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TITLE PAGE

Silibinin induces cell death through ROS-dependent down-regulation of Notch-1/ERK/Akt signaling in human breast cancer cells

Thae Hyun Kim, Jae Suk Woo, Yong Keun Kim and Ki Hyung Kim

Department of Physiology (TH Kim, JS Woo and YK Kim), Department of Obstetrics and Gynecology (KH Kim), Pusan National University School of Medicine, Yangsan, 626-870

Pusan National University Hospital Biomedical Research Institute and Pusan Cancer Center (KH Kim), Pusan, 602-739, Republic of Korea

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Corresponding authors:

Ki Hyung Kim, MD, Ph.D.

Department of Obstetrics and Gynecology, College of Medicine, Pusan National University Hospital, Pusan, 602-739, Republic of Korea.

Tel: 82-51-510-8070

Fax: 82-51-510-8076

E-mail: ghkim@pusan.ac.kr

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Abbreviations:

ROS, reactive oxygen species; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DCFH-DA, 2', 7'-dichlorofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; NAC, *N*-acetylcysteine; ERK, extracellular signal-regulated kinase; MEK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase1; caMEK, constitutively active MEK; caAkt, constitutively active Akt; AIF, apoptosis inducing factor; siAIF, small interfering AIF

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Abstract

The present study was undertaken to determine the underlying mechanism of silibinin-induced cell death in human breast cancer cell lines MCF7 and MDA-MB-231. Silibinin-induced cell death was attenuated by antioxidants, *N*-acetylcysteine (NAC) and Trolox, suggesting that the effect of silibinin was dependent on generation of reactive oxygen species (ROS). Western blot analysis showed that silibinin induced down-regulation of ERK and Akt. When cells were transiently transfected with constitutively active MEK (caMEK) and Akt (caAkt), they showed resistance to silibinin-induced cell death. Silibinin decreased the cleavage of Notch-1 mRNA and protein levels. Notch-1-overexpressed cells were resistant to the silibinin-induced cell death. Inhibition of Notch-1 signaling was dependent on ROS generation. Overexpression of Notch-1 prevented silibinin-induced inhibition of ERK and Akt phosphorylation. Silibinin-induced cell death was accompanied by increased cleavage of caspase-3 and was prevented by caspase-3 inhibitor in MDA-MB-231 cells, but not in MCF7 cells. Silibinin induced translocation of apoptosis inducing factor (AIF), which was blocked by NAC, and transfection of caMEK and caAkt. Silibinin-induced cell death was prevented by silencing of AIF expression using small interfering AIF (siAIF) RNA in MCF7 cells, but not in MDA-MB-231 cells. In conclusion, Silibinin induces cell death through an AIF-dependent mechanism in MCF7 cells and a caspase-3-dependent mechanism in MDA-MB-231 cells and ROS generation and Notch-1 signaling act upstream of the ERK and Akt pathway. These data suggest that silibinin may serve as a potential agent for induction of apoptosis in human breast cancer cells.

Introduction

Breast cancer is the most common cancer in Korea women (Jung et al., 2012). Although, effective treatment includes surgery, radiation and chemotherapy, breast cancer frequently shows resistance to these therapies (Trape and Gonzalez-Angulo, 2012). Therefore, there is a great need for the new agents to treat breast cancer is necessary.

Silibinin is a major bioactive component of silymarin, which was isolated from the milk thistle (*Silybum marianum*), and has been used as a traditional medicine for hepatoprotective therapeutics in Europe and Asia (Singh and Agarwal, 2002). Many studies have shown that silibinin has the anticancer activities in various cancer cells such as colon, prostate, skin, glioma, and breast cancer cells and the effect mainly results from targeting the proliferation, inflammation, apoptosis, and cancer cell metabolism (Deep et al., 2011; Jeong et al., 2011; Kim et al., 2011; Kauntz et al., 2012). However, underlying mechanism of changes induced by silibinin has not been clearly elucidated.

ERK and Akt signaling pathways serve to coordinate the cellular response to a variety of extracellular stimuli (Steelman et al., 2011). These pathways are implicated in cancer cell proliferation, invasion, and tumorigenesis and drug resistance in many different cell types (Gan et al., 2010; Huynh et al., 2010; Roy et al., 2010; Cheng et al., 2011). Silibinin induces cancer cell death through the increase of ERK and Akt activities in human glioma (Kim et al., 2009), prostate (Singh et al., 2007), and melanoma cancer cell line (Jiang et al., 2011). However, inhibition of ERK and Akt signaling by silibinin induces human lung (Chen et al., 2005), and renal cancer cell death (Li et al., 2008). These studies suggest that the effect of silibinin on ERK and Akt activation may depend on cell types.

Notch signaling is important for the cell-cell communications, which control multiple processes of cell differentiation during embryonic and adult cell fate decision (Allenspach et al., 2002). Recent studies suggest that Notch signaling plays critical roles in numerous

cancers. When Notch signaling and related transcriptional factors are up-regulated in invasive human cancer, cells become resistant to apoptosis. Previous reports have shown that suppression of Notch-1 expression inhibits human breast cancer cell tumorigenesis and metastasis (Wang et al., 2011; Simmons et al., 2012). Flavonoids such as, genistein and quercetin induces cancer cell death by inhibition of Notch signaling pathway (Wang et al., 2006; Zhou et al., 2012). However, whether Notch-1 signaling pathway is involved in silibinin-induced breast cancer cell death has not been defined.

The aim of the present study is to determine the molecular mechanisms of the silibinin-induced cell death in MCF7 and MDA-MB-231 breast cancer cell lines. Our data demonstrated that silibinin induced cell death through the ROS/Notch-1/ERK/Akt pathway, followed by the nuclear translocation of AIF in MCF7 cells, and the caspase-3 activation in MDA-MB-231 cells.

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Material and Methods

Reagents

Silibinin, 3-[4,5-dimethylthiazol2-yl]-2,5-diphenyltetrazolium bromide (MTT), *N*-acetylcysteine (NAC), Trolox, and propidium iodide were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences (San Jose, CA, USA). 2,7-dichlorofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR, USA). Constitutively active MEK and Akt expressing pCMV plasmids were kindly provided by Dr. Suh (Pohang University, Pohang, Korea). Tween 20, z-VAD-FMK, and Ac-DEVD-CHO were purchased from Calbiochem (San Diego, CA, USA). Plasmid EF.hlCN1.CMV.GFP was purchased from AddGene (Cambridge, MA, USA). All antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). All other chemicals were of the highest commercial grade available.

Cell culture

MCF7 and MDA-MB-231 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by serial passages in 75-cm^2 culture flasks (Costar, Cambridge, MA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Invitrogen, Carsbad, CA, USA) containing 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA) at 37 ℃ in humidified 95% air/5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using 0.02% EDTA-0.05% trypsin solution. The cells were grown on well tissue culture plates and used 1-2 days after plating. Unless otherwise stated, cells were treated with silibinin in serum-free medium.

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Measurement of cell viability

Cell viability was evaluated using a MTT assay. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 2 hr at 37 ℃, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 0.11 ml of dimethyl sulfoxide. A 0.1 ml aliquot of each sample was then transferred translated to 96-well plates and the absorbance of each well was measured at 550 nm with ELISA Reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany). Data were expressed as the percentage of control measured in the absence of silibinin. Unless otherwise stated, the cells were exposed to 30 μM silibinin for 48 hr. Test reagents were added to the medium 30 min before exposure silibinin.

Measurement of apoptosis

Cell apoptosis was estimated using an FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). The annexin V binding assay was performed as described in the manufacturer's manual. The cells were treated with 30 μM silibinin for 24 hr. Cells were harvested with 0.025% trypsin and washed with cold PBS and then resuspended in annexin V binding buffer. The cells were then incubated for 15 min with binding solution containing FITC annexin V and propidium iodide in the dark and used FACSort Becton Dickinson Flow Cytometer (Becton-Dickinson Bioscience, San Jose, CA, USA) and data were analyzed with CELLQuest Software. The percentage of apoptotic cells was calculated as the quadrant statistics of the early and late apoptotic region to the entire cell population (1 x 10^4 cells).

Measurement of reactive oxygen species (ROS)

The intracellular generation of ROS was measured using DCFH-DA. The nonfluorescent ester penetrates into the cells and is hydrolyzed to DCFH by the cellular esterases. The probe (DCFH) is rapidly oxidized to the highly fluorescent compound DCF in the presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Cells cultured in 24-well plate were preincubated in the culture medium with 30 μM DCFH-DA for 1 hr at 37 ℃. The cells were then exposed to 30 μM silibinin for various periods. Changes in DCF fluorescence was assayed using FACSort Becton Dickinson Flow Cytometer (Becton-Dickinson Bioscience, San Jose, CA, USA) and data were analyzed with CELLQuest Software.

Western blot analysis

Cells were harvested at various times after silibinin treatment and disrupted in lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4). Cell debris was removed by centrifugation at 10,000g for 10 min at 4 ℃. The resulting supernatants were resolved on a 10% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30 min and incubated with different primary antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. The signal was visualized using an enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Transfection

To modulate the activity of ERK and Akt, a transient transfection of constitutively active forms of MEK1, an upstream kinase of ERK, and Akt was performed. Cells were seeded in six well plates and grown to 70% confluence. Each 2 μg cDNA was transiently transfected using Lipofectamine (Invitrogen, Carsbad, California, USA) according to manufacturer's guidelines. After 4 hr incubation at 37 ℃, cells were maintained in normal culture media for 24 hr. To overexpress intracellular Notch-1, we transferred EF.hlCN1.CMV.GFP (Addgene, Cambridge, MA, UK) according to the manufacturer's instructions.

Measurement of AIF nuclear translocation

Cells were harvested at various times after silibinin treatment and washed twice with PBS. To measure AIF nuclear translocation, the cells were incubated with cytosol lysis buffer (10 mM HEPES, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.05% digitonin, and 1 mM phenylmethylsulfonyl fluoride) at 4℃ for 30 min with vortexing each 10 min, then centrifuged at 10,000 g for 10 min at 4℃. The pellet was incubated in the nuclear lysis buffer (350 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4, and protease inhibitors) at 4℃ for 30 min with vortexing each 10 min, then centrifuged at 10,000 g for 10 min at 4 ℃. Western blot analysis of AIF nuclear fractions obtained was performed as described above.

Immunocytochemistry

Cells were cultured on cover glasses and treated with silibinin. Cells were fixed with 4% paraformaldehyde for 15 min and blocked with 1% BAS in PBS for 1 hr. Remove the

blocking solution and incubated overnight at 4 ℃ with rabbit anti-AIF (1:500 dilution). Cells were washed and incubated with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA) at room temperature for 1 hr. After washing, counterstaining was carried out with Hoechst 33258 for 15 min. Cells were viewed under a fluorescent microscope (Leica, Wetzlar, Germany).

RNA interference

Silencing of AIF expression was achieved by the small interfering (siRNA) technique. We used The BLOCK-iT^{TM} Pol miR RNAi Expression Vector Kits (Invitrogen, Carlsbad, CA, USA) to facilitate the expression of microRNA (miRNA). miRNA sequences for AIF were designed using online software (BLOCK-iT RNAi Designer from Invitrogen). The target sequence was 5'-ATGCAGAACTCCAAGCACGTT-3'. This single-stranded oligonucleotide generated a double-stranded oligonucleotide, which instructed into pcDNA^{TM} 6.2-GW/EmGFP-miR vector. Cells were transiently transfected with these plasmids using Lipofectamine (Invitrogen).

Reverse-transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from MCF7 and MDA-MB-231 cell treated or untreated with silibinin. Reverse transcription was according conventional protocols. cDNA was synthesized in a reaction mixture containing oligo dT primer, dNTP mixture, RNase inhibitor, reverse transcriptase, RT buffer and total cellular RNA. cDNA amplification was performed using the primer sets. Notch-1 5'- GCAACAGCTCCTTCCACTTC-3, 5'-GCCTCAGACACTTTGAAGCC-3' GAPDH 5'-TCCATGACAACTTTGGTATCG-3', 5'-TGTAGCCAAATTCGTTGTCA-3'. The primer sequences were determined using

established GenBank sequences. We performed PCT for 30 cycles and the products were electrophoresed on 1.5% agarose gels, and stained with ethidium bromide and visualized (EtBr) with ultraviolet illumination. The optical density of the bands was quantified using an image analysis system.

Statistical analysis

The data are expressed as means ± SEM and the difference between two groups was evaluated using Student's t-test. Multiple group comparison was done using one-way analysis of variance followed by the Tukey post hoc test. A probability level of 0.05 was used to establish significance.

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Results

Silibinin induces inhibition of cell viability

To investigate the effect of silibinin on cell viability in MCF7 and MDA-MB-231 cells, cells were exposed to various concentrations of silibinin for 24 and 48 hr and cell viability was measured by MTT assay. Silibinin significantly decreased cell viability in both cell lines in a dose-dependent manner (Figure 1A).

To evaluate whether silibinin-induced decrease of cell viability is attributed to the induction of apoptosis, cells were treated with 30 μM silibinin, and the apoptosis was evaluated using an annexin V binding assay. As shown in Figure 1B, 34.2% of the MCF7 cells and 22.1% of the MDA-MB-231 cells were the annexin V-positive. These results suggest that silibinin decreases cell viability through the induction of apoptosis.

Silibinin induces ROS generation

Many studies have shown that oxidative stress has anti-tumor effects in cancer cells (Dewaele et al., 2010; Sesti et al., 2012). To examine the effect of silibinin on ROS generation, cells were exposed to silibinin and the changes in DCF fluorescence was measured with the flow cytometry. Silibinin increased ROS generation in a time-dependent manner, and it was blocked by an antioxidant, NAC (Figure 2A and 2B). To determine whether ROS generation is involved in the silibinin-induced cell death, we tested the effect of antioxidants on the cell viability. The silibinin-induced cell death was significantly prevented

by antioxidants NAC and Trolox (Figure 2C). These results suggest that the silibinin-induced cell death is associated with ROS generation.

Silibinin induces down-regulation of cell survival kinases

ERK and Akt play critical roles in cell proliferation, survival, and differentiation. It is well known that ROS generation is closely related to ERK and Akt signaling. Therefore, we examined whether the silibinin-induced cell death is associated with these kinases. Cells were exposed to 30 μM silibinin for various duration and the activities of these kinases were estimated by measuring their phosphorylation by Western blot analysis. Silibinin induced down-regulation of phospho-ERK and phospho-Akt in a time dependent manner (Figure 3A).

To confirm that the decrease of phosphorylation in these kinases is involved in silibinin-induced cell death, the effect of silibinin on cell death was evaluated in cells transfected with caMEK and caAkt. The transfection efficiency was evaluated over than 70% by immunofluorescence (data not shown) and phospho-ERK and phospho-Akt expression were determined by Western blot analysis. Expression of both kinases was increased compared with cells transfected with the empty vector (EV) (Figure 3B). The cells were transfected with caMEK and caAkt showed increased resistance to silibinin-induced cell death (Figure 3C).

To investigate whether the silibinin-induced down-regulation of phospho-ERK and phospho-Akt was attributed to ROS generation, cells were exposed to silibinin in the presence or absence of the NAC. The decrease of phospho-ERK and phospho-Akt by silibinin was prevented by NAC in both cells (Figure 3D). These data suggest that the down-regulation of phospho-ERK and phospho-Akt are dependent on the ROS generation and play a critical role in silibinin-induced cell death.

Silibinin inhibits Notch-1 signaling in breast cancer cells

We investigated whether silibinin-induced cell death is associated with Notch signaling pathway. The expression of Notch-1 in silibinin-treated cells was assessed by RT-PCR and Western blot analysis. As shown in Figure 4A, silibinin induced down-regulation of Notch-1 mRNA expression. Likewise, it decreased Notch-1 protein synthesis time-dependently in both cancer cells (Figure 4B).

To evaluate whether the down-regulation of Notch-1 is involved in the silibinin-induced cell death, cell viability was measured in cells transfected with EF.hlCN1.CMV.GFP. The effective transfection was confirmed by observing the fluorescence of GFP, which was inserted into the CMV promoter, under a fluorescence microscope (data not show). Both breast cancer cells overexpressing Notch-1 exhibited increased resistance to silibinin-induced cell death (Figure 4C), indicating that the silibinin-induced cell death is associated with down-regulation of Notch-1 signaling pathway in both MCF7 and MDA-MB-231 cells.

To determine whether silibinin inhibits Notch-1 signaling through the increase of ROS generation, cells were pretreated with NAC before the exposure to silibinin and the changes in Notch-1 expression were determined by Western blot analysis. As shown in Figure 4D, silibinin-induced inhibition of Notch-1 signaling was prevented by the treatment of NAC.

To determine whether silibinin-induced inhibition of Notch-1 signaling is responsible for down-regulation of ERK and Akt, the cells were transfected with EF.hlCN1.CMV.GFP, and the changes in phospho-ERK and phospho-Akt were determined by Western blot analysis. Phospho-ERK and phospho-Akt were increased by the EF.hlCN1.CMV.GFP transfection in both breast cancer cells and transfected cells were recovered the silibinin-induced down-regulation of phospho-ERK and phospho-Akt (Fig. 4E). Results suggest that silibinin-induced inhibition of phospho-ERK and phospho-Akt was due to down-regulation of Notch-1 signaling in both breast cancer cells.

Silibinin induces caspase-dependent cell death in MDA-MB-231 cell

Caspases are essential in the apoptosis process. Among them, caspase-3 plays a central role in the execution phase of apoptosis. To determine whether silibinin induces apoptosis through a caspase-dependent pathway, the effect of silibinin on the activation of caspase-3 was examined. Silibinin induced the caspase-3 cleavage in a time-dependent manner in MDA-MB-231 cells. The caspase-3 was not detected in MCF7 cells (Figure 5A).

To evaluate if caspase is involved in the silibinin-induced cell death, the effect of caspase inhibitors on the cell viability was examined. Cells were exposed to silibinin in the presence or absence of the caspase-3 specific inhibitor Ac-DEVD-CHO and the general caspase inhibitor z-VAD-FMK. Silibinin-induced cell death was prevented by these inhibitors in MDA-MB-231 cells. In MCF7 cells, the cell death was prevented by the general caspase inhibitor, whereas it was not affected by the caspase-3 specific inhibitor (Figure 5B).

To determine whether the effect of silibinin on the activation of caspase-3 is dependent on down-regulation of Notch-1 signaling, ERK, and Akt in MDA-MB-231 cells, cells were transfected with EF.hlCN1.CMV.GFP, caMEK, and caAkt and exposed to silibinin and the activation of caspase-3 was determined. The silibinin-induced caspase-3 activation was prevented by the transfection of EF.hlCN1.CMV.GFP, caMEK, and caAkt (Figure 5C). These data suggest that silibinin cause caspase-3 activation through down-regulation of Notch-1, ERK, and Akt in MDA-MB-231 cell.

Silibinin induces MCF7 cell death through AIF nuclear translocation

To determine whether nuclear translocation of AIF is involved in the silibinin-induced cell death, the effect of silibinin on the AIF nuclear translocation was measured by Western blot analysis. Silibinin increased the translocation of AIF into the nucleus in a time-dependent

manner in MCF7 cells, whereas only a small amount of AIF was translocated into the nucleus in MDA-MB-231 cells as compared with MCF7 cells (Figure 6A). The translocation induced by silibinin was confirmed by immunocytochemistry analysis (Figure 6B).

To determine whether the AIF nuclear translocation was associated with ROS generation and phosphorylation of ERK and Akt, cells were pretreated NAC and transfected with caMEK and caAkt before exposure to silibinin. The nuclear translocation of AIF was blocked by the treatment of NAC, and the transfection of caMEK and caAkt (Figure 6B).

To evaluate the role of AIF in the silibinin-induced cell death, AIF mRNA expression was down-regulated by transfection with small interfering AIF RNA (siAIF). Transfection with siRNA effectively prevented the silibinin-induced cell death in the MCF7 cells, but not in MDA-MB-231 cells (Figure 6C).

To determine whether silibinin-induced nuclear translocation of AIF was attributed to down-regulation of Notch-1 signaling, phospho-ERK, and phospho-Akt in MCF7 cells, cells transfected with EF.hlCN1.CMV.GFP, caMEK, and caAkt were exposed to silibinin. Silibinin-induced nuclear translocation of AIF was prevented by EF.hlCN1.CMV.GFP, caMEK, and caAkt (Figure 6D), suggesting that silibinin induces AIF nuclear translocation through down-regulation of Notch-1, ERK, and Akt.

Discussion

Silibinin has been shown to have anti-tumor and anti-inflammation activity in various cancer models. In our previous study, silibinin inhibited glioma cell proliferation *in vitro* and the tumor growth *in vivo* (Kim et al., 2009). Silibinin is a dietary ingredient which we can take easily from foods such as fruits, vegetables, grains, and tea. It has a wide range of pharmacological effects such as inhibition of DNA synthesis, cell proliferation, cell cycle progression, and apoptosis in various cancer cell lines including breast cancer cells (Cheung et al., 2010). However, molecular mechanism of silibinin-induced cell death has not been clearly defined.

ROS is a natural byproduct of the normal metabolism of oxygen and has important roles in cell signaling and homeostasis (Sena and Chandel, 2012). However, the increased ROS level by environmental stress induces DNA damage and thus enhance the tumor development (Alfadda and Sallam, 2012). It is well known that flavonoids exhibit anti-oxidant actions (Agati et al., 2012). However, it may also behave as a pro-oxidant which is responsible for cell death in some cancer cells (Chen et al., 2013). In the present study, silibinin induced ROS generations and the anti-oxidant NAC prevented the silibinin-induced cell death (Figure 2). These results indicate that the silibinin-induced cell death is associated with ROS generation. These results are consistent with those reported in MCF7 cells (Wang et al., 2010). In the present study, we did not define the mechanism by which silibinin induces ROS generation. However, in our previous study, silibinin increased ROS generation through an intracellular Ca^{2+}-dependent mechanism in glioma cells (Kim et al., 2009). Ca^{2+} influx induces accumulation of Ca^{2+} in mitochondria (Orrenius et al., 2003) and leading to an

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increased production of ROS by the respiratory chain (Thor et al., 1984). Silibinin may cause an increase in ROS generation through Ca^{2+}-dependent mechanism in breast cancer cells.

Thus, we can suppose that silibinin has been shown to induce cell death through down-regulation of ERK1/2 and Akt activity in various cancer cells as well (Li et al., 2008; Kim et al., 2010; Wang et al., 2012). Therefore, ERK and Akt expression level may be critical in silibinin-induced cell death. Indeed, the present study showed that silibinin caused inhibition of ERK and Akt phosphorylation time-dependently (Figure 3A). The silibinin-induced cell death was blocked by the transfection with caMEK and caAkt (Figure 3C). Similar results have been reported from hepatocellular carcinoma (Cui et al., 2009) and human fibrosarcoma (Duan et al., 2011).

ROS activates multiple signaling pathways such as ERK and Akt, leading to cell survival or cell death (Martindale and Holbrook, 2002). On the other hand, ROS can trigger down-regulation of the ERK and Akt pathway (Jeong et al., 2009). In the present study, silibinin-induced inhibition of ERK and Akt was blocked by the antioxidant NAC (Figure 3D), suggesting that silibinin induces down-regulation of ERK and Akt through a ROS-dependent mechanism.

Our previous studies have shown that down-regulation of ERK and Akt is involved in flavonoid-induced apoptosis in various cancer cells (Jeong et al., 2009; Cho et al., 2013). However, underlying mechanism of such a down-regulation of kinases remains unclear. Notch signaling promotes cell growths migration, invasion, and apoptosis in various cancer cells. It has been reported that cell survival kinases such as ERK and Akt are involved in Notch signaling pathway (Miyamoto et al., 2003; Dang, 2012). Therefore, Notch signaling may be involved in the silibinin-induced down-regulation of ERK and Akt. To test the possibility, we examined role of Notch signaling in the silibinin-induced cell death. In the present study, silibinin decreased Notch-1 mRNA and protein level. In addition, silibinin-

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induced cell death was prevented by overexpression of Notch-1 in both cell lines (Figure 4A, B and C), indicating involvement of Notch1 signaling in the silibinin-induced cell death. The cleavages of Notch by a γ-secretase releases Notch intracellular domain (NICD) into the cytoplasm where it activates the ERK pathway. Activated ERK also up-regulates Notch signaling pathway, resulting the positive feedback mechanism (Takeshita et al., 2007; Kang et al., 2011). Released NICD into the cytoplasm can also inhibit Akt signaling by PTEN (Cornejo et al., 2011). Therefore, the silibinin-induced down-regulation of ERK and Akt may be attributed to inhibition of Notch signaling. Indeed, in the present study, the silibinin-induced inhibition of phospho-ERK and phospho-Akt was prevented by transfection with EF.hlCN1.CMV.GFP (Figure 4E).

The correlation between ROS generation and Notch1 signaling is very intricate and follows many steps. Notch-1 suppresses ROS generation (Kim et al., 2007) and reversely ROS generation could regulate Notch-1 signaling (Coant et al., 2010). In the present study, silibinin increased ROS generation (Figure 2B) and inhibited Notch-1 protein expression (Figure 4B). Furthermore, the silibinin-induced down-regulation of Notch signaling and cell death were prevented by NAC (Figure 4D). These results suggest that silibinin-induced down-regulation of ERK and Akt was due to the inhibition of Notch-1 signaling pathway which is dependent on ROS generation.

It has been well known that cancer cell apoptosis is associated with the caspase family, especially, caspase-3 plays a key role during the execution phase in various forms of apoptosis (Fadeel et al., 2008; Fiandalo and Kyprianou, 2012). Curiously, MCF7 breast cancer cells do not express caspase-3 and caspase-3 is not essential during the apoptosis (Mc Gee et al., 2002). In the present study, silibinin induced MDA-MB-231 breast cancer cell death through the caspase-3 cleavage as demonstrated by Western blot analysis. However, caspase-3 expression was not detected in MCF7 cells (Figure 5A). Selective caspase-3

inhibitor Ac-DEVD-CHO and the general caspase inhibitor z-VAD-FMK prevented silibinin-induced MDA-MB-231 cell apoptosis. Although silibinin-induced cell death was prevented by z-VAD-FMK in MCF7 cells, the cell death was not affected by Ac-DEVD-CHO (Figure 5B). It indicates that other caspase mechanisms are involved in the silibinin-induced MCF7 cells death. These results are similar to reports that silibinin induces apoptosis through a mechanism independent of caspase-8 activation in MCF7 and T47D breast cancer cell (Tiwari et al., 2011).

AIF is released from mitochondria, and translocate from the cytosol to the nucleus. In nucleus, it induces oligonucleosomal DNA fragmentation, a hallmark of caspase-independent apoptosis (Chou et al., 2009; Vittar et al., 2010; Liu and Chang, 2011). Our data show that silibinin increased nuclear translocation of AIF in the both breast cancer cells as demonstrated by the Western blot analysis and immunocytochemistry (Figure 6A and B). AIF knockdown prevented the cell death in MCF7 cells, but not in MDA-MB-231 cells (Figure 6C). Although AIF nuclear translocation was induced by silibinin treatment in MDA-MB-231 cells, the quantity of translocation may be not sufficient to induce apoptosis or AIF does not interact with molecules of apoptosis induction in the nucleus. Taken together, these data indicate that silibinin induces MCF7 cell apoptosis through the nuclear translocation of AIF. Although silibinin induced nuclear translocation of AIF in MDA-MB-231 cell, it did not play an important role in the silibinin-induced cell death.

In conclusion, silibinin induces ROS-dependent down-regulation of Notch/ERK/Akt signaling, which leads to the cell death through a caspase-3-dependent mechanism in MDA-MB-231 cells and an AIF-dependent mechanism in MCF7 cells. The data suggest that silibinin induces cell death through different mechanisms between MCF7 and MDA-MB-231 cells (Figure 7).

Authorship Contributions

Participated in research design: Thae Hyun Kim, Jae Suk Woo, Yong Keun Kim,

Ki Hyung Kim

Conducted experiments: Thae Hyun Kim

Contributed new reagents or analytic tools:

Performed data analysis: Thae Hyun Kim, Yong Keun Kim

Wrote or contributed to the writing of the manuscript: Thae Hyun Kim, Jae Suk Woo,

Yong Keun Kim, Ki Hyung Kim

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Footnotes

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Figure legends

Figure 1. Effects of silibinin on cell viability and apoptosis. Cells were treated with various concentration of (5-50 μM) silibinin for 24 and 48 hr. Cell viability was determined by a MTT assay (A) and cell apoptosis was estimated by FITC-conjugated Annexin V binding assay (B). Data in (A) are mean ± SEM of three independent experiments performed in duplicate. *$p<0.05$ compared with control without silibinin. C, control.

Figure 2. Role of ROS generation in silibinin-induced cell death. Cells were loaded with DCFH-DA for 1 hr and treated with 30 μM silibinin for 12 hr (A) in the presence or absence of 2 mM NAC and indicated various times (B). DCF fluorescence intensity was measured by flow cytomerty. (C) Cells were 30 μM silibinin in the presence or absence of 2 mM NAC and 1mM Trolox for 48 hr. Cell viability was determined by MTT assay. Data in (B and C) are mean ± SEM of three independent experiments performed in duplicate. *$p<0.05$ compared with silibinin alone. C, control.

Figure 3. Role of cell survival kinases in silibinin-induced cell death. Cells were exposed to 30 μM silibinin for various times. (A) Expression of phospho-ERK (p-ERK), total-ERK (t-ERK) phospho-Akt (p-Akt), and total-Akt (t-Akt) were evaluated using the specific antibodies. C, control. (B) Effect of modulation of ERK and Akt signaling on silibinin-induced cell death. Cells were transfected with empty vector (EV), and constitutively active forms of MEK (caMEK) and Akt (caAkt). Cells were exposed to 30 μM silibinin for 48 hr. Expression of phospho-ERK (p-ERK), total-ERK (t-ERK) phospho-Akt (p-Akt), and total-Akt (t-Akt) was estimated by Western blot analysis and quantitative densitometry data. Data are mean ± SEM of three independent experiments. *$p<0.05$ compared with EV with

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silibinin. (C) Cell viability was estimated by MTT assay. Data are mean ± SEM of three independent experiments performed in duplicate. *p<0.05 compared with EV with silibinin. (D) Relationship between cell survival kinases and ROS generation on silibinin-induced cell death. Cells were treated with 30 μM silibinin in the presence or absence of 2 mM NAC for 48 hr. Expression of phospho-ERK (p-ERK), total-ERK (t-ERK) phospho-Akt (p-Akt), and total-Akt (t-Akt) was estimated by Western blot analysis and quantitative densitometry data. Data are mean ± SEM of three independent experiments. *p<0.05 compared with silibinin alone. C, control.

Figure 4. Role of Notch-1 signaling pathway in silibinin-induced cell death. (A) Cells were incubated for 48 hr in the presence (Sili) or absence (Con) of 30 μM silibinin and Notch-1 mRNA level was detected by RT-PCR in breast cancer cells. GAPDH was used as a loading control. (B) The cleavage (C.N) and total (T.N) form of Notch-1 protein level was measured by Western blot analysis for various times. C, control. (C) Effect of Notch-1 in silibinin-induced cell death. Cells were transfected with empty vector (EV) and EF.hlCN1.CMV.GFP (Notch1) and the cell viability was measurement by MTT assay. Data are mean ± SEM of three independent experiments performed in duplicate. *p<0.05 compared with EV with silibinin. (D) Relationship between Notch-1 signaling and ROS generation in cells with silibinin. Cells were treated with 30 μM silibinin in the presence or absence of 2 mM NAC for 48 hr. Expression of cleavage (C.N) and total (T.N) form of Notch-1 protein level was measured by Western blot analysis and quantitative densitometry data. Data are mean ± SEM of three independent experiments. *p<0.05 compared with silibinin alone. C, control. (E) The association between Notch-1 signaling pathway and cell survival kinases. Cells were transfected with empty vector (EV) and EF.hlCN1.CMV.GFP (Notch1) and changes in phospho-ERK (p-ERK), total-ERK (t-ERK), phospho-Akt (p-Akt), and total-Akt (t-Akt) were

evaluated by Western blot analysis and quantitative densitometry data. Data are mean ± SEM of three independent experiments. *p<0.05 compared with EV with silibinin.

Figure 5. Role of caspase-3 in silibinin-induced cell death. (A) Cells were exposed to 30 μM silibinin for various times and the uncleaved caspase-3 forms (Cas-3) and cleaved caspase-3 forms (Cl. Cas-3) were measured by Western blot analysis using the specific antibody. GAPDH was used as a loading control. C, control. (B) Effect of caspase inhibitor on silibinin-induced cell death. Cells were exposed to 30 μM silibinin for 48 hr in presence or absence of each 10 μM Ac-DEVD-CHO (CHO) and z-VAD-FMK (FMK). Cell viability was estimated by MTT assay. Data are mean ± SEM of three independent experiments performed in duplicate. *p<0.05 compared with silibinin alone. C, controls. (C) Relationship between caspase-3 cleavage and upstream signaling such as Notch-1, ERK, and Akt on silibinin-induced cell death. Cells were transfected with empty vector (EV), EF.hlCN1.CMV.GFP (Notch1), caMEK, and caAkt. Cells were exposed to 30 μM silibinin for 48 hr. The uncleaved caspase-3 forms (Cas-3) and cleaved caspase-3 forms (Cl. Cas-3) were estimated by Western blot analysis and quantitative densitometry data. Data are mean ± SEM of three independent experiments. *p<0.05 compared with EV with silibinin. GAPDH was used as a loading control.

Figure 6. Role of nuclear translocation of AIF (Nu-AIF) in silibinin-induced cell death. (A) Cells were exposed to 30 μM silibinin for various times and the expression levels of cytosol AIF (Cyto-AIF) and nuclear AIF (Nu-AIF) were determined by Western blot analysis using the specific antibody. β-Actin and Histon H1 were used as a loading control. C, control. (B) Nuclear localization of AIF was estimated by immunocytochemistry. Cells were exposed to 30 μM silibinin for 48 hr in the presence or absence 2 mM NAC or in cells transfected with

caMEK and caAkt. Nuclei were counterstained with hoechst33258. Green and blue fluorescence indicated AIF and nuclei, respectively. (C) Effect of nuclear translocation of AIF in silibinin-induced cell death. Cells transfected with siAIF were treated with 30 μM silibinin for 48 hr and cell viability was determined by MTT assay. Data are mean ± SEM of three independent experiments performed in duplicate. *$p<0.05$ compared with siCon. (D) Effect of Notch signaling, ERK, and Akt pathway on silibinin-induced AIF nuclear translocation. Cells were transfected with EV, EF.hlCN1.CMV.GFP (Notch1), caMEK, and caAkt. Cells were exposed to 30 μM silibinin for 48 hr. The expression levels of Nuclear AIF (Nu-AIF) were estimated by Western blot analysis and quantitative densitometry data. Data are mean ± SEM of three independent experiments. *$p<0.05$ compared with EV with silibinin. Histon H1 was used as a loading control.

Figure 7. Proposed model of silibinin-induced cell death in human breast cancer cell lines. Silibinin increased ROS generation which inhibited Notch-1 signaling pathway. These signal transductions lead to down-regulation of phosphorylation of ERK and Akt. Down-regulation of ERK and Akt induced cell death through a caspase-3-dependent mechanism in MDA-MB-231 cell and an AIF-dependent mechanism in MCF7 cell.

Fig-1

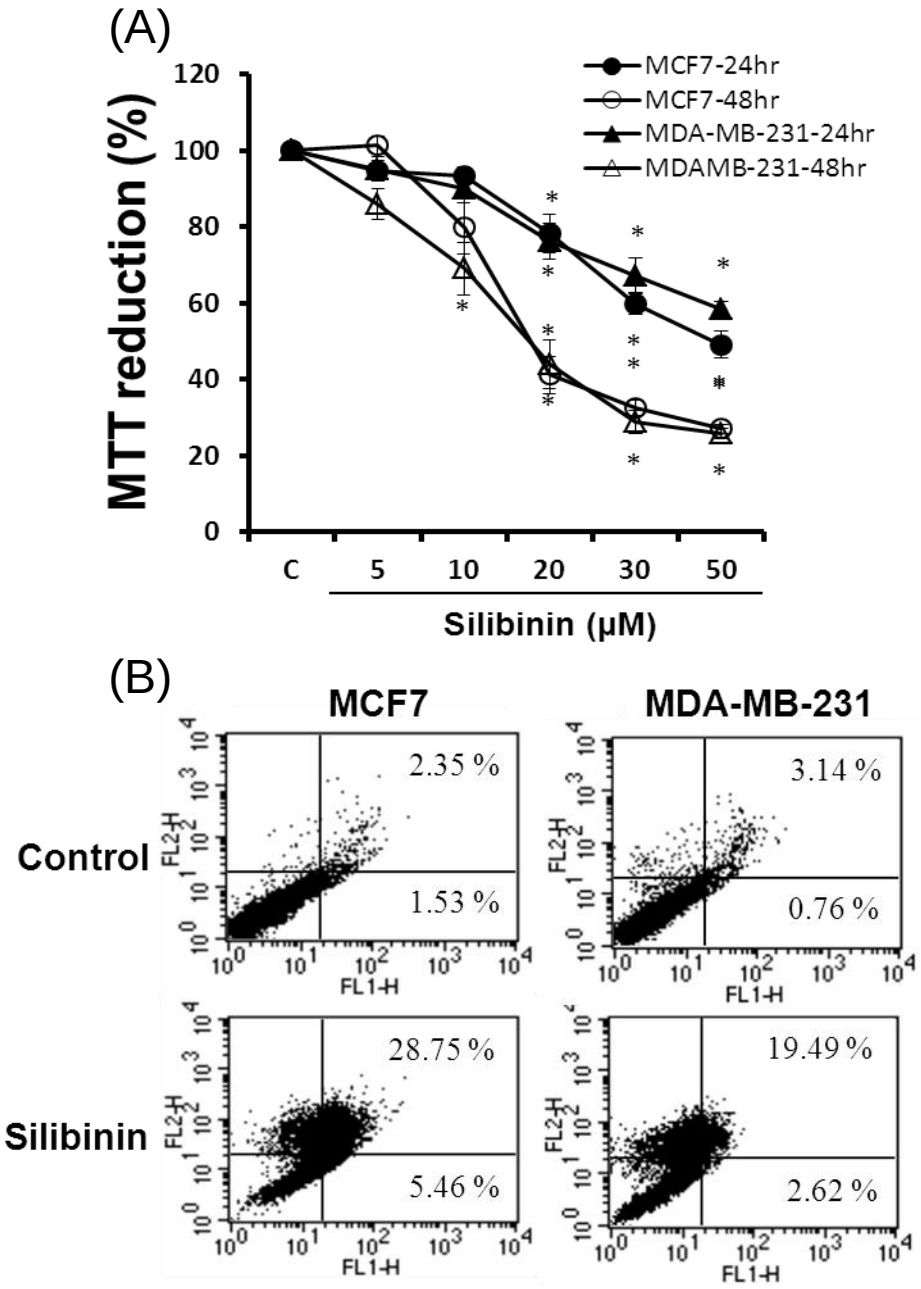

(A)
MTT reduction (%)
120
100
80
60
40
20
0
MCF7-24hr
MCF7-48hr
MDA-MB-231-24hr
MDAMB-231-48hr
C
5
10
20
30
50
Silibinin (μM)
(B)
MCF7
MDA-MB-231
Control
Silibinin
2.35 %
1.53 %
3.14 %
0.76 %
28.75 %
5.46 %
19.49 %
2.62 %
FL2-H
FL1-H

Fig-2

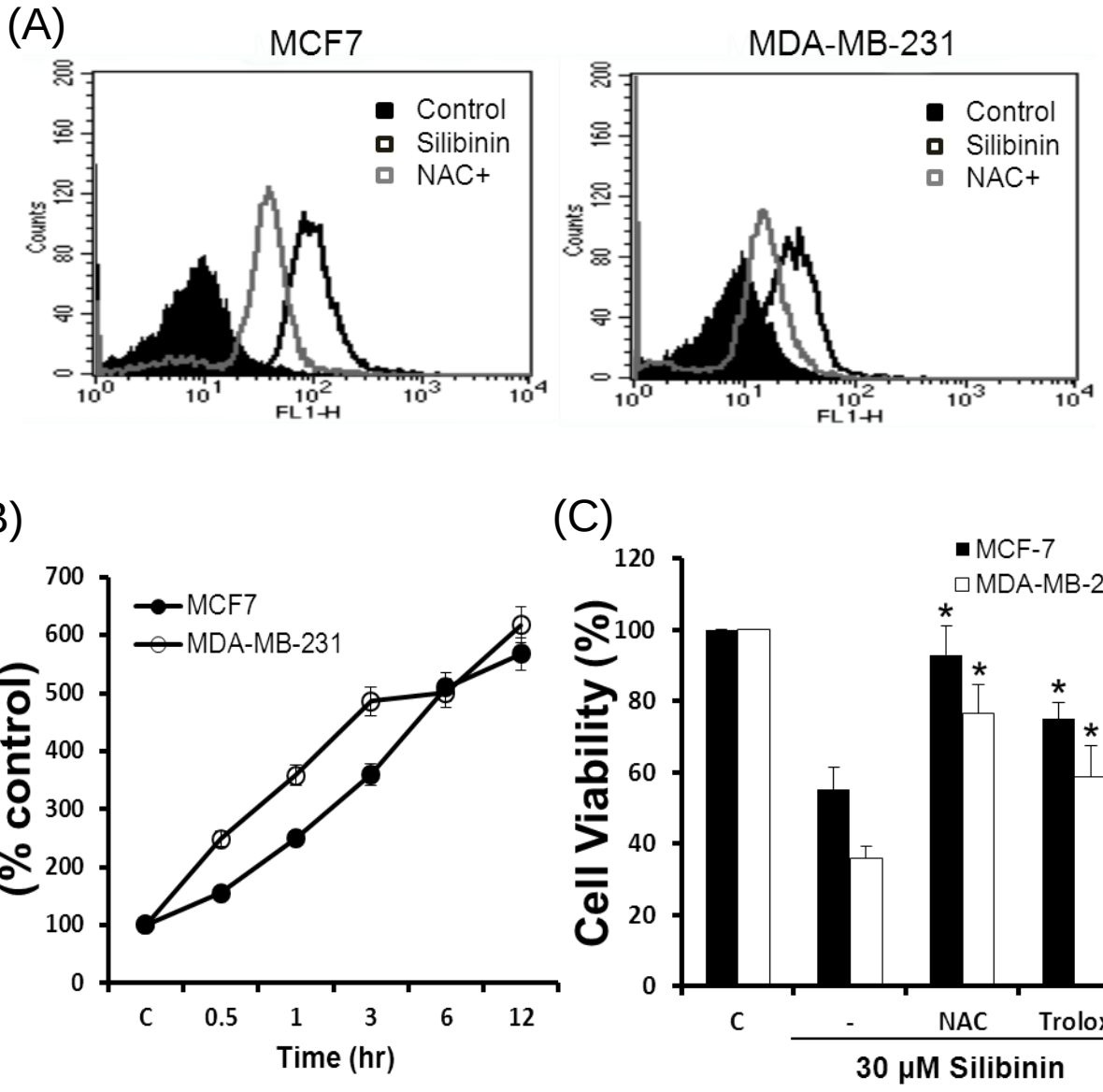

(A)
MCF7
MDA-MB-231
Control
Silibinin
NAC+
Counts
FL1-H
(B)
DCF fluorescence (% control)
MCF7
MDA-MB-231
Time (hr)
C
0.5
1
3
6
12
(C)
Cell Viability (%)
MCF-7
MDA-MB-231
C
-
NAC
Trolox
30 μM Silibinin

Fig-3

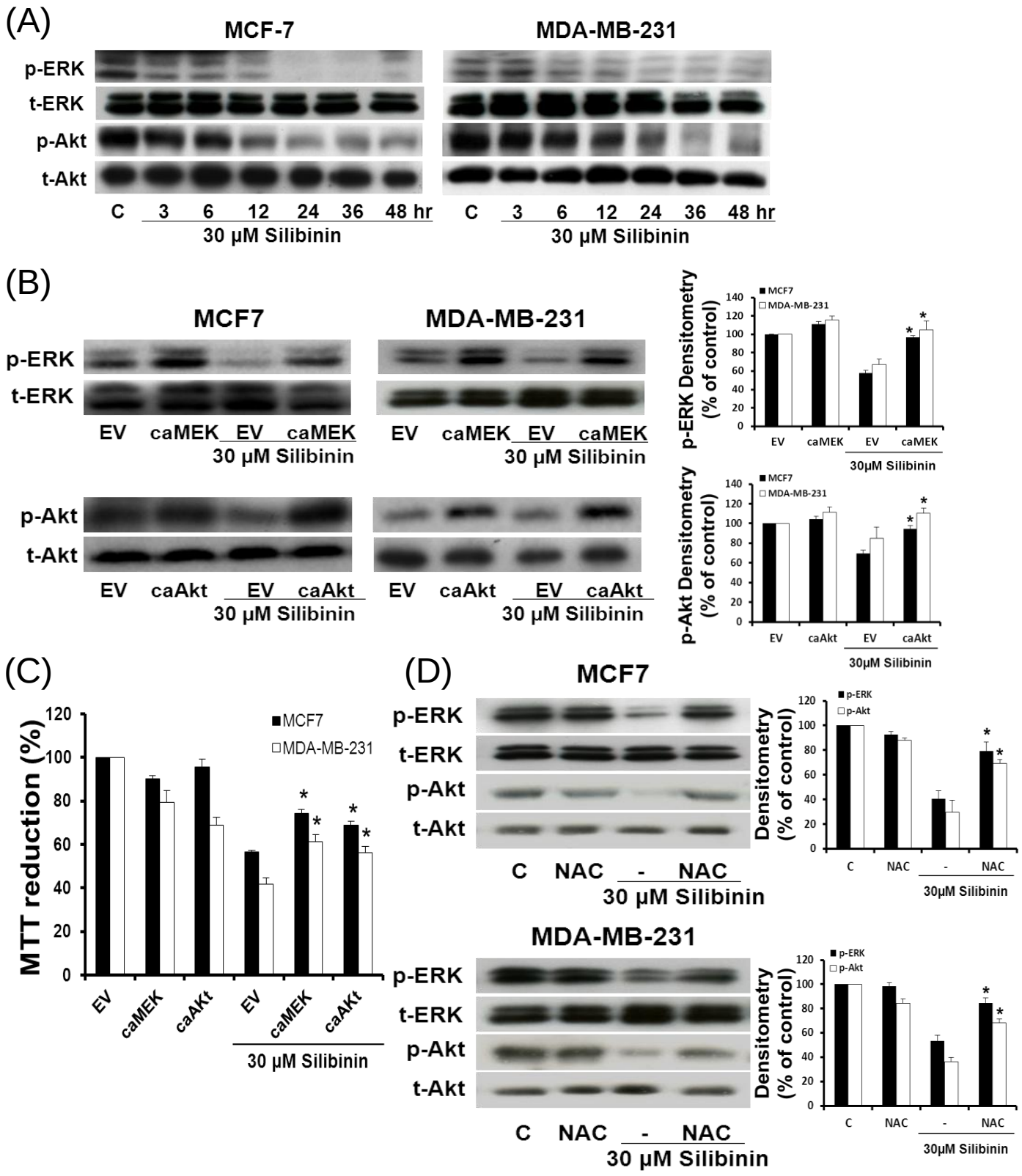

(A)
MCF-7
MDA-MB-231
p-ERK
t-ERK
p-Akt
t-Akt
C 3 6 12 24 36 48 hr
30 μM Silibinin
(B)
MCF7
MDA-MB-231
EV caMEK EV caMEK
EV caAkt EV caAkt
30 μM Silibinin
p-ERK Densitometry (% of control)
p-Akt Densitometry (% of control)
30μM Silibinin
(C)
MTT reduction (%)
MCF7
MDA-MB-231
EV caMEK caAkt EV caMEK caAkt
30 μM Silibinin
(D)
MCF7
MDA-MB-231
C NAC - NAC
30 μM Silibinin
Densitometry (% of control)
p-ERK
p-Akt
30μM Silibinin

Fig-4

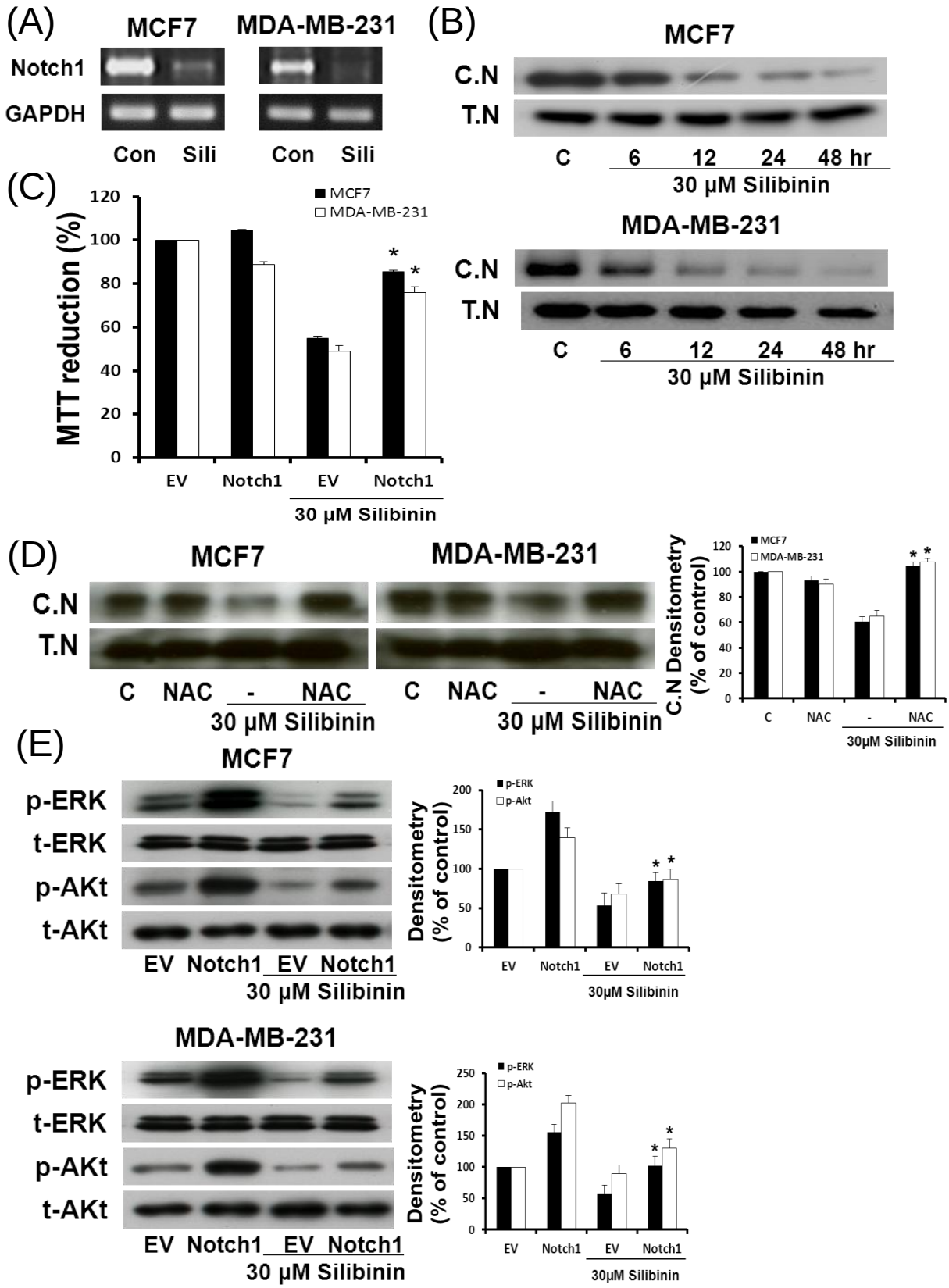

(A)
MCF7
MDA-MB-231
Notch1
GAPDH
Con
Sili
Con
Sili
(B)
MCF7
C.N
T.N
C
6
12
24
48 hr
30 μM Silibinin
MDA-MB-231
C.N
T.N
C
6
12
24
48 hr
30 μM Silibinin
(C)
MTT reduction (%)
MCF7
MDA-MB-231
EV
Notch1
EV
Notch1
30 μM Silibinin
(D)
MCF7
MDA-MB-231
C.N
T.N
C
NAC
-
NAC
30 μM Silibinin
C.N Densitometry (% of control)
MCF7
MDA-MB-231
C
NAC
-
NAC
30μM Silibinin
(E)
MCF7
p-ERK
t-ERK
p-AKt
t-AKt
EV
Notch1
EV
Notch1
30 μM Silibinin
Densitometry (% of control)
p-ERK
p-Akt
EV
Notch1
EV
Notch1
30μM Silibinin
MDA-MB-231
p-ERK
t-ERK
p-AKt
t-AKt
EV
Notch1
EV
Notch1
30 μM Silibinin
Densitometry (% of control)
p-ERK
p-Akt
EV
Notch1
EV
Notch1
30μM Silibinin

Fig-5

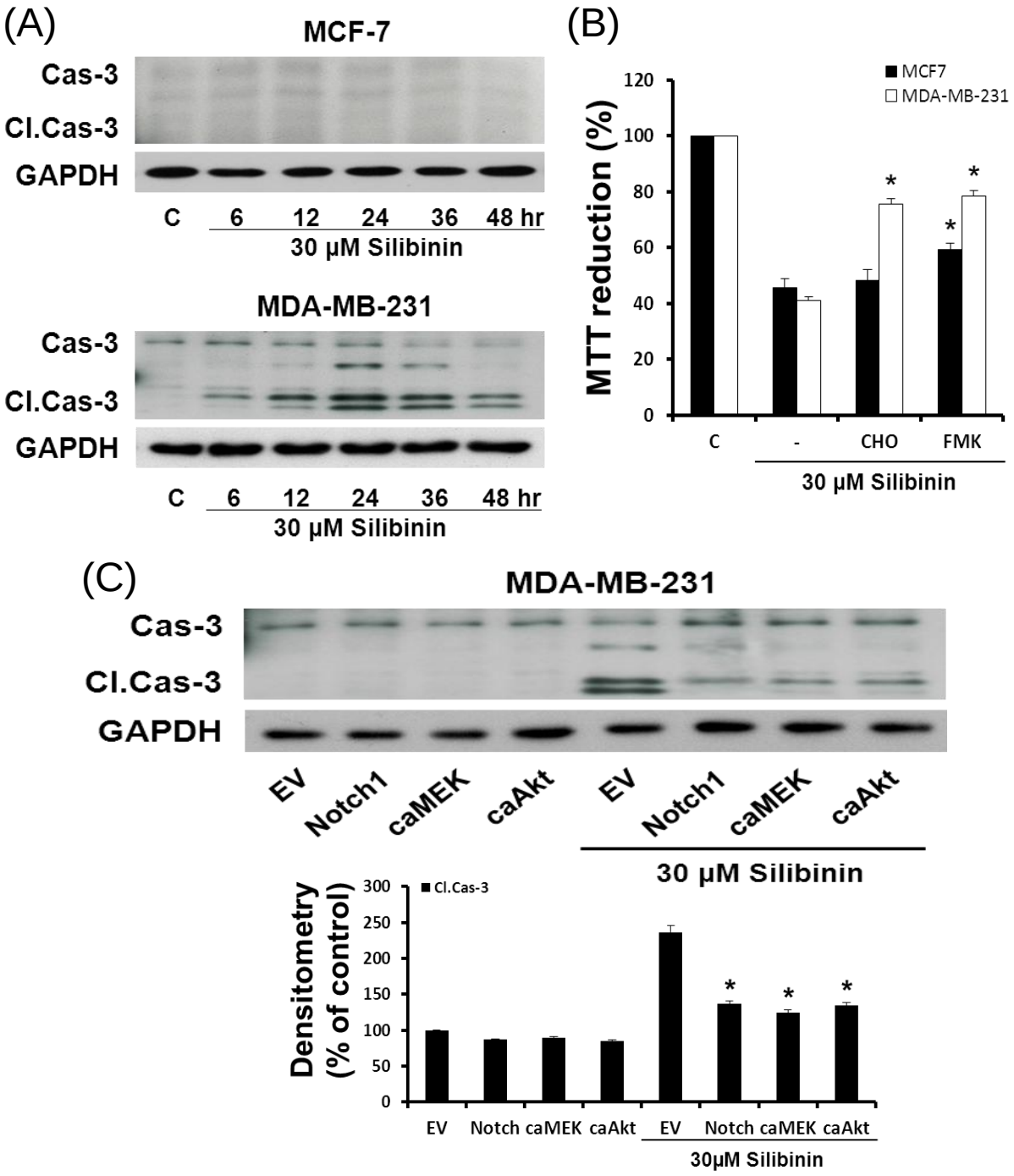
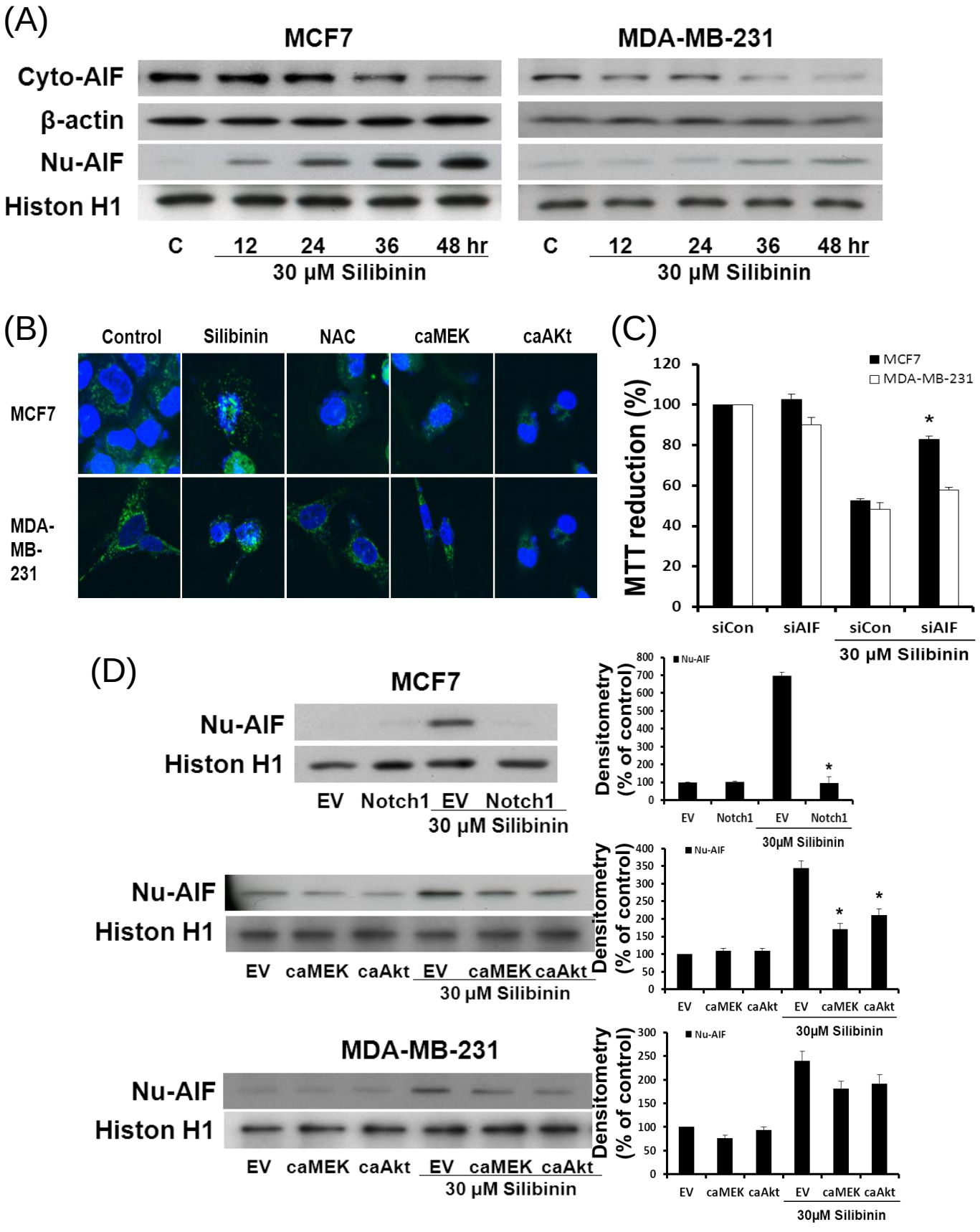

(A)
MCF-7
Cas-3
Cl.Cas-3
GAPDH
C 6 12 24 36 48 hr
30 μM Silibinin
MDA-MB-231
Cas-3
Cl.Cas-3
GAPDH
C 6 12 24 36 48 hr
30 μM Silibinin
(B)
MCF7
MDA-MB-231
MTT reduction (%)
120
100
80
60
40
20
0
C
-
CHO
FMK
30 μM Silibinin
(C)
MDA-MB-231
Cas-3
Cl.Cas-3
GAPDH
EV
Notch1
caMEK
caAkt
EV
Notch1
caMEK
caAkt
30 μM Silibinin
Densitometry (% of control)
Cl.Cas-3
300
250
200
150
100
50
0
EV
Notch
caMEK
caAkt
EV
Notch
caMEK
caAkt
30μM Silibinin

Fig-6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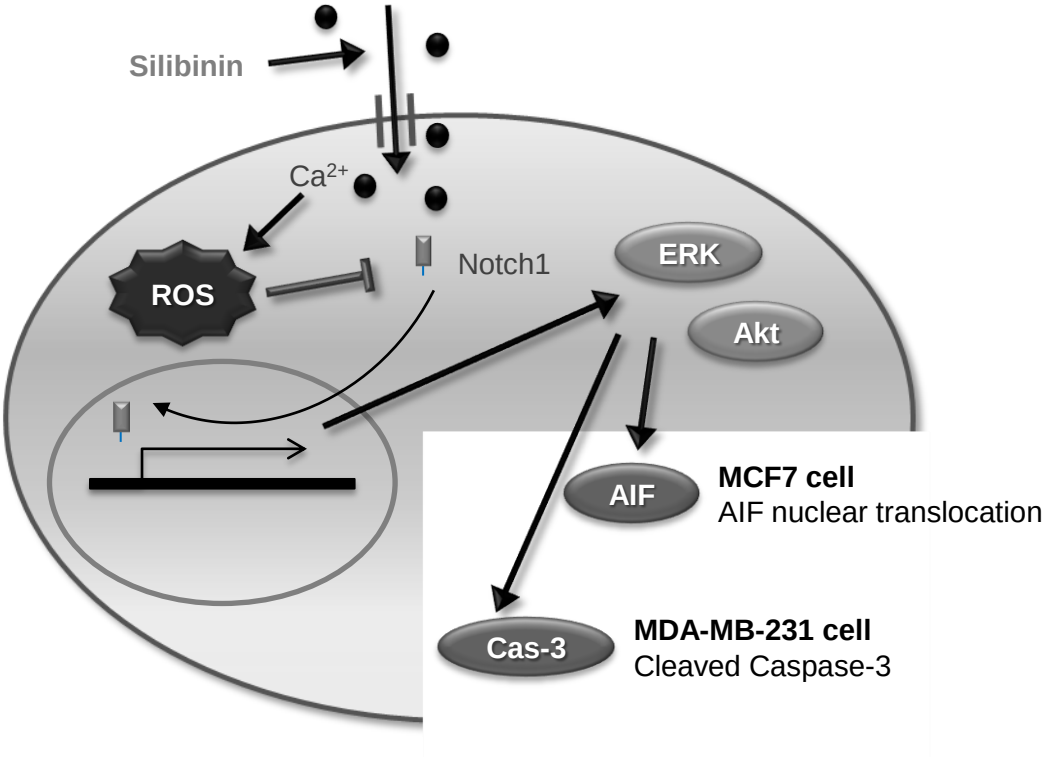
MCF7
MDA-MB-231
Cyto-AIF
β-actin
Nu-AIF
Histon H1
C 12 24 36 48 hr
30 μM Silibinin
C 12 24 36 48 hr
30 μM Silibinin
(B)
Control
Silibinin
NAC
caMEK
caAKt
MCF7
MDA-MB-231
(C)
MCF7
MDA-MB-231
MTT reduction (%)
siCon siAIF siCon siAIF
30 μM Silibinin
(D)
MCF7
Nu-AIF
Histon H1
EV Notch1 EV Notch1
30 μM Silibinin
Densitometry (% of control)
Nu-AIF
EV Notch1 EV Notch1
30μM Silibinin
Nu-AIF
Histon H1
EV caMEK caAkt EV caMEK caAkt
30 μM Silibinin
Densitometry (% of control)
Nu-AIF
EV caMEK caAkt EV caMEK caAkt
30μM Silibinin
MDA-MB-231
Nu-AIF
Histon H1
EV caMEK caAkt EV caMEK caAkt
30 μM Silibinin
Densitometry (% of control)
Nu-AIF
EV caMEK caAkt EV caMEK caAkt
30μM Silibinin

Fig-7


Silibinin
Ca2+
ROS
Notch1
ERK
Akt
AIF
MCF7 cell
AIF nuclear translocation
Cas-3
MDA-MB-231 cell
Cleaved Caspase-3