$ cells/well in the presence of silibinin or AG1024. Floating dead cells were collected from culture medium by centrifugation (240 x g at 4°C for 10 min), and LDH content from the pellets lysed in 0.1% NP-40 at 37°C for 30 min was used as an index of apoptotic cell death (LDHa); the LDH content released in the culture medium (LDHn) was used as an index of necrotic cell death. The adherent and viable cells were lysed in 0.1% NP-40 at 37°C for 30 min to release LDH (LDHi). Then the substrate reaction buffer of LDH [50 mM L(+)-sodium lactate, 0.66 mM INT, 0.28 mM PMS, and 1.3 mM NAD$^+$ in pH 8.2 Tris-HCl solution] was added. The absorbance (A) at 490 nm was assayed after reacting for 1 and 5 min, respectively. LDH activity-based cytotoxicity assay

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LDH = (A_{5min} - A_{1min}) / 4.
\]

The percentage of apoptotic and necrotic cell death was calculated as follows:

\[
\%\text{ apoptosis} = \frac{LDHa}{(LDHa + LDHn + LDHi)} \times 100
\]

\[
\%\text{ necrosis} = \frac{LDHn}{(LDHa + LDHn + LDHi)} \times 100
\]

**Flow cytometric analysis of cell apoptosis**

MCF-7 cells ($1 \times 10^6$ cells) were harvested and washed once in cold PBS. The cell pellets were fixed in 75% 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 FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

**Nuclear damage observed by fluorescence morphologic examination**

Apoptotic morphology was studied by staining cells with the fluorescent, DNA-binding dye AO. MCF-7 cells were plated in the wells of a 6-well plate at a density of $1.0 \times 10^5$ cells per well. Cells were harvested and washed three times with PBS after being incubated with silibinin or AG1024, and then they were stained with 20 μg/ml AO at 37°C for 15 min. Apoptotic cells were identified as cells with condensed and fragmented nuclei by using fluorescence microscopy (Olympus, Tokyo).

**Western blot analysis**

Both adherent and floating MCF-7 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 EDTA, 1 mM EGTA, and 1 mM PMSF, supplemented with the proteinase inhibitors 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml pepstatin at 4°C for 1 h. After 9,500 x g centrifugation at 4°C for 15 min, the protein concentration was determined by a Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of total proteins were separated by SDS-polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose membranes. Proteins were detected with the indicated primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibody and then visualized by the HRP substrate 3,3-diaminobenzidine tetrahydrochloride (DAB).

**Preparation of mitochondria and cytosolic extracts**

The extract process was based on the previously described method (17). The cells were collected by centrifugation at 200 x g at 4°C for 5 min and then washed twice with ice-cold PBS. The cell pellets were resuspended in ice-cold homogenizing buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1.5 mM MgCl$_2$, 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The cells were homogenized with 20 strokes of a Dounce homogenizer at 4°C. Nuclei and intact cells were removed by centrifugation at 500 x g for 12 min at 4°C. The supernatants were subjected to centrifugation for 30 min (9,500 x g, 4°C) to precipitate the mitochondria. The resulting supernatants were used as the cytosol fraction, and the pellets were lysed in lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF) at 4°C for 1 h. The lysates were subjected to centrifugation for 30 min (9,500 x g, 4°C), and the supernatants were used as the mitochondria fraction.

**Statistical analyses**

All data represent at least three independent experiments and are expressed as the mean ± S.D. The data were analyzed by ANOVA using Statistics Package for Social Science (SPSS) software (version 13.0; SPSS Inc., 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

Determination of the cytotoxicity of silibinin to the MCF-7 cell line

The MTT cell-viability assay demonstrated that silibinin inhibited MCF-7 cell growth in a time- and dose-dependent manner with an IC_{50} (at 24 h) of 202.4 μM (Fig. 2A). To determine whether treatment with silibinin resulted in apoptotic cell death, MCF-7 cells were treated with 200 μM silibinin for 24 h and detected by flowcytometry. The result showed that the sub-G0/G1-phase peak (apoptotic peak) was strikingly boosted in the silibinin-treated group (Fig. 2B).

Inhibition of IGF-1R signaling enhanced growth-inhibitory and proapoptotic effects of silibinin in MCF-7 cells

MTT cell-viability assay showed that the growth inhibitory ratio for 200 μM silibinin at 24 h was 44%. Combined treatment with tyrphostin AG1024 or genistein (a tyrosine kinase inhibitor) and silibinin exerted higher growth inhibiton. The inhibitory ratio was 67% for 200 μM silibinin in combination with 4 μM AG1024 and 64% for 200 μM silibinin with 50 μM genistein (Fig. 3A). The effect of combination of silibinin and AG1024 was assessed by calculation of the combination index (CI). The result (CI = 0.697) showed a synergistic effect between silibinin and AG1024 in cell growth inhibition.

MCF-7 cells were induced to undergo apoptosis by cotreatment with silibinin and AG1024. Apoptosis was morphologically confirmed by examining the nuclear morphology after AO staining. As shown in Fig. 3B, the cells in control medium were stained homogeneously with AO, whereas treatment with silibinin resulted in remarkable chromatin condensation and nucleus fragment in MCF-7 cells, a hallmark of apoptosis, and AG1024-treated cells displayed a more significant number of apoptotic nuclei. LDH analyses of MCF-7 cells treated with AG1024, silibinin, or both, are shown in Fig. 3C. Combined treatment with silibinin and AG1024 caused more significant apoptosis, and the

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**Fig. 2.** Induction of growth-inhibition and apoptosis by silibinin in MCF-7 cells. A: Silibinin inhibited MCF-7 cell growth in a time- and dose-dependent manner. The cells were treated with different doses of silibinin for 12 and 24 h in media containing 10% FBS, and inhibitory ratio was assayed by the MTT method. n = 3. Mean ± S.D. B: Flowcytometric analysis of silibinin-treated MCF-7 cells. The cells were treated with 200 μM silibinin for 24 h, harvested, stained with PI, and analyzed by flowcytometry as detailed in “Materials and Methods”. The M1 area indicates the sub-G0/G1 peak. a: medium control, b: silibinin-treated group, c: the flowcytometric quantification result of apoptotic cells. n = 3, mean ± S.D. **P<0.01 vs control group.
apoptotic ratio was increased by more than 9% over treatment with silibinin alone.

Effect of treatment with AG1024 and silibinin on activity of IGF-1R

The signaling activity of IGF-1R is crucial to the control of apoptosis and proliferation processes, and IGF-1R activation results in autophosphorylation. Therefore, the effect of silibinin on IGF-1R expression and phosphorylation was investigated. The protein level of IGF-1R was not changed in 200 μM silibinin–treated MCF-7 cells, whereas phosphorylation of IGF-1R was remarkably inhibited by silibinin, as well as in the presence of AG1024 (Fig. 4: A and B).

Inhibition of IGF-1R signaling enhanced silibinin-induced activation of Fas proapoptotic pathway in MCF-7 cells

MCF-7 cells were treated with different concentrations of silibinin for 24 h, or with 200 μM silibinin for 6, 12, and 24 h. Figure 5A shows Western blot analysis of apoptosis-associated proteins in whole-cell lysates. Expression level of FasL and FADD, which initiate the apoptotic cascades, were increased in MCF-7 cells in a silibinin-time and -dose dependent manner. In untreated MCF-7 cells, caspase-8 was present primarily as two isoforms of 55 and 53 kDa. Induction of apoptosis by silibinin resulted in a dose-dependent processing of pro-caspase-8 to two active fragments of 43 and 41 kDa (Fig. 5A). These results demonstrated that the extrinsic
apoptosis pathway was activated by silibinin. In the presence of AG1024, the expression level of FADD was further upregulated in silibinin-treated cells, whereas FasL expression was not affected. In addition, administration of AG1024 induced more obvious cleavage of pro-caspase-8 in the silibinin-treated cells (Fig. 5B). Therefore, induction of FADD expression contributed to enhancement of silibinin-induced apoptosis exaggerated by AG1024.

Effects of silibinin on expression and translocation of Bax in MCF-7 cells

The intrinsic apoptotic pathway is controlled at the level of the mitochondrial membrane by the Bcl-2 superfamily proteins. A significant increase in expression level of Bax was observed in silibinin-treated MCF-7 cells in a time- and dose-dependent manner, while expression of the antiapoptotic protein Bcl-2 was not altered by silibinin (Fig. 6A). Bcl-2 is integral membrane protein of the mitochondria, whereas Bax resides in the cytosol. The death signal causes Bax translocation from the cytosol into the mitochondrial membrane, where it would disrupt outer membrane integrity and induce apoptosis. Our result demonstrated that the protein level of Bax in mitochondria was clearly increased in silibinin-treated MCF-7 cells (Fig. 6B).
Down-regulation of SIRT1 and translocation of Bax contributed to AG1024-mediated reinforcement of apoptosis in silibinin-treated MCF-7 cells

SIRT1 has been shown to be associated with apoptosis, and inactivation of SIRT1 could promote translocation of Bax from the cytosol to mitochondria (18). As shown in Fig. 7 (A and B), SIRT1 expressions were markedly repressed in MCF-7 cells treated with AG1024, silibinin, or both. In contrast, Bax levels in mitochondria were clearly boosted by cotreatment with silibinin and AG1024, compared with treatment with silibinin alone (Fig. 7C). In contrast, no apparent change was observed in the protein level of Bcl-2 in mitochondria. These results demonstrated that AG1024 enhanced silibinin-induced translocation of Bax by inhibiting expression of SIRT1.

Inhibition of IGF-1R activity promoted release of cytochrome c

Disruption of outer mitochondrial membrane integrity leads to the release of cytochrome c from the mitochondrial intermembrane space to the cytosol and subsequent initiation of apoptosis. To investigate the effect of silibinin on release of cytochrome c, the protein level of cytochrome c in the cytosol was increased after cotreatment with silibinin and AG1024. In contrast, the level of cytochrome c in mitochondria was apparently decreased (Fig. 8).
The anticancer activity of silibinin has been described in various cancer types such as hepatoma, bladder, and lung cancer but mainly on the steroid hormone-dependent malignancies like prostate cancer (19–22). Silibinin affects various processes involved either in cancerogenesis or in cancer proliferation, e.g., silibinin inhibited erbB1 signaling and DNA synthesis in human prostate cancer cells (22).

Induction of apoptosis has become a predominant mechanism by which chemotherapeutic agents exerted cytotoxicity. The data presented here provided evidence of the proapoptotic effect of silibinin in human breast cancer MCF-7 cells. The induction of apoptosis by silibinin in MCF-7 cells was confirmed by occurrence of condensed nuclear chromatin (Fig. 3B) and DNA hypoploidy (Fig. 2B), as well as analysis of lactate dehydrogenase activity (Fig. 3C). Our results suggested that silibinin induced MCF-7 cell apoptosis by upregulating the expressions of FasL and FADD, which would initiate the extrinsic apoptosis pathway, and the active forms of caspase-8 were detected (Fig. 5A). We also found that treatment with silibinin resulted in activation of the intrinsic apoptosis pathway by increasing the Bax/Bcl-2 ratio (Fig. 6A) and promoting release of cytochrome c (Fig. 8) in MCF-7 cells.

IGF-1R levels have been found to be elevated in breast cancer compared with non-malignant tumors or normal epithelium (23). In breast cancer cell lines, IGF-1R is often co-expressed with autocrine IGF-like mitogens that promote proliferation (24). The results presented here show the effects of the strategy of combining anti-IGF-1R and silibinin treatment in MCF-7 cells. According to our Western blotting data, the phosphorylation level, but not protein level of IGF-1R, was suppressed by silibinin in MCF-7 cells (Fig. 4A), indicating an inhibitory effect on the IGF-1R-mediated signaling pathway. IGF-1R signaling was primarily an antiapoptotic signaling (11, 12). Therefore, we supposed that silibinin induced MCF-7 cell apoptosis, as well as inhibited antiapoptotic IGF-1R activity. It was reported that silibinin inhibited IGF-1R activity by increasing the expression of insulin-like growth factor binding protein 3 (IGFBP-3) (25, 26). Whether silibinin inhibits IGF-1R phosphorylation in MCF-7 cells by the same mechanism still needs further study.

AG1024 is a synthetic tyrphostin that inhibits ligand-stimulated IGF-1R autophosphorylation. Tyrphostins bind to the active site of receptors and modify its conformation to prevent the substrate and ATP from binding (27). Cotreatment with AG1024 and silibinin resulted in more apparent inhibition of IGF-1R phosphorylation in MCF-7 cells (Fig. 4B). In subsequent results we showed that AG1024 enhanced silibinin-induced apoptosis through the Fas pathway in MCF-7 cells. This finding is in line with another report that inhibition of action of IGF-1 sensitizes human adipocytes for death ligand (CD95/Fas antibody, TNFα and TRAIL)-induced apoptosis (28). In the case of FasL-induced apoptosis, the classical paradigm involves a direct connection between the death receptor and caspase-8 via the intermediary adaptor molecule FADD. We showed that the protein level of FADD, but not FasL, was clearly upregulated by AG1024 and silibinin (Fig. 5B). Therefore, augmentation of caspase-8 activation by AG1024 was due to the upregulation of FADD.

Recent advances had provided a mechanistic basis for the life prolonging effect of caloric restriction (CR). A class of evolutionarily conserved molecules with protein deacetylation activities known as sirtuins (29), which are homologues of the yeast Sir2, appear to play a pivotal role in longevity. Studies that focused on the mammalian Sir2 homologue, SIRT1, had yielded clues suggesting that the deacetylase might function in the context of prolonging life in mammals. SIRT1 could attenuate apoptotic signals by deacetylating the DNA repair factor Ku70, which was found to have a role in sequestering the proapoptotic factor Bax away from the mitochondria (18, 30). Bax can exert proapoptotic action through complex formation with its antiapoptotic counterpart Bcl-2, disrupting outer membrane integrity and leading to the release of cytochrome c and ATP from the mitochondrial intermembrane space and subsequent formation of the apoptosome. Our data show that silibinin promoted apoptosis by inducing translocation of Bax to mitochondria and the release of cytochrome c into the cytosol. In addition to the mitochondrial
apoptotic pathway, decreased SIRT1 protein level might provide such a trigger. The previous research indicated that silibinin protects against rat cardiac myocyte injury through the induction of SIRT1 concomitant with decreased Bax concentration in mitochondria and cytochrome c efflux (4). This might provide evidence that SIRT1 played a pivotal role in regulating translocation of Bax. However, silibinin has completely the opposite action in regulating SIRT1 expression in normal cardiac myocytes and breast cancer MCF-7 cells. The direct target of silibinin that is important for understanding of this apparent paradox remains unknown. Histone modifier genes have tissue-type specific patterns of expression, and Sirt1 is among the essential genes discriminating the various tissue types (31). Therefore, there might be different expression mechanisms of SIRT1 in normal cardiac myocytes and breast cancer MCF-7 cells. Assessing the potential mechanism by which silibinin regulates the expression of SIRT1 is helpful for specific chemoprevention against breast cancer. Down-regulation of SIRT1 protein level was observed in MCF-7 cells treated by silibinin or AG1024. It is yet unclear which step or aspect of IGF-1 signaling regulates SIRT1 protein level and whether this occurs primarily through regulation of SIRT1 transcription. It is also unknown whether SIRT1 activity has a reciprocal effect on the expression of components of the IGF-1 signaling system. The regulatory relationship among IGF-1, SIRT1, and silibinin is undoubtedly a problem that is worth further investigation.

Taken together, silibinin induced conspicuous apoptosis in MCF-7 cells. Combined treatment of silibinin with AG1024 resulted in synergistic growth inhibition in MCF-7 cells. AG1024 enhanced silibinin-induced apoptosis accompanied by increased FADD expression and Bax mitochondrial translocation. Silibinin also activated apoptotic pathways apart from inhibition of the IGF-1R-mediated signals. Silibinin induced apparent expression of Bax and FasL, whereas AG1024 treatment alone did not have the similar effect. The findings described here suggested that silibinin exerted a strong anticarcinogenic effect against MCF-7 cells, and blocking of IGF-1R signaling might be developed as a therapeutic strategy to increase the antitumor efficacy of silibinin.

References

21 Singh RP, Deep G, Chittezhath M, Kaur M, Dwyer-Nield LD,


