Original Article

Autophagy inhibition enhances apigenin-induced apoptosis in human breast cancer cells

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Abstract: Apigenin (4',5,7-trihydroxyflavone) is a member of the flavone subclass of flavonoids present in fruits and vegetables. The involvement of autophagy in the apigenin-induced apoptotic death of human breast cancer cells was investigated. Cell proliferation and viability were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and clonogenic assays. Flow cytometry, fluorescent staining and Western blot analysis were employed to detect apoptosis and autophagy, and the role of autophagy was assessed using autophagy inhibitors. Apigenin dose- and time-dependently repressed the proliferation and clonogenic survival of the human breast cancer T47D and MDA-MB-231 cell lines. The death of T47D and MDA-MB-231 cells was due to apoptosis associated with increased levels of Caspase3, PARP cleavage and Bax/Bcl-2 ratios. The results from flow cytometry and fluorescent staining also verified the occurrence of apoptosis. In addition, the apigenin-treated cells exhibited autophagy, as characterized by the appearance of autophagosomes under fluorescence microscopy and the accumulation of acidic vesicular organelles (AVOs) by flow cytometry. Furthermore, the results of the Western blot analysis revealed that the level of LC3-II, the processed form of LC3-I, was increased. Treatment with the autophagy inhibitor, 3-methyladenine (3-MA), significantly enhanced the apoptosis induced by apigenin, which was accompanied by an increase in the level of PARP cleavage. Similar results were also confirmed by flow cytometry and fluorescence microscopy. These results indicate that apigenin has apoptosis- and autophagy-inducing effects in breast cancer cells. Autophagy plays a cyto-protective role in apigenin-induced apoptosis, and the combination of apigenin and an autophagy inhibitor may be a promising strategy for breast cancer control.

Key Words: Apoptosis; autophagy; apigenin; breast cancer; 3-methyladenine

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Introduction

Breast cancer is the second leading cause of cancer deaths in women worldwide. The prevalence of breast cancer continues to increase by approximately 2% each year (1,2). Although chemotherapy produces objective responses in patients with breast cancer, it is far from completely effective (3,4). Numerous studies have indicated that environmental and lifestyle-related factors, especially dietary factors, play a crucial role in breast cancer etiology (3-5). In addition, epidemiological studies have suggested benefits of the consumption of cruciferous vegetables, namely a reduced risk of developing cancer (6). Substances contained in fruits and vegetables called phytochemicals may suppress the occurrence and progression of malignant tumors, including breast cancer (7). Thus, identifying

these phytochemicals is important in the prevention and treatment of breast cancer.

Apigenin (4',5,7-trihydroxyflavone) is a member of the flavone subclass of flavonoids present in fruits and vegetables and is considered to be a potent dietary phytochemical effective in cancer chemoprevention (8-10). Studies of malignant human cancer cell lines have shown that apigenin inhibits cancer cell growth via the promotion of cell cycle arrest and apoptosis (11-13). As a candidate anticancer agent, apigenin is of particular interest because it selectively induces cell cycle arrest and apoptosis in human prostate carcinoma cells without affecting normal cells (14). Apigenin is also reported to be nonmutagenic and of low toxicity compared to related flavonoids. Various *in vivo* and *in vitro* laboratory investigations have demonstrated that apigenin exhibits potent activity against breast cancer by inducing apoptosis and cell cycle arrest (15-17). There are, however, no reports describing the autophagy-inducing effects of apigenin, and we have found that autophagy plays a key role in apigenin-induced apoptosis and may contribute to the effectiveness of apigenin in breast cancer treatment.

Autophagy is an evolutionarily conserved catabolic process for degrading damaged proteins and/or organelles and recycling the materials to maintain the quality of the cellular components (18). Autophagy involves the formation of double-membrane vacuoles, termed autophagosomes, containing cytosol and organelles. Autophagosomes then fuse with endosomes and lysosomes to form autolysosomes, whose contents are degraded by hydrolytic enzymes (19). Autophagosome formation is a complex mechanism, and various autophagy-related (Atg) proteins participate, including Beclin 1 and light chain 3(LC3) (20). Autophagy occurs at basal levels in almost all cells, and its major function is the degradation of cellular components, including proteins and organelles that are aged, damaged, potentially dangerous or no longer needed (21,22). However, recent studies have shown that autophagy also plays an important role in human disease, including cancer (23). Furthermore, emerging evidence indicates that chemotherapeutic agents induce autophagy in various types of cancer cells (24-26). Our previous studies have revealed that apigenin can induce autophagy accompanied by the induction of apoptosis in breast cancer cells. Because autophagy and apoptosis occur simultaneously, it is unclear what relationship exists between them. In this study, we examined the apoptosis- and autophagy- inducing effects of apigenin and further discussed the role of autophagy in apigenin-induced apoptosis in breast cancer cells.

Materials and methods

Cell lines and chemicals

The T47D and MDA-MB-231 breast cancer cell lines were obtained from American type culture collection (ATCC). Fetal bovine serum (FBS) was obtained from Life Technologies (Gaithersburg, MD, USA). Apigenin (>95% purity) was obtained from A.G. Scientific (San Diego, CA, USA). 3-Methyl adenine (3-MA) and acridine orange were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hochest/MitoTracker-Red/YO-PRO-1 was purchased from Invitrogen (Carlsbad, CA, USA). LC3-GFP cDNA plasmid was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Propidium iodide (PI), Annexin V and MTT, trypsin-EDTA and DMSO were purchased from Sigma Chemical (St. Louis, MO, USA). Caspase3, PARP, Bcl-2, Bcl-xl, Bax, and LC3 antibodies were obtained from Cell Signaling Technology (Fremont, CA, USA).

Cell culture

T47D and MDA-MB-231 breast cancer cells were routinely maintained in RPMI 1640 (Gibco) media supplemented with 10% FBS and 1% antibiotics (50 U/mL of penicillin and 50 μg/mL streptomycin, Gibco) at 37 ℃ in a humidified atmosphere containing 5% CO_2. The total concentration of DMSO in the medium did not exceed 0.2% (v/v) during the treatments, which had no effect on cell growth.

Cell proliferation and colony-formation assay

The effects of apigenin on cell proliferation were determined by MTT assays. Briefly, 1×10^4 cells/well were plated in 96-well culture plates. After an overnight incubation, the cells were treated with varying concentrations of apigenin (0, 10, 20, 40, and 80 μM) for 24 and 48 h. The cells were treated with 50 μL of 5 mg/mL MTT, and the resulting formazan crystals were dissolved in DMSO (200 μL). The absorbance was recorded at 570 nm. The results were calculated as the percentage of inhibition by the following formula: % inhibition = [1−(At/As)]×100%. At and as indicate the absorbance of the test substance and the solvent control, respectively.

The colony-formation assay was conducted by plating two hundred cells in each well of a 6-well plate. After a 12-h incubation, the cells were treated with apigenin at different concentrations (0, 10, 20, 40, and 80 μM) for 1 h. DMSO (0.1%) was added to a control group. After rinsing with

fresh medium, the cells were allowed to grow for 14 days to form colonies, which were then stained with crystal violet. Colonies containing more than 50 cells were counted. The clonogenic assay was used to elucidate the possible differences in the long-term effects of apigenin on human breast cells.

Flow cytometry

Apoptosis was analyzed by detecting phosphatidylserine externalization by flow cytometry. Two-color analysis with FITC-labeled Annexin V/PI double staining was used. Briefly, breast cancer cells were treated with either DMSO or apigenin for 24 or 48 h. The cells were harvested, washed in cold PBS, stained with 1 mg/L FITC-labeled Annexin V and 0.2 mg/L PI, and analyzed by flow cytometry. Apoptosis was determined by measuring the Annexin V(+)/PI (-)versus Annexin V(+)/PI(+) events.

Hochest/Mito Tracker-Red/YO-PRO-1 fluorescent staining

Ten thousand cells per well were plated in 6-well plates. After overnight incubation, the cells were treated with varying concentrations of apigenin with or without 3-MA. After 24 h, a final concentration of 5 μg/mL Hoechst, 250 nM MitoTracker Red and 1 μM Yo-pro-1 were added and incubated for 30 min at room temperature. The morphologic changes were observed under an inverted fluorescence microscope.

Western blot analysis

Cells were harvested, washed with ice-cold PBS, and solubilized using sodium dodecyl sulfate (SDS) lysis buffer by passing the cell suspension through a 20-gauge syringe needle. The protein concentration in the lysate was determined by Bradford assay. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for 1 h at 100 V at 4 ℃. The membranes were blocked in tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% nonfat milk (w/v) for 1 h at room temperature (RT), followed by incubation with the indicated primary antibody (1:1,000 v/v) in TBST containing 5% nonfat milk overnight at 4 ℃. The membranes were washed three times for 10 min with TBST, followed by incubation with the appropriate secondary antibody (1:10,000) in TBST containing 5% nonfat milk for 1 h at RT. The detected proteins were visualized by chemiluminescent autoradiography using the Amersham ECL plus detection system (GE Healthcare, Buckingham-shire, UK).

Acridine orange staining

Autophagy is characterized by the formation of acidic vesicular organelles (AVOs). To detect AVOs, vital staining with acridine orange was performed. Briefly, 5×10^5 cells were seeded in 50-mm culture dishes and allowed to attach. The cells were treated with apigenin for 24 h then incubated with 1 mg/L acridine orange for 15 min. The dye was removed, and fluorescence micrographs were captured using an inverted microscope equipped with a 100-W mercury lamp, 490-nm bandpass blue excitation filters, a 500-nm dichroic mirror, and a 515-nm long-pass barrier filter. The cells were then collected by trypsin-EDTA and resuspended. The stained cells were then analyzed using an EPICS flow cytometer.

Transient transfection of activated LC3-GFP cDNA plasmid

Transfection of the breast cancer cells was carried out using Lipofectamine 2000 reagent. The breast cancer cells were exposed to a mixture of Lipofectamine 2000 reagent and LC3-GFP cDNA plasmid for 6 h. Then, 8 mL of RPMI 1640 containing 20% fetal bovine serum was added to each culture dish. After 18 h of incubation, fresh RPMI 1640 was added, and the cells were incubated for another 2 days. The transfection efficiencies were determined by measuring the β-galactosidase activity using the β-galactosidase ELISA kit (Roche). The cells were treated with apigenin at different concentrations for 24 h, and the formation of autophagosomes was observed by fluorescence microscopy.

Autophagy inhibition by autophagy inhibitors

To investigate the role of apigenin-induced autophagy, 3-MA, a specific autophagy inhibitor, was analyzed. 3-MA, a specific inhibitor of class III phosphatidylinositol-3 kinase (PI3K), inhibits autophagy at an early stage by inhibiting autophagic sequestration. The T47D breast cancer cells were treated with 60 M apigenin and/or 50 μM 3-MA. The cells were incubated for 24 h, and the AVO-positive cells were quantified.

Statistical analysis

All discrete values, expressed as the means ± SD, were analyzed

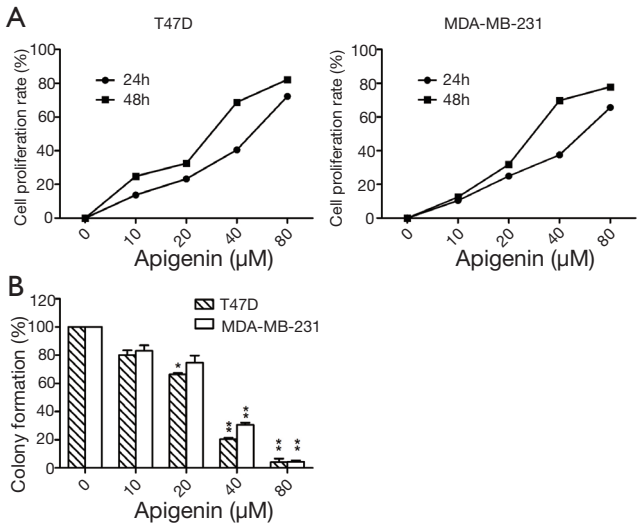

Figure 1 Dose- and time- dependent effect of apigenin on the proliferation and viability of human breast cancer cells. A. For the proliferation assay, MDA-MB-231 and T47D cells were plated in 96-well plates at 5×10^3 cells/well and treated with different concentrations of apigenin. The cell proliferation after the indicated amounts of time was determined by MTT assay. The data are presented as the means ± SD of three independent experiments; B. Cell viability was determined by a colony-formation assay. Cells were plated in 6-well plates at 200 cells/well and treated with different concentrations of apigenin for 4 h. Colonies with over 50 cells were counted. The data are presented as the means ± SD, *P<0.05 and **P<0.01 compared with the controls

using Student's t-test. P-values less than 0.05 and 0.01 were considered as significant and highly significant, respectively.

Results

Apigenin inhibits cell proliferation and clonogenic survival in T47D and MDA-MB-231 cells

To investigate the potential cell growth inhibition by apigenin in breast cancer, we first examined the effect of apigenin on cell proliferation and clonogenic survival in T47D and MDA-MB-231 cells. As shown in *Figure 1A*, apigenin significantly inhibited cell growth in both cancer cell lines in a concentration-dependent and time-dependent manner.

Additional experiments were performed to determine the long-term antitumor activities of apigenin. *Figure 1B* shows the effects of apigenin on the relative clonogenicity of the control and apigenin-treated T47D and MDA-MB-231 cells. The clonogenicity of both cancer lines was reduced in a concentration- and time-dependent manner after exposure to apigenin. Interestingly, the clonogenic rate of MDA-MB-321 cells was lower than that of the T47D cells at each concentration point, suggesting that MDA-MB-231 cells are more resistant to the inhibitory effect of apigenin than the T47D cells. Because *in vitro* clonogenic assays have been shown to correlate very well with *in vivo* assays of tumorigenicity in nude mice (27,28), these results could suggest antitumor effects of apigenin on breast cancer *in vivo*.

Apigenin induces apoptosis in T47D and MDA-MB-231 breast cancer cell lines

Apoptotic cell death plays an important role in the regulation of the killing of cancer cells by anticancer agents. To evaluate whether the cell death observed in T47D and MDA-MB-231 cells after apigenin treatment might occur through apoptosis, Hochest/MitoTracker-Red/YO-PRO-1 fluorescent staining was utilized to observe the morphologic changes in both cell lines in response to apigenin. The cell nuclei can be dyed blue using Hoechst stain, a DNA-specific dye. The cytoplasm can emit red fluorescence under a fluorescence microscope, for the reason that MitoTracker-Red can combined to the mitochondria in living cells. In addition, YO-PRO-1 can pass through the cell membrane of apoptotic cells, but not of living cells. Therefore, the yellow fluorescent points under the fluorescence microscope represent the apoptotic cells stained with YO-PRO-1. The results indicated that apigenin could effectively induce apoptosis in both cell lines in a concentration-dependent manner (*Figure 2A*).

FITC annexin-V/PI double staining was also performed to examine the apoptotic rate of both cell lines treated with apigenin. After apigenin treatment for 24 or 48 h, the rate of inhibition in both cell types significantly increased in a dose- and time-dependent manner compared with the control group (*Figure 2B*). Western blotting was performed to examine the changes in the levels of apoptosis-related molecules, such as Caspase3, PARP, Bcl-2, Bcl-xl and Bax. The cells treated with apigenin exhibited an increase in the levels of Bax, increased Caspase3 and PARP cleavage and a decrease in the Bcl-2 and Bcl-xl levels (*Figure 2C*).

Apigenin induces autophagy in T47D and MDA-MB-231 cells

To ascertain whether apigenin induces autophagy in breast

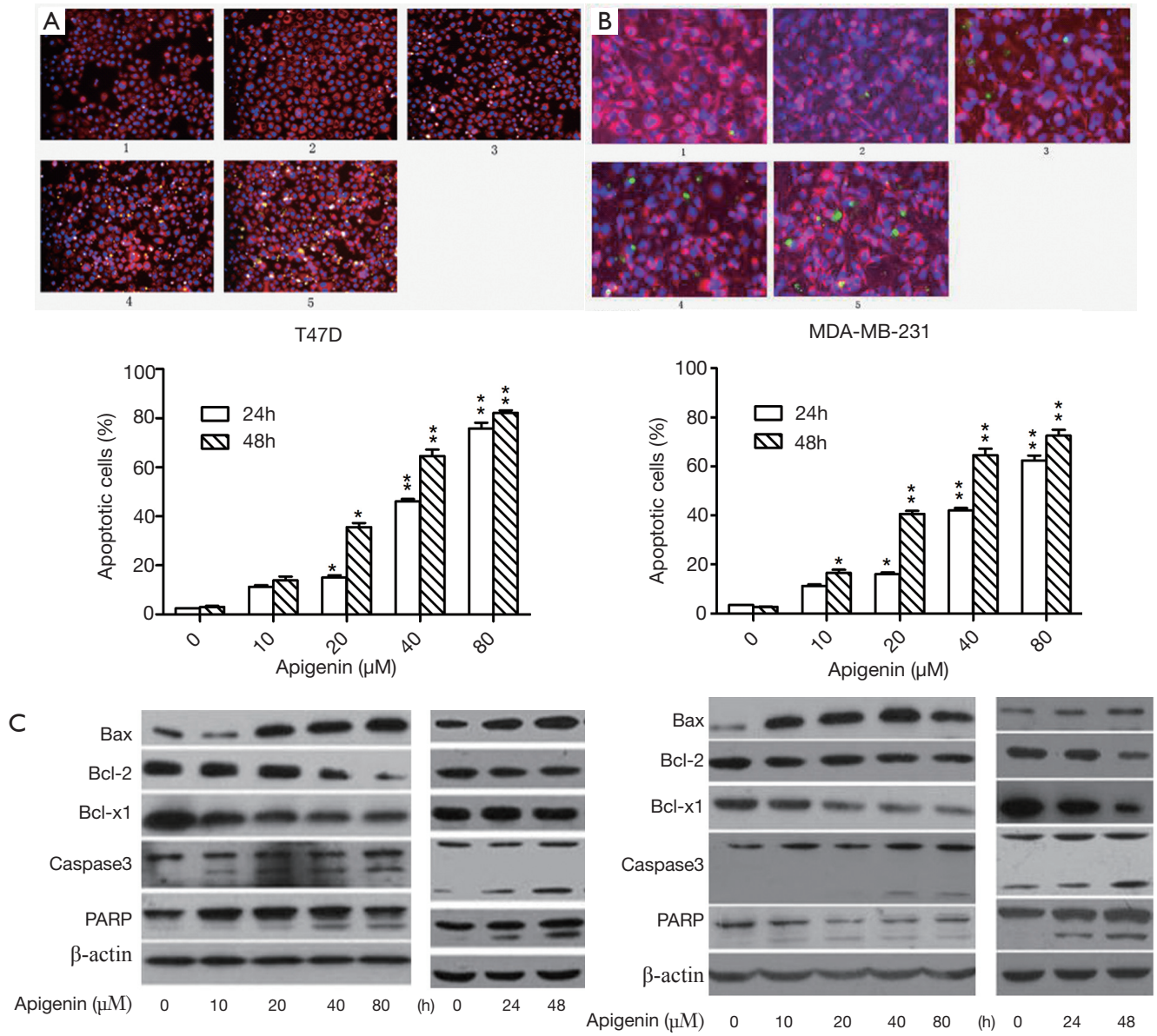

Figure 2 Induction of apoptosis in human breast cancer cells after treatment with apigenin. A. Hochest/MitoTracker-Red/YO-PRO-1 fluorescent staining was examined to observe the morphologic changes in both cell lines in response to apigenin. B. Apoptotic assays were performed by flow cytometry after staining with Annexin V-FITC/PI. The cells were treated with apigenin at different concentrations for different amounts of time. The data are presented as the means ± SD, *P<0.05 and **P<0.01 compared with the controls. C. The expression levels of apoptosis-related molecules, such as Bax, Bcl-2, and Caspase-3 and PARP, were analyzed by Western blotting

cancer cells, we examined the apigenin-treated cells by fluorescence microscopy. We first determined the effect of apigenin treatment on the formation of AVOs in breast cancer cells by fluorescence microscopy after staining with the acridine orange. As shown in *Figure 3A*, the apigenin treatment resulted in the appearance of AVOs when the cells were stained with acridine orange after 24 h of treatment.

To quantify the accumulation of the acidic component, we performed fluorescence-activated cell sorting analysis of the acridine orange-stained cells using the FL3 channel to evaluate bright red fluorescence and the FL1 channel

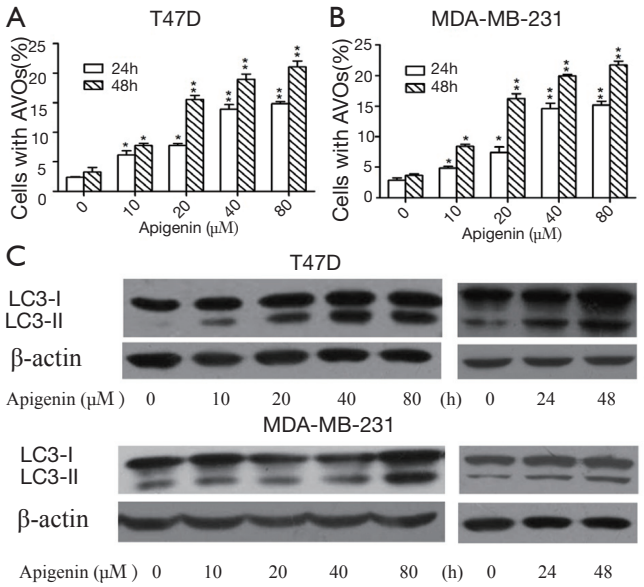

Figure 3 Determination of the induction of autophagy in MDA-MB-231 and T47D breast cancer cells. A. MDA-MB-231 and T47D cells were treated with apigenin at concentrations of 0, 10, 20, 40 and 80 μM, and AO staining was performed to detect the formation of acidic vesicular organelles (AVOs) in autophagic cells. The microscopic images indicate progressive increases in AVOs in the cells as the apigenin concentration increased; B. Flow cytometric analysis of the apigenin-treated cells after staining with AO for the quantification of AVOs. Data are presented as the means ± SD, *P<0.05 and **P<0.01 compared with the controls; C. Western blotting to examine the expression of LC3 I/II. Cell lysates from DMSO controls and cells treated with different apigenin concentrations were subjected to SDS-PAGE and immunoblotted with anti-LC3 antibody

to evaluate green fluorescence. As shown in *Figure 3B*, in contrast to the control cells, the apigenin-treated cells accumulated a significant number of AVOs in a dose- and time-dependent manner.

LC3 is known to be one of the most important autophagy-related proteins participating in the process of autophagosome formation, which also requires the cellular cytoskeleton. During autophagy, the cytoplasmic LC3-I (18 kDa) form is processed to generate LC3-II (16 kDa), and tracking this conversion is indicative of the autophagic activity. Once autophagy is initiated, LC3-I is transformed into LC3-II and translocates to the surface of autophagosomes from the cytoplasm. We examined the changes in the protein levels of LC3. The results indicated that the cells treated with apigenin exhibited a significant increase in both the LC3-I and LC3-II protein levels compared with the control cells (*Figure 3C*). These results suggest that apigenin can induce autophagy by modulating LC3 expression and autophagosome formation in breast cancer cells.

Autophagy inhibition enhances apigenin-induced apoptosis in breast cancer T47D cells

3-MA, a specific autophagy inhibitor, was used to investigate the role of autophagy in apigenin-induced apoptosis in breast cancer cells. Representative histograms of the acridine orange staining after 24 h of treatment with apigenin in the presence or absence of 3-MA are presented in *Figure 4A*. The acridine orange staining after 24 h of treatment with 6 μM apigenin indicated a significant accumulation of AVOs, compared with the control group in the T47D breast cancer cell line. However, this accumulation was significantly inhibited by 3-MA (P<0.01).

We transfected a plasmid carrying the LC3-GFP (green fluorescence protein) gene into T47D breast cancer cells. Cells containing the LC3-GFP gene, which encodes LC3-I-GFP, evenly emit green fluorescence. Once autophagy is initiated, LC3-I is transformed into LC3-II, which translocates to the surface of autophagic vacuoles from the cytoplasm. Therefore, the green fluorescence points under the fluorescence microscope are evidence of the existence of autophagy. We observed that the green fluorescence points appeared within the cytoplasm of apigenin-treated cells, while relatively few of these structures were observed in the cytoplasm of cells exposed to 3-MA, indicating that 3-MA effectively inhibits the autophagy induced by apigenin (*Figure 4A*).

Next, to investigate the effect of 3-MA on cell apoptosis, Hochest/MitoTracker-Red/YO-PRO-1 fluorescent staining was performed. We observed that cells exposed to both apigenin and 3-MA exhibited a higher rate of apoptosis compared to those treated with apigenin alone or the control group (*Figure 4B*). Furthermore, FACS analysis following double staining with PI and Annexin V was also performed. As showed in *Figure 4B*, the cells treated with apigenin exhibited a significant increase in apoptosis compared with the control group (P<0.05). Interestingly, the apigenin treatment in the presence of 3-MA significantly reduced cell viability in T47D breast cancer cells compared to the apigenin treatment alone (P<0.05).

We next examined the alterations in the protein levels of Beclin1, LC3 and PARP. We observed an increase in the

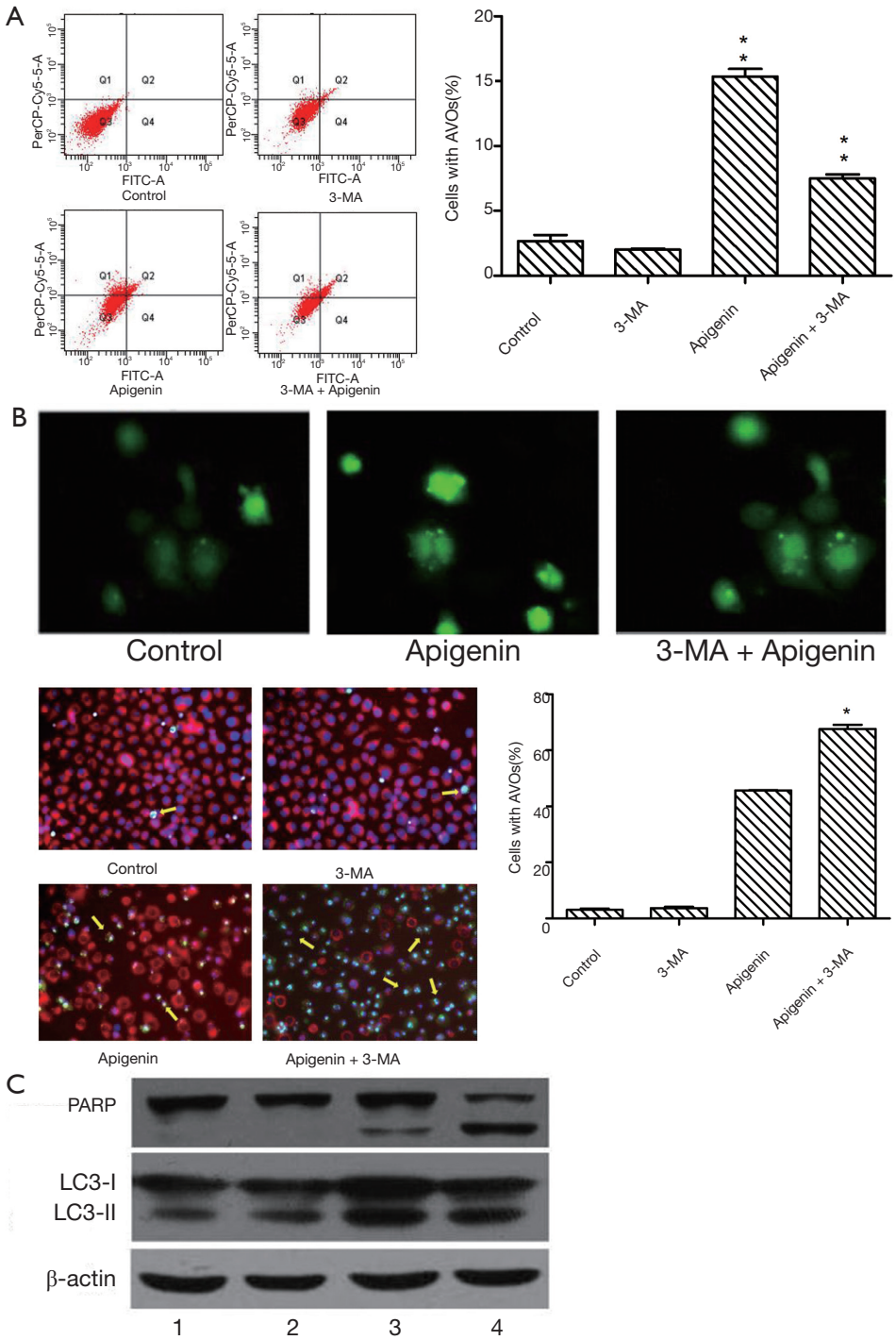

Figure 4 Effects of 3-methyladenine on apigenin-induced autophagy in MDA-MB-231 cells. A. Cells were treated with 60 and 50 mM 3-MA for the indicated periods of time, and the acridine orange-positive cells were quantified by flow cytometry. Data are presented as the means ± SD, *P<0.05 and **P<0.01 compared with the controls; B. Apoptotic assays were performed by flow cytometry after staining with Annexin V-FITC/PI. The cells were treated with different concentrations of apigenin for different time periods. The data are presented as the means ± SD, *P<0.05 and **P<0.01 compared with the controls; C. Cell lysates from the DMSO controls and apigenin-treated cells with or without 3-MA were subjected to SDS-PAGE, and apoptosis- and autophagy-associated molecules were analyzed by Western blotting

levels of LC3-II and increased cleavage of PARP upon apigenin treatment in the absence of 3-MA. However, the combination of 3-MA and apigenin resulted in a significant increase in the levels of cleaved PARP and a decrease in Beclin1 compared to the apigenin treatment alone. These results suggested enhanced apoptosis in response to the combined treatment with 3-MA and apigenin. Treatment with 3-MA alone had no effect on the apoptotic rate or the levels of apoptosis-related molecules (*Figure 4C*).

Discussion

Numerous studies have demonstrated the anticancer effects of apigenin on various cancer cells. In our study, we found that apigenin effectively inhibited breast cancer cell growth by inducing apoptosis. Autophagy was also determined to occur concurrently with apoptosis in breast cancer cells. The aim of our research was to uncover the role of autophagy in the process of apoptosis and to elucidate the crosstalk between autophagy and apoptosis, which might represent a novel strategy for breast cancer therapy. To evaluate the effects of apigenin on apoptosis, fluorescence microscopy, flow cytometry and Western blot analysis were performed, revealing that apigenin induced apoptosis of breast cancer cells in a dose- and time-dependent manner. The molecules involved in apigenin-induced apoptosis included increased levels of Bax, cleaved Caspase 3 and PARP and decreased levels of Bcl-2.

Increasing evidence indicates that autophagy can provide cancer cells with a protective response under unfavorable conditions, such as hypoxia and nutrient deprivation. However, it has also been reported that autophagy is triggered in response to various anticancer agents, including As_2O_3 (29,30), tamoxifen (31,32), and epirubicin (33), in some cancer types. The results of our research indicate that apigenin can induce autophagy in breast cancer cells, which was characterized by the formation of autophagic vacuoles and increased levels of LC3-II. Flow cytometry further indicated that the autophagy-inducing effect of apigenin occurred in a dose- and time-dependent manner.

Thus, apigenin can simultaneously induce apoptosis and autophagy. This may be explained by the inhibitory effect of apigenin on the PI3K/Akt/mTOR pathway. Several studies have indicated that the anticancer effect of apigenin is related to the inhibition of the PI3K/Akt/mTOR pathway (34,35), which is also an essential pathway that negatively regulates autophagy (36). As the autophagy and apoptosis induced by apigenin occur at the same time in breast cancer cells, the question arises of whether there is any crosstalk between these two processes, and specifically, what role does autophagy play in the process of apigenin-induced apoptosis.

Studies on autophagy in chemotherapy indicate that the induction of autophagy plays a protective role in the resistance to apoptosis induced by anticancer drugs (37). In addition, increasing evidence has demonstrated that autophagy prevents cancer cells from cell death by removing the damaged proteins and/or organelles in the cells exposed to chemotherapeutic agents, and the inhibition of autophagy leads to increased apoptosis. Therefore, the combination of autophagy inhibitors and chemotherapeutic agents is considered a promising strategy for cancer therapy. For example, Li *et al.* (38) reported that the inhibition of autophagy augmented 5-fluorouracil chemotherapy in human colon cancer in *in vitro* and *in vivo* models. In addition, targeting autophagy augments *in vitro* and *in vivo* anti-myeloma activity of DNA-damaging chemotherapy (39). The autophagy inhibitors 3-MA and Baf were also shown to enhance the cytotoxicity of anthocyanin in PLC/PRF/5 human hepatocellular carcinoma (40) and the cytotoxicity of As_2O_3 in U373-MG human glioblastoma cells (41). Through our research, we arrived at a similar conclusion as these two studies, revealing that apigenin could induce not only apoptosis but also autophagy, and the treatment with 3-MA significantly increased apigenin-induced apoptosis in T47D breast cancer cells. Although numerous studies have focused on the antineoplastic effects of apigenin and the possible mechanisms, such as inducing apoptosis and cell cycle arrest, few studies have reported the autophagy-inducing effect of apigenin or the role of autophagy in apigenin-induced apoptosis.

Crosstalk does occur between apoptosis and autophagy, which determines cell fate, but the molecular mechanism is not fully understood. Different proteins that belong to the mitochondrial pathway of apoptosis have also been shown to crosstalk with Atg proteins and to regulate autophagy in cultured breast cancer cells. For example, the activation of an apoptosis promoter, Bid protein, also affects apoptosis and autophagy in opposing directions because it not only stimulates apoptosis but also reduces autophagy by inhibiting Beclin 1 (42). In contrast, the anti-apoptotic protein Bcl-2 regulates both processes in the same direction because it negatively regulates the levels of three Atg proteins, including Beclin 1, Atg5 and LC3-II, which play an important role in the process of autophagy (43).

Several investigations, however, have arrived at the completely opposite conclusion, indicating that autophagy could mediate cell death and not only protect cells from apoptosis (44). Persistent stress can also promote extensive autophagy, leading to cell death. More recently, a second type of cell death pathway has emerged: autophagic cell death, also called type II programmed cell death. For example, Park *et al.* (45) demonstrated that MHY218, a new synthetic HDAC inhibitor, induces apoptosis or autophagy-related cell death in tamoxifen-resistant MCF-7 breast cancer cells. These results are possible because the effect of autophagy may vary dependent on the type of cancer, the individual characteristics of the cancer cells, the microenvironment, and the therapeutic treatment. Autophagy is a highly complex process, which can exert both cyto-protective and death-promoting effects. Furthermore, its mechanism requires further investigation.

In conclusion, apigenin, a naturally occurring plant flavone, could inhibit the proliferation of T47D and MDA-MB-231 breast cancer cells by inducing apoptosis. In addition, the autophagy induced by apigenin also increased simultaneously with apoptosis. The inhibition of autophagy by 3-MA enhanced the apoptosis induced by apigenin. Therefore, the combination of apigenin and autophagy inhibitors represents a novel and promising strategy for breast cancer therapy. The relationships between autophagy and apoptosis are quite complex, but we predict that a better understanding of the underlying molecular mechanisms could contribute to anticancer therapy in the near future.

Acknowledgements

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