Silibinin inhibits translation initiation: implications for anticancer therapy

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Abstract
Silibinin is a nontoxic flavonoid reported to have anticancer properties. In this study, we show that silibinin exhibits antiproliferative activity on MCF-7 breast cancer cells. Exposure to silibinin leads to a concentration-dependent decrease in global protein synthesis associated with reduced levels of eukaryotic initiation factor 4F complex. Moreover, polysome profile analysis of silibinin-treated cells shows a decrease in polysome content and translation of cyclin D1 mRNA. Silibinin exerts its effects on translation initiation by inhibiting the mammalian target of rapamycin signaling pathway by acting upstream of TSC2. Our results show that silibinin blocks mammalian target of rapamycin signaling with a concomitant reduction in translation initiation, thus providing a possible molecular mechanism of how silibinin can inhibit growth of transformed cells. [Mol Cancer Ther 2009;8(6):1606–12]

Introduction
Deregulated protein synthesis is emerging as a key event in human oncogenesis. Regulation of mRNA translation is required for cell growth, proliferation, differentiation, and cellular homeostasis (1, 2). The recruitment of ribosomes to the 5′-end of mRNAs during translation initiation in eukaryotes is generally thought to be the rate-limiting step of protein synthesis and is under regulation of the mammalian target of rapamycin (mTOR) protein kinase. Cap-dependent translation initiation is stimulated by eukaryotic initiation factor (eIF) 4F, a complex consisting of three subunits: eIF4E, which interacts directly with the mRNA cap structure; eIF4A, a RNA helicase that prepares the mRNA template for ribosome binding; and eIF4G, a large molecular scaffold that mediates mRNA binding of the 43S preinitiation complex (40S ribosome and associated factors; ref. 3). Elevated eIF4F complex levels preferentially increase translation of “weak” mRNAs, which encode growth factors and proto-oncogenes such as vascular endothelial growth factor, c-Myc, cyclin D1, and ornithine decarboxylase (4).

TOR functions by integrating extracellular signals (growth factors and hormones), with amino acid availability and intracellular energy status to control several metabolic processes, including translation (5). The two best-characterized targets of mTOR phosphorylation are the eIF4E binding proteins, 4E-BP’s (of which there are three and the best-characterized one is 4E-BP1), and ribosomal protein S6 kinases (S6K) 1 and 2. Hypophosphorylated 4E-BP1 inhibits cap-dependent translation initiation by competing with eIF4G for binding to eIF4E, whereas mTOR-mediated phosphorylation of 4E-BP1 liberates eIF4E from this inhibitory complex, allowing it to bind eIF4G and enter the eIF4F complex (6). S6K1 directly phosphorylates the 40S ribosomal protein S6 and a known tumor suppressor gene product, programmed cell death 4 (PDCD4). PDCD4 binds eIF4A (7) to inhibit cap-dependent protein synthesis and phosphorylation of PDCD4 targets it for degradation, allowing eIF4A assembly into the eIF4F complex (8). Therefore, mTOR regulates translation initiation by controlling eIF4F assembly.

Silibinin, a naturally occurring polyphenolic flavonoid, constitutes a major biologically active portion of the plant extract from milk thistle (Silybum marianum), which is widely consumed as a dietary supplement (9). Recent studies have shown that silibinin exerts both preventive and anticarcinogenic effects in different skin tumor models and inhibits the proliferation of human breast, lung, colon, pancreas, and prostate cancer cells in vitro and in vivo (10), apparently by inducing G1 cell cycle arrest (11). Furthermore, synergistic anticancer effects of silibinin are observed with conventional cytotoxic agents, such as doxorubicin, against MCF-7 and MBA-MD-468 cells (12). Recently, silibinin has been reported to repress the Akt/mTOR signaling pathway in HeLa and Hep3B cells, although the level at which inhibition was occurring was not determined (13). In this study, we have investigated the ability of silibinin to inhibit cancer cell growth by targeting the Akt/mTOR/eIF4F signaling pathway and show that silibinin likely targets this pathway upstream of TSC2 but downstream of PTEN.

Materials and Methods
Cell Lines and Cell Culture
MCF-7 cells (American Type Culture Collection) were cultured in DMEM/F-12 (Cellgro/Mediatech) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100 μg/mL penicillin-streptomycin. TSC2+/+ and TSC2−/− mouse embryonic fibroblasts (MEF) were cultured in DMEM...
supplemented with 10% fetal bovine serum, 2 mmol/L l-glutamine, and 100 μg/mL penicillin-streptomycin.

For proliferation assays, cells were seeded in 96-well plates (20,000 per well) 24 h before silibinin treatment. Cells were treated with increasing doses of silibinin (25-200 μmol/L) in DMEM/F-12 containing 10% fetal bovine serum or with vehicle (DMSO) only for 24 h. Cell growth was determined using the sulforhodamine B assay (14).

**Cell Cycle Analysis**

MCF-7 cells at 80% confluency were either treated with DMSO alone or the indicated concentrations of silibinin. Cells were then washed with PBS and trypsinized. Cell pellets were incubated with 1 mL DNA staining buffer (0.3% Triton-X 100, 50 μg/mL propidium iodine, 20 μg/mL RNase A, and 4 mmol/L sodium citrate). Cell cycle distribution was analyzed by flow cytometry using the Becton Dickinson FACS system.

**Western Blot Analysis**

Cells were harvested in radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L DTT, 0.1% SDS, 1% NP-40, and 0.5% sodium deoxycholate] with addition of 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL each of leupeptin, pepstatin, and aprotinin. Protein concentrations were quantified using a Bio-Rad protein assay. Total protein lysates (30 μg) were resolved by SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes (Millipore) and analyzed using the indicated antisera and enhanced chemiluminescence detection (Amersham).

**Metabolic Labeling**

MCF-7 cells (5 × 10⁶) were seeded in 24-well plates 1 day before the experiment. Cells were incubated with the indicated concentrations of silibinin for 24 h. Cells were cultured for 15 min in methionine-free medium followed by 15 min in [³⁵S]methionine-containing medium supplemented with 10% dialyzed FCS, washed, and lysed in radioimmunoprecipitation assay buffer. Proteins were TCA-precipitated onto 3 MM paper and the amount of incorporated radioactivity was quantitated by scintillation counting.

**7-Methyl-GTP Sepharose Pull-Down**

Cells were harvested in 300 μL buffer A [20 mmol/L HEPES (pH 7.5), 100 mmol/L KCl, 1.0 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 0.2% Tween 20, 10 mmol/L NaF, and 20 mmol/L β-glycerophosphate] and then subjected to three cycles of freeze-thaw. One milligram of cell extract was then incubated with 50 μL of 50% slurry of 7-methyl-GTP Sepharose 4B (GE Healthcare) for 2 h at 4°C. The resin was washed three times with 1 mL buffer A and one time with buffer A containing 200 μmol/L GDP. Finally, proteins bound to the resin were eluted with 80 μL 7-methyl-GTP (1 mmol/L) for 10 min on ice. Aliquots of the eluted fractions (25 μL) were resolved by SDS-PAGE (10% polyacrylamide) and analyzed by Western blotting.

**Polysome Analysis and Quantitative Reverse Transcription-PCR**

Polysome analyses were done on ∼2 × 10⁷ cells for each gradient. Total RNA from recovered fractions was isolated using Trizol (Invitrogen). The amount of cyclin D1 mRNA was detected by quantitative reverse transcription-PCR using the Roche Diagnostics LightCycler instrument and LightCycler RNA Master SYBR Green I kit according to the manufacturer’s instructions. The human cyclin D1 primers used for quantitative reverse transcription-PCR were 5′-CTCCCTCCTCCGGACCATTTGAT-3′ and 5′-ACCCGCT-CAGGGTTATGCAA-3′. The human eEF1A primers were 5′-GATGGTGCTGCTGTGGTGTGGA-3′ and 5′-TCA-CACCCAGTGTTAGCAAGCGAA-3′.

**Immunofluorescence**

MCF-7 cells were seeded in Lab-Tek chamber slides (Nunc) at 60% confluency 24 h before treatment. After silibinin and/or rapamycin treatment, cells were fixed, permeabilized, and blocked as described previously (15). Fixed cells were incubated with an anti-eIF4E mouse monoclonal antibody (1:200; a kind gift from S.R. Kimball) overnight at 4°C. After washing with PBS, cells were incubated with Alexa Fluor 594-conjugated secondary antibodies (1:1,000; Molecular Probes) for 35 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (1:2,000; Sigma-Aldrich). Images were obtained using a ∼0 objective of a Zeiss LSM 510 confocal microscope. The details about the confocal analysis of nuclear versus cytoplasmic eIF4E are described in the Supplementary Data.

**Results**

**Silibinin Inhibits Cell Growth and eIF4F Complex Formation in MCF-7 Human Breast Cancer Cells**

MCF-7 cells were exposed to increasing concentrations of silibinin (Fig. 1A). The sulforhodamine B assay showed a dose-dependent inhibition of cell proliferation with an IC₅₀ ∼ 200 μmol/L (Fig. 1B). To determine the potency of silibinin on protein synthesis in vivo, we performed metabolic labeling studies and the consequences on protein synthesis were assessed by monitoring incorporation of [³⁵S]methionine into TCA-precipitable material. Silibinin treatment led to a reduction in translation with a maximal inhibition of 42% at 200 μmol/L (Fig. 1C). To assess whether the silibinin-mediated decrease in translation was associated with altered levels of eIF4F complex, we perform 7-methyl-GTP pull-down assays. Analysis of the 7-methyl-GTP eluents revealed that silibinin, like rapamycin, a specific mTOR inhibitor, caused a decrease in the levels of eIF4E-associated eIF4G1 and eIF4A1 and an increase in the levels of eIF4E-associated 4E-BP1 in silibinin-treated cells (Fig. 1D). These results indicate that silibinin reduces the levels of the eIF4F complex. Taken together, our results indicate that silibinin exhibits antiproliferative activity against MCF-7 human breast cancer cells by inhibiting translation initiation.

**Silibinin Induces G₁ Cell Arrest and Inhibits Translation of Cyclin D1 mRNA in MCF-7 Cells**

We examined the effect of silibinin on cell cycle progression in MCF-7 cells. As shown in Fig. 2A, silibinin treatment showed G₁ arrest (76.5% versus 63.69% in control). The effect of silibinin on G₁ arrest was accompanied by a reduction of cells in S phase (26% versus 17.25%) and G₂-M phase.

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Silibinin did not appear to induce apoptosis because there was no significant change in the sub-G0 fraction of cells treated with silibinin (data not shown). The cyclin D1 proto-oncogene is an important regulator of G1-S-phase transition and is an eIF4F-responsive mRNA (4). Therefore, we next assessed the effect of silibinin on cyclin D1 expression and observed that cyclin D1 protein levels were decreased in MCF-7 cells exposed to silibinin (Fig. 2B). To determine if this inhibition was at the level of translation, we performed polysome profile analysis (Fig. 2C). Treatment of MCF-7 cells with 100 μmol/L silibinin caused a partial collapse of polysome with a concomitant increase in 80S ribosomes (Fig. 2C), consistent with silibinin exerting an inhibitory effect on translation initiation. We determined the distribution of cyclin D1 mRNA in polysomes from vehicle- and silibinin-treated MCF-7 cells (Fig. 2D, top). Quantitative reverse transcription-PCR indicated that cyclin D1 mRNA shifted to lighter polysome fractions in silibinin-treated cells (Fig. 2D, fractions 8-10). In contrast, the distribution of β-actin was similar among polysomes of vehicle- and silibinin-treated cells (Fig. 2D, bottom). These results indicate that translation of cyclin D1 mRNA in silibinin-treated cells is reduced. We also observed a decrease in translation of elongation factor eEF1A in response to silibinin exposure, a 5′-TOP mRNA known to be rapamycin-responsive (Supplementary Fig. S1).3

Silibinin Inhibits mTOR Signaling

Silibinin has been reported to inhibit mTOR signaling (13). To confirm this in MCF-7 cells, we performed Western blot analysis to assess the phosphorylation status of two direct downstream targets of mTOR: S6K and 4E-BP1. In MCF-7 cells, silibinin treatment led to a dose- and time-dependent decrease in the phosphorylation of S6K and its target, ribosomal protein S6 (Fig. 3A). We also examined the phosphorylation of 4E-BP1. Three isoforms of 4E-BP1 were detected (Fig. 3A), which represent differentially phosphorylated forms of the protein, with the slowest migrating band (labeled γ) corresponding to the more hyperphosphorylated form and the fastest migrating band (labeled α) corresponding to the hypophosphorylated form of the protein (6). Silibinin treatment caused a shift in the phosphorylation status of 4E-BP1 from the γ to the α form (Fig. 3A). The inhibitory effect of silibinin on mTOR activity was observed as early as 1 h at 50 μmol/L and continued through 16 h of treatment (Fig. 3A). The serine/threonine protein kinase Akt is a major regulator of mTOR and we noted that phosphorylated Akt status was increased on silibinin treatment in MCF-7 cells (Fig. 3A). As silibinin treatment inhibited activation of the mTOR/p70S6K pathway, we sought to determine its effects on PDCD4 protein expression, the stability of which has been shown to be under mTOR control (8). Treatment of MCF-7 cells with silibinin resulted in an increase in PDCD4 protein, prominently at 16 h (Fig. 3A). We did not detect changes in expression of poly(ADP-ribose) polymerase cleavage status in MCF-7 cells treated with silibinin (Fig. 3A), indicating that the observed silibinin-mediated mTOR
inhibition is not an indirect consequence of cell death. Inhibition of mTOR activity was also observed in MDA-MB-468 breast cancer cells, a line deficient for PTEN expression (Fig. 3B). Therefore, silibinin acts downstream of PTEN to down-regulate mTOR. We also observed inhibition of mTOR activity in HeLa cells on silibinin treatment, thus excluding a cell-line specific effect (Supplementary Fig. S2).3

Silibinin Down-Regulates mTOR Activity by Acting Upstream of TSC2

To further elucidate the mechanism of action by which silibinin inhibits mTOR, we examined the effect of silibinin on established TSC2+/+ and TSC2−/− MEFs (ref. 16; Fig. 3C). In TSC2−/− MEFs, mTOR activity is elevated relative to TSC2+/+ cells as judged by endogenous levels of phosphorylated S6K, pS6, and phosphorylated 4E-BP1 and as documented previously (16). As expected, in both TSC2+/+ and TSC2−/− MEFs, rapamycin led to a decrease in phosphorylation of S6K and ribosomal protein S6 and a shift from hyperphosphorylated 4E-BP1 to the hypophosphorylated form in response to serum and insulin (Fig. 3C). However, silibinin was only able to diminish phosphorylation of S6K, ribosomal protein S6, and 4E-BP1 levels in TSC2+/+ but not in TSC2−/− MEFs (Fig. 3C). The results indicated that silibinin is interdicting the Akt/mTOR pathway upstream of the TSC1/TSC2 complex.

Silibinin Induces eIF4E Localization to the Nucleus

Recent studies in MEFs have shown that nuclear 4E-BPs sequester eIF4E in the nucleus in a manner that is dependent on the phosphorylation status of 4E-BP1, a reduction in phosphorylated 4E-BP1 status being associated with increased nuclear retention of eIF4E (17). Because our data suggest that silibinin causes hypophosphorylation of 4E-BP1, we reasoned that it should increase the nuclear content of eIF4E. Cells were treated with either rapamycin or silibinin for 6 h and the amount of cytoplasmic/nuclear eIF4E was quantified in MCF-7 cells using immunofluorescence.

Figure 2. A, silibinin induces G1 arrest in cell cycle progression of MCF-7 cells. Cells were treated with either DMSO or silibinin at 50 or 100 μmol/L for 24 h. Cell cycle distribution was then determined as described in Materials and Methods. Mean ± SD of three independent experiments. *, P < 0.01; **, P < 0.05 (calculated using Student’s t test) compared with DMSO control. B, silibinin inhibits cyclin D1 protein production. MCF-7 cells were treated with the indicated doses of silibinin and harvested, and lysates were resolved on 13% SDS-polyacrylamide gels. Quantification of cyclin D1 was analyzed by NIH ImageJ software. C, analysis of polysome profiles in silibinin-treated MCF-7 cells. Cells were treated with 100 μmol/L silibinin or DMSO for 2 h. D, documenting the polysome distribution of cyclin D1 and β-actin mRNA in cells after exposure to silibinin. RNA was purified from the indicated fractions in the polysome profile from C and quantified using quantitative reverse transcription-PCR for cyclin D1 and β-actin. The relative amount of mRNA in each fraction is expressed as a percentage of its total in polysomes. Values are from one of the three independent experiments done in duplicate. Bars, SE.
(Fig. 4A and B). eIF4E relocalized from the cytoplasm to the nucleus on silibinin and rapamycin treatment (Fig. 4), consistent with nuclear 4E-BP1 phosphorylation status being affected by silibinin treatment.

**Discussion**

The phosphatidylinositol 3-kinase/Akt signaling is activated in many breast cancers and plays a variety of physiologic roles, including cell growth, cell cycle regulation, migration, and survival (18). It is activated in breast cancer cells via HER-2/neu overexpression as well as activation of insulin-like growth factor and integrins (19, 20). Hence, interfering mTOR as a downstream kinase in the phosphatidylinositol 3-kinase/Akt pathway is an attractive therapeutic target for breast cancer therapy. Our study confirms the antiproliferative effect of silibinin as shown by inhibition of MCF-7 breast cancer cell growth. Specifically, we find that silibinin inhibits translation initiation by down-regulating the Akt/mTOR/eIF4E signaling pathway.

To determine if the effect was at the level of translation initiation, polysome profiles were obtained from MCF-7 cells treated with vehicle or silibinin. A partial collapse of polysomes was observed in silibinin-treated cells (Fig. 2C) and was accompanied by more pronounced consequences on the translation of specific mRNAs as we have shown for cyclin D1 (Fig. 2D). We show here that translation initiation on cyclin D1 mRNA was preferentially decreased (Fig. 2B and C) when eIF4F complex levels were down-regulated in silibinin-treated cells (Fig. 1D). The silibinin-mediated decrease in cyclin D1 protein expression was also associated with mTOR inhibition and a decrease in phosphorylation of 4E-BP1 (Fig. 3A). This is consistent with the findings that expression of constitutively active 4E-BP1 in MCF-7 cells leads to cell cycle arrest, which is also associated with down-regulation of cyclin D1 (21). The cyclin D1 proto-oncogene is an important regulator of G1-S-phase transition in numerous cell types from diverse tissues and its overexpression is reported in 30% to 50% of primary human breast cancers, is noted early during tumorigenesis, and is associated with a better response to tamoxifen in estrogen receptor-α-positive tumors (22). Therefore, targeting cyclin D1 expression by silibinin could be of potential benefit for breast cancer therapy.

Treatment of cells with silibinin has also been shown to inhibit expression of Mcl-1 and survivin (23, 24), two eIF4E-responsive genes (25, 26). Identification of other mRNAs, the translation of which may be affected by silibinin, will require more intensive microarray analysis of transcripts in polysomes of silibinin-treated cells. However, we expect the candidates will also include critical growth factors and proto-oncogenes such as vascular endothelial growth factor, c-Myc, and ornithine decarboxylase, which have been shown previously to be eIF4F-responsive transcripts (4).

Deregulation of protein synthesis has been strongly implicated in the pathogenesis of cancer and metastasis. Alterations in the levels of the eIF4F subunits are associated with oncogenesis. eIF4AI is overexpressed in melanoma (27) and hepatocellular carcinoma (28). The availability of the eIF4A subunit for incorporation into the eIF4F complex is regulated by its association with the tumor suppressor gene product PDCD4, a protein with levels reduced in human lung, renal, and glial tumors (29). Moreover, overexpression of PDCD4 in the epidermis delays tumor onset and progression in a chemically induced murine skin tumor model.

**Figure 3.** A, silibinin inhibits mTOR signaling in a dose- and time-dependent manner. MCF-7 cells were treated with the indicated doses of silibinin for indicated time. Cells were harvested and lysates were resolved on 13% SDS-polyacrylamide gels. Western blot analysis was carried out using the indicated antibodies. B, silibinin acts downstream of PTEN. Breast cancer MDA-MB-468 cells were treated with indicated doses of silibinin for 16 h, harvested, and subjected to SDS-PAGE following by immunoblotting with antibodies against indicated proteins. C, silibinin inhibits mTOR signaling upstream of TSC2. TSC2+/+ and TSC2−/− MEFs were deprived of serum for 24 h. Cells were then exposed to 100 μmol/L silibinin or 20 nmol/L rapamycin for another 16 h and stimulated with either 10% serum or 100 nmol/L insulin for 30 min. Total cell extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies against the indicated proteins.
In our study, we found that treatment of MCF-7 cells with silibinin led to a decrease in phosphorylation of S6K (Thr389) with a corresponding increase in PDCD4 protein levels (Fig. 3A), indicating one of the molecular mechanisms by which silibinin may inhibit translation initiation and cancer cell growth maybe via increased sequestration of eIF4A. A recently study has shown that PDCD4 down-regulation contributes to the decreased sensitivity of MCF-7 cells to tamoxifen and geldanamycin (29). Therefore, therapeutic strategies to up-regulate PDCD4 expression with geldanamycin and that target other aspects of eIF4F assembly, such as with silibinin, in combination, may offer promise to target breast cancer.

Ectopic expression of eIF4E or eIF4GI can transform cells in culture (31, 32). In particular, overexpression of eIF4E co-operates with c-Myc during lymphomagenesis (33) and leads to rapamycin resistance in vivo (34). Moreover, increased eIF4F levels are essential for maintenance of the malignant phenotype in human mammary epithelial cells (35). 4E-BP1 is a cell signaling hallmark in breast cancer that correlates with pathologic prognosis (36). Phosphorylated 4E-BP1 expression in breast tumors is associated with malignant progression and poor prognosis (37).

Some reports have documented that silibinin inhibits Akt phosphorylation (23, 38). However, in our study, silibinin was found to induce the activation of the prosurvival kinase Akt in MCF-7 breast cancer cells (Fig. 3A). This result is also consistent with recent findings that silibinin activates Akt in human cervical and hepatoma cancer cells (13). Our results do not exclude the possibility that silibinin exerts additional effects on signal transduction pathways; indeed, silibinin has been shown to also down-regulate the extracellular signal-regulated kinase 1/2 pathway (39). Our results indicate that, at pharmacologically relevant concentrations (~60 μmol/L; ref. 40), silibinin inhibits cell growth (Fig. 1B) and protein translation (Fig. 1C) and inhibits mTOR activity (Fig. 3). These findings may provide a rationale for the development of silibinin as an anticancer drug.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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