Original Article

Silibinin Induces Apoptosis and Inhibits Proliferation of Estrogen Receptor (ER)-Negative Breast Carcinoma Cells through Suppression of Nuclear Factor Kappa B Activation

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Abstract

Background: Silibinin is a traditionally well-known drug for its hepatoprotective efficacy against various types of liver afflictions. In addition, it has recently been considered broadly as a potential chemopreventive agent against many types of cancers. The current study was designed to evaluate the restrictive effects of pharmacological doses of silibinin on SKBR3, an ErbB2-overexpressed and ER-negative human breast carcinoma cell line.

Methods: Effect of silibinin on metabolic activity and proliferation of human breast carcinoma (SKBR3) cell line were evaluated by MTT and BrdU assays respectively. Furthermore, the proapoptotic effect of silibinin was investigated using flow cytometry. The NF-κB phosphorylation assay was also used to assess the effect of silibinin on NF-κB activation. The alkalizing effect of silibinin on SKBR3 cell line was evaluated by measuring *p*H of media of the silibinin-treated cells compared to control.

Results: Our results indicate that silibinin inhibited metabolic activity and cell proliferation of SKBR3 cells in a dose-dependent manner. Moreover, silibinin significantly induced apoptosis in SKBR3 cells. On the other hand, silibinin significantly inhibited activation of NF-κB which is known to be highly active in this cell line. Alkalizing effect of silibinin was also observed.

Conclusion: The results obtained here indicate that silibinin may be an efficacious therapeutic agent against ER-negative breast carcinomas with high inhibitory effect on NF-κB.

Keywords: Estrogen receptor, NF-κB, silibinin,

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Introduction

Breast cancer is the most common cancer in women with an incidence of 95 per 100,000 persons in developed countries.[1] There are about 184,000 new cases of breast cancer detected annually in the US and it is estimated that one in every eight women in the US will develop breast cancer during their lifetime.[2] Therefore, breast cancer is becoming a common disease and needs to be addressed very seriously.

Therapeutic and prognostic classification of breast cancer is based primarily on expression of two important growth factor receptors, the nuclear estrogen receptor (ER) and the membrane receptor tyrosine kinase, ErbB2, although there are some other classifications.[3–5] ErbB2 is a member of tyrosine kinase-coupled dimeric receptors which binds to ligands, including the heregulins, by forming heterodimers with ErbB1, ErbB3, and ErbB4.[4,6–7] More than 20% all of human breast carcinomas express high levels of the ErbB2 receptor protein, and at least 30% of ER-negative breast cancers contain overexpressed ErbB2.[4,6–7] ER-positive breast cancers respond to hormonal therapy. However, the prognosis for ER-negative breast carcinomas is poor.[8–10] Therefore, improvements in treatment strategies against ER-negative cancer cells remain a major priority and mostly depend on discovering new therapeutic agents which might shed a light on treatment of specific subclasses of human breast cancers.

NF-κB signaling is an important pathway frequently deregulated in human malignancies.[11] This pathway governs all hallmarks of cancer such as proliferation, survival, evasion from apoptosis, and metastasis through transcription regulation of the genes involved in these processes.[11] It has been reported that ER-negative human breast cancer cells harboring overexpressed ErbB2 have activated NF-κB. In addition, it has been reported that inhibition of NF-κB activation in ER-negative cancer cells inhibits tumor formation in murine models.[12–13] On the other hand, *in vitro* studies on SKBR3 indicate that suppression of NF-κB inhibits proliferation of this cell line and induces apoptosis.[14] Therefore, it has been supposed that NF-κB is a potential therapeutic target for ER-negative tumors harboring amplified ErbB2.[12–13,15]

Silibinin is the major active component of silymarin, the mixture of polyflavonoids extracted from milk thistle. Recently, silibinin has been introduced as a prominent anti-neoplastic agent against various tumor cells including hepatocellular carcinoma,[16] prostate,[17] glioblastoma,[18] renal cell carcinoma,[19] bladder,[20] colon,[21] lung,[22] and skin cancers.[23] Currently, silibinin is under phase I/II clinical trial in patients with prostate cancer.[24] On the other hand,

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there is a large body of evidences showing anti-proliferative effects of silibinin on breast carcinomas through different mechanisms.[25] Among the targets through which silibinin inhibits proliferation of cancer cells, NF-κB is of paramount importance.[25] The inhibitory role of silibinin on NF-κB is well established in human prostate carcinoma[26] and hepatocellular carcinoma cells.[27] In addition, in our previous study, we reported that silibinin suppresses invasion of glioma cells through suppression of NF-κB.[28]

Putting aside several reports showing the effects of silibinin on discrepant properties of human breast cancer cells, no study, has to date addressed the anticancer efficacy of this therapeutic agent on ER-negative breast carcinoma cells in which NF-κB is highly activated. The present study aims to investigate the effect of silibinin on SKBR3, an ER-negative human breast carcinoma cell line.

Materials and Methods

Cell line and silibinin treatment

The human breast cancer cell line, SKBR3 (ErbB2-overexpressed and ER-negative breast carcinoma cell line), was obtained from Pasteur Institute of Iran (Tehran, Iran) and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) in 5% CO_2 at 37 °C. Stock solutions of silibinin (Sigma, St. Louis, Missouri, USA) were prepared in concentration of 0.1 M in DMSO. Then, the cultures were treated with varying concentrations of silibinin.

Microculture tetrazolium (MTT) assay

MTT assay was used to assess the inhibitory effect of silibinin on metabolic activity of SKBR3 cells. For this, seeded cells were plated onto 96-well plates at a density of 10,000 cells/200 µl PRMI in each well and then, exposed to either control or varying concentrations of silibinin (25 µM – 300 µM) for different time intervals (24, 48, and 72 h). Thereafter, the control medium and the media containing silibinin were replaced by MTT solution (0.5 mg/ml). After 3 h, DMSO was added and the color absorbance was read at wavelength of 578 nm in an ELISA reader. The percentage metabolic activity was calculated as: $(OD_{exp}/OD_{con}) \times 100$, where OD_{exp} and OD_{con} are the optical densities of exposed and control cells, respectively.

BrdU cell proliferation assay

The suppressive effect of silibinin on DNA synthesis (which is proportional to cell proliferation) was measured using a colorimetric bromodeoxyuridine (BrdU)-based Cell Proliferation ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) in accordance with the manufacturer's instructions. Briefly, the cells (at a density of 5000 cells/100 µl/well) were treated with varying concentrations of silibinin (0 µM – 200 µM) and then incubated with 10 µl of BrdU solution at 37 °C for 48 h. Thereafter, the fixation was carried out using 200 µl of FixDenat solution for each well. Then, the cells were incubated with peroxidase-conjugated anti-BrdU antibody at room temperature for 1 h followed by exposure to 100 µl of substrate tetramethylbenzidine for 30 min at room temperature. To stop the peroxidase reaction, 25 µl of 1 M H_2SO_4 was added and the samples were read at 450 nm.

Cell-based quantification of NF-κB phosphorylation

To evaluate the effect of silibinin on NF-κB activation, a Cellular Activation of Signaling ELISA kit (CASE Kit, Super Array Bioscience, Frederick, Maryland, USA) was used in accordance with the manufacture's protocol. First, the cells were pleated in 96-well plates and exposed to different concentrations of silibinin for 48 h. Then, the cells were fixed in 8% formaldehyde: PBS solution and incubated with primary and secondary antibodies. Subsequently, the cells were exposed to developing and stop solutions provided with the kit. For normalization, the relative cell number was obtained by a cell staining buffer at 578 nm. Lastly, the ratio of phosphorylated NF-κB to total NF-κB was obtained at 450 nm.

Analysis of gene expression by real-time PCR

To assess mRNA expressions of IκB kinase genes (IKK), real-time PCR was performed. First, RNA was extracted by Fast Pure RNA kit (Takara Bio Inc., Otsu, Japan) from cultured cells and quantified by Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, Delaware, USA). Then, 1 µg of RNA from each sample was applied to reverse transcription using the Prime Script RT reagent kit (Takara Bio Inc). Real-time PCR was performed with a light cycler instrument (Roche Diagnostics, Germany) using SYBR Premix Ex Taq technology (Takara Bio Inc.). In a total volume of 20 µl, 10 µl SYBR Green master mix, 2 µl of cDNA samples, 0.5 µl of forward and reverse primers (10 pmol) and 7 µl of nuclease-free water (Qiagen, Hilden, Germany) were added to each capillary tube. Thermal cycling conditions were an initial activation step of 30 s at 95°C followed by 40 cycles including a denaturation step of 5s at 95 °C and a combined annealing/extension step of 20 s at 60 °C. Melting curves were analyzed to validate single PCR product of each primer. Hypoxanthine phosphoribosyltransferase1 (HPRT1) was amplified as internal control and the fold change in relative expression of each target mRNA was calculated on the basis of comparative C_t ($2^{-\Delta\Delta Ct}$) method. The primer sequences are as follow: HPRT1-F: TGGACAGGACTGAACGTCTTG, HPRT1-R: CCAGCAGGTCAGCAAAGAATTTA, IKK1-F: AAGTTGAACCATGCCAATGTTGT, IKK1-R: TCTCCTCCAGAACAGTATTCCAT, IKK2-F: CACCATCCACACCTACCCTG, IKK2-R: CTTATCGGGGATCAACGCCAG.

Media pH assessment

To evaluate the effect of silibinin on acidity of media containing SKBR3 cells, the pH of control and silibinin-treated media were measured using a laboratory pH meter (827 pH lab, Metrohm, Swiss) before and after incubation time (48 h).

Quantification of apoptosis by flow cytometry

In order to evaluate the effect of silibinin on apoptosis in SKBR3 cells, a double staining kit with Hoechst 33342 and Propidium iodide (PI) was used (Invitrogen). After treatment of cells with silibinin for 48 h, the cells were harvested and washed in cold PBS and the cell density was adjusted to 0.5×10^6 cells/mL in PBS. One microliter of the Hoechst 33342 stock solution (5.0 mg/mL solution in water) and 1 µL of the Propidium iodide stock solution (1.0 mg/mL solution in water) were added to each 1 mL of cell suspension. After 15 min, the stained cells were evaluated by flow cytometry instrument (Partec PasIII, Germany), using excitation/emission ~ 350/461 and ~ 535/617 nm for Hoechst 33342 and PI, respectively. The data were then analyzed using FlowMax software.

Statistical analysis

The results were expressed as mean ± SD. All experiments were performed in triplicate. Statistical significances of difference throughout this study were calculated using a Student's *t*-test and one-way variance analysis. *P* values ≤ 0.05 were considered significant. 50% lethal concentration (LC_{50}) values of silibinin on SKBR3 cell line at different time intervals were determined by analyzing dose-dependent inhibition using the GraphPad Prism 5 statistical package.

Results

Silibinin inhibits metabolic activity of SKBR3 cells

The inhibitory effect of silibinin on metabolic activity of SKBR3 cell line was investigated through MTT assay at different concentrations and time intervals (24, 48 and 72 h). As shown in Figure 1, treatment of the cell line with silibinin at 100, 200, and 300 µM for 48 h, reduced metabolic capabilities of SKBR3 cells by 21.02%, 42.45% and 68.13% respectively ($P < 0.01$). In addition, LC_{50} values for 48 and 72 h were 228, and 196 µM, respectively. The treatment of SKBR3 cell line at concentrations of 200 and below for 48 h was used for further evaluations throughout the study.

Inhibitory effect of silibinin on DNA synthesis

To explore if silibinin treatment has inhibitory effect on DNA synthesis in SKBR3 cells, a colorimetric BrdU proliferation assay was applied. A dose-dependent reduction in proliferation of SKBR3 cells was observed after silibinin treatment for 48 h. As shown in Figure 2, 20.44% and 24.6% reduction in cell proliferation was observed by silibinin at 100 and 200 µM, respectively ($P < 0.05$).

Silibinin suppresses activation of NF-κB in SKBR3 cells

To assess whether silibinin suppresses NF-κB signaling cascade in SKBR3 cells, we evaluated the effect of silibinin on the NF-κB activation using a cell-based ELISA assay for NF-κB protein phosphorylation. As shown in Figure 3, silibinin exposure at 50, 100, and 200 µM reduced NF-κB phosphorylation by 66.2%, 76.5%, and 67.55%, respectively ($P < 0.01$), compared with the control. However, there was no significant decrease observed in mRNA expression of IKK1 and IKK2 (data not shown).

Silibinin-induced media pH alkalization

Acidification of extracellular pH has roles in driving protease-mediated digestion and remodeling of the ECM. In this study, as displayed in Figure 4, we observed that silibinin significantly increased the pH of the media in a dose-dependent manner ($P < 0.05$).

Silibinin stimulates apoptosis in SKBR3 cells

To investigate the impeding effects of silibinin on apoptosis rate in SKBR3 cells, flow cytometry analysis was performed. As shown in Figure 5, silibinin stimulated apoptotic rate of SKBR3 cells from 21% in the control to 33.7% and 30.4% at 100 and 200 µM, respectively ($P < 0.01$).

Discussion

Human breast cancers are heterogeneously different in histological patterns, pathological grades and progression behaviors.[29] Therapeutically, breast cancers are commonly classified based on expression of two important growth factor receptors: the nuclear

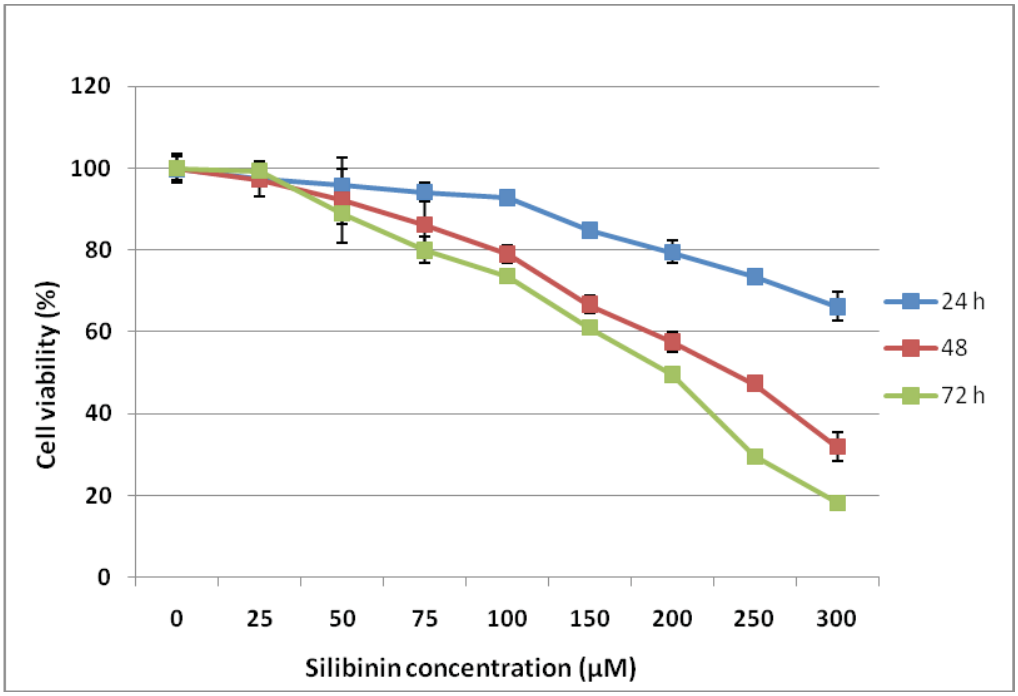

Figure 1. Evaluation of silibinin on the metabolic activity of SKBR3 cells. The cells were treated with various concentrations of silibinin for 24, 48 and 72 h and their metabolic activity was assessed by MTT assay. Results are expressed as percentage of metabolic activity compared to untreated control and are presented as mean ± SD from three independent experiments ($P < 0.05$ by one-way variance analysis).

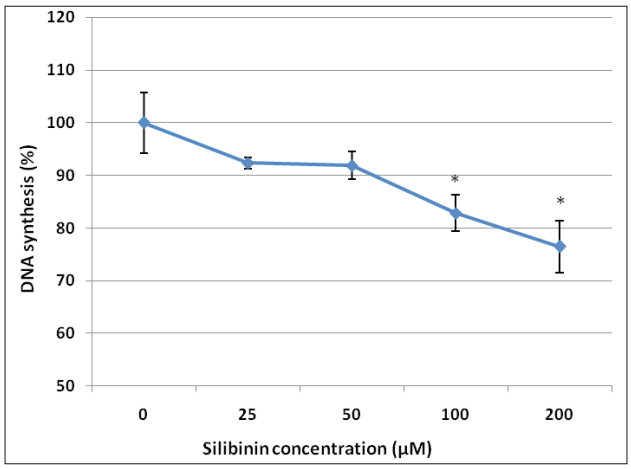

Figure 2. Evaluation of silibinin on the DNA synthesis in SKBR3 cells. The cells were treated with various concentrations of silibinin for 48 h and their proliferation (which is proportional to DNA synthesis) was measured by BrdU assay. Results are expressed as percentage of cell proliferation compared to control and are presented as mean ± SD from three independent experiments ($P < 0.05$ by one-way variance analysis).

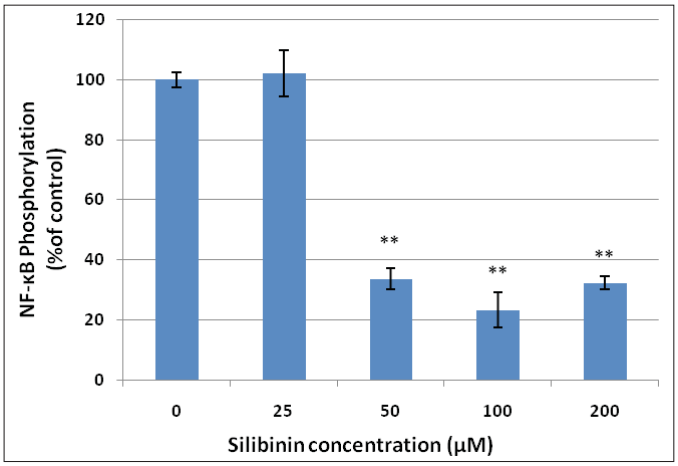

Figure 3. Effect of silibinin on phosphorylation of NF-κB in SKBR3 cells. Relative phosphorylated levels of NF-κB were measured using Cellular Activation of Signaling ELISA kit as described in materials and methods section. Values are given as mean ± SD from three independent experiments and $P < 0.05$ is considered as significant.

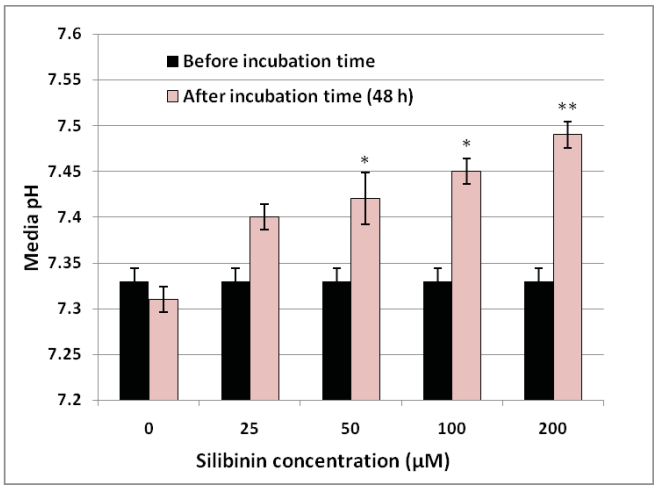

Figure 4. Effect of silibinin on media pH. The media pH was measured immediately after treatment with desired concentrations of silibinin, and then measured again after 48 h. As shown in figure, silibinin alkalizes the extracellular media of SKBR3 cells in a dose-dependent manner. Values are given as mean ± SD from three independent experiments and $P < 0.05$ is considered as significant.

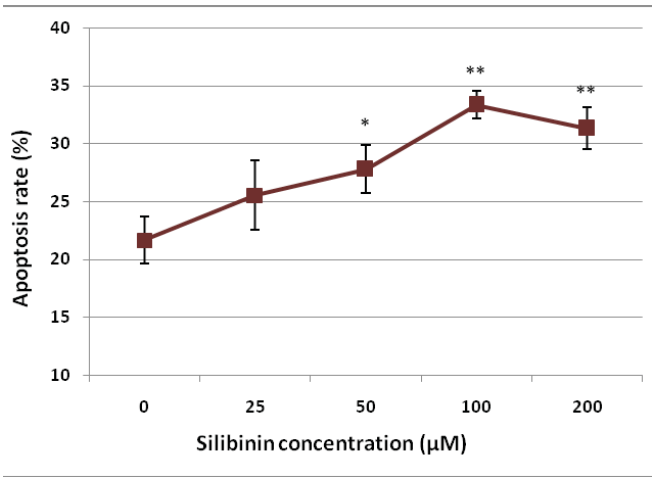

Figure 5. Effect of silibinin on apoptosis in SKBR3 cells. Hoechst 33342 stains the condensed chromatin in apoptotic cells more brightly than the chromatin in normal cells and Propidium iodide (PI) is only permeate to dead cells (FL2: PI, FL6: Hoechst 33342). As shown in figure, silibinin significantly stimulated the apoptotic rate of SKBR3 cells compared to the control ($P < 0.01$). Values are given as mean ± SD from three independent experiments.

estrogen receptor (ER) and the membrane receptor tyrosine kinase (ErbB2).[3–4,30] Prognosis of ER-negative breast carcinomas is significantly poorer compared to ER-positive breast carcinomas.[8–10] Therefore, outlining new strategies for treatment of ER-negative cancer cells remains a major priority. In this study for the first time, we evaluated the restrictive potentials of various concentrations of silibinin on SKBR3, an ER-negative human breast carcinoma cell line. We first assessed the effects of silibinin on metabolic activity of SKBR3 cell line and demonstrated that silibinin highly inhibits the metabolic activity of SKBR3 cells in a time- and dose-dependent manner. The IC50 value of silibinin for this cell line at the time interval 48 h was determined and concentrations below IC50 were used for further evaluations. In parallel to the results of metabolic activity assay, our results also show that silibinin inhibits proliferation of SKBR3 cells in a dose-dependent manner.

A wealth of evidence indicates that silibinin stimulates apoptosis in many types of cancer cells, including breast carcinomas.[25] Wang et al reported that silibinin up regulates the expression of Fas ligand (FasL), Fas-associated death domain protein (FADD), and Bax in MCF-7 breast carcinoma cell line.[31] Moreover, they demonstrated that this therapeutic agent induces translocation of Bax to mitochondria and release of cytochrome *c* to the cytosol.[31] Also, it has been reported that silibinin is able to alter mitochondrial transmembrane potential.[32] All these findings suggest that silibinin has the ability to activate apoptosis in human breast cancer cells through both extrinsic and intrinsic pathways. Therefore, we decided to assess the effects of varying concentrations of silibinin

on apoptosis in SKBR3 cells. We observed that silibinin stimulates apoptosis in SKBR3 cell line. Although this observation is in line with previous studies, it is very interesting that silibinin may be a potent pro-apoptotic agent in ER-negative breast carcinomas which respond poorly to many therapies. However, further studies on other ER-negative breast carcinoma cell lines are required to confirm if silibinin is an efficient pro-apoptotic drug for treatment of this subtype.

Silibinin has recently been suggested as a well-known drug for its anti-invasive and anti-metastatic functions in cancer cells.[25,33] This role is mediated through different mechanisms including *p*H alkalization. Both *in vitro* and *in situ* tumor studies have reported that tumor cells have acidic interstitial extracellular *p*H which serves protease-mediated digestion and the remodeling of extracellular matrix (ECM), followed by the migration of cancer cells.[34] In our previous study, we reported that silibinin alkalizes the media pH of human glioma U87MG cell line and suppresses gene expression of carbonic anhydrase IX (CA9) which is an important mediator for the cancer cell microenvironment acidification.[35] In this regard, we examined the alkalizing effect of silibinin on SKBR3 cell line. Our results demonstrate that silibinin alkalizes the extracellular *p*H of this cell line in a dose-dependent manner. This finding suggests that silibinin may affect SKBR3 cells through an anti-invasive mechanism. Regarding the fact that silibinin is a well-known anti-invasive agent in many cancer cell types,[25] this observation may show promise to consider silibinin as a potent anti-invasive agent against ER-negative breast cancer; still, further studies are needed to confirm this observation.

Nuclear factor kappa B signaling is a pivotal pathway which is currently beginning to shed a light on our understanding of the cellular basis of tumorigenesis.[11] There are frequent reports suggesting NF-κB as a signaling pathway which stimulates growth and proliferation of different types of cancer cells, including human breast cancer.[11,13,36–37] Moreover, NF-κB activation is suggested to be correlated with enhanced tumor aggressiveness in recurrent and metastatic lesions that show resistant phenotype to anti-cancer therapy , a major barrier to improving overall survival of cancer patients,[38] and may have prognostic value for breast cancer subtype and overall survival.[39] In addition, it has been reported that ER-negative human breast cancer cells harboring overexpressed ErbB2 have activated NF-κB. Further studies indicate that inhibition of NF-κB activation with dominant negative inhibitor κB kinase (dnIKKβ) in ER-negative cancer cells incapacitates tumor formation in murine models. Furthermore, NF-κB expression has been found to change depending on expression of ER as well as molecular subtype.[40] Therefore, NF-κB is an approved therapeutic target for ER-negative tumors harboring amplified ErbB2. Moreover, *in vitro* studies on SKBR3 show that NF-κB essential modulator (NEMO) binding domain (NBD) peptide, an established selective inhibitor of IκB-kinase (IKK), induces apoptosis and inhibits proliferation of this cell line.[14] On the other hand, it has been frequently reported that silibinin selectively inhibits the activation of NF-κB in several types of cancers, including human prostate carcinoma,[26] hepatocellular carcinoma[27] and glioblastoma cells.[28] Considering the paramount importance of NF-κB in proliferation of ER-negative human breast cancer cells[14] and the reported roles of silibinin in suppressing this signaling pathway,[25–28] we next surveyed the effect of varying concentrations of silibinin on phosphorylated status of NF-κB in SKBR3 cells. Our results indicate that silibinin significantly suppresses the activation of the p56 subunit of NF-κB. This finding suggests that silibinin may be an efficient therapeutic agent for targeting ER-negative breast cancer cells harboring amplified ErbB2.

The activation of NF-κB signaling pathway is induced through phosphorylation of inhibitory proteins (IκB) by IκB kinase (IKK) complex which is suggested to be formed by two catalytic subunits, IKK1 (IKKα), and IKK2 (IKKβ), and a regulatory protein called NEMO.[41–42] In this regard, we evaluated the mRNA expression of IKK1 and IKK2 in SKBR3 cells. In contrast to our expectations, no significant inhibition was observed in silibinin-treated SKBR3 cells compared to the control. The reason may be that these genes function on protein level.

Collectively, our results indicate that silibinin highly inhibits proliferative potentials of SKBR3 and induces apoptosis in this ER-negative breast carcinoma cell line. On the other hand, silibinin highly suppresses phosphorylation of NF-κB, which is a well-established therapeutic target for this subtype of breast carcinoma. However, more studies are needed to clarify the detailed mechanisms through which suppression of NF-κB is linked to inhibition of cell proliferation and induction of apoptosis in ER-negative human breast carcinomas.

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Conflict of interest statement: *None declared.*

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