

Parthenolide induces autophagy and apoptosis of breast cancer cells associated with the PI3K/AKT/mTOR pathway

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Research Article

Keywords: parthenolide, autophagy, apoptosis, breast cancer cells

Posted Date: March 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-289764/v1>

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Abstract

Background: Breast cancer is an aggressive malignancy that is unresponsive to conventional therapies. Parthenolide has been demonstrated to have anticancer effects against various types of cancer, including breast cancer. The aim of the present study was to investigate the effect and underlying mechanism of parthenolide in human breast cancer.

Methods: Autophagy was measured through immunofluorescence and western blotting. DAPI staining and flow cytometry analysis were used to measure apoptosis. Western blot analysis was used to investigate the mechanism of autophagy induced by parthenolide on the expression levels of phosphoinositide 3-kinase (PI3K), AKT, phosphorylated (p-) AKT, mammalian target of rapamycin (mTOR), ATG13 and ATG14. Furthermore, apoptosis was confirmed via western blot analysis.

Conclusion: Parthenolide inhibits breast cancer cell proliferation and induces autophagy and apoptosis, and suggested that the PI3K/AKT/mTOR pathway serves an important role in autophagy and apoptosis.

Introduction

Breast cancer is the most commonly occurring type of cancer in women, and the risk of contracting it increases with age. Breast cancer has five subtypes, comprising luminal A, luminal B, basal epitheliallike, ERBB2overexpressing, and normal breastlike group. From the perspective of clinical therapeutic interventions, antiestrogen therapy, chemical therapy, and radiotherapy have led to increased survival rates. However, the occurrence rate of breast cancer continues to increase. Compared with other types of breast cancer, triplenegative breast cancer (TNBC), owing to lack of estrogen receptors, is not sensitive to traditional therapies [1], and there are only limited treatment strategies available for TNBC.

Parthenolide has a variety of pharmacological activities and is used to treat a variety of diseases including arthritis, fever and headache [2, 3]. PTL was reported in vitro to inhibit proliferation and induce apoptosis in various human cancers, including those of osteosarcoma, pancreatic cancer and prostate cancer [46]. Although effects of parthenolide on breast cancer have been reported, its detailed mechanism and the biological target(s) are yet to be fully elucidated.

To date, three main cell death pathways have been defined, namely, apoptosis, autophagy, and necrosis. Autophagy and apoptosis have both been termed 'programmed cell death' pathways. Apoptosis serves an important role as a protective mechanism against carcinogenesis by eliminating either damaged cells or an abnormal excess of cells that have proliferated owing to various chemical inductions. Autophagy comprises three subtypes: macroautophagy, microautophagy, and chaperonemediated autophagy. Of these, the most prevalent form of autophagy is macroautophagy [7]. Autophagy is a constitutively active, evolutionarily conserved, and physiologically selfdegradative process that serves to remove unnecessary or dysfunctional cellular components through the fusion of autophagosomes with lysosomes to form autolysosomes, and it is extremely important for sustaining cellular homeostasis and inducing survival mechanisms under various types of stresses, such as radiation and chemotherapy [8]. Under normal

conditions, autophagy levels remain low; autophagy may be induced only via an internal or external source of stimulation [9]. Intrinsic or acquired therapeutic resistance may result because of various factors, and autophagy may exert a significant role in these processes. Although the role of autophagy in suppressing or promoting cell death is yet to be fully elucidated, it remains a putative target for therapeutic intervention. Recently, the interplay between autophagy and apoptosis in terms of the response to chemotherapy has become a subject of intense debate. Basic research data have suggested that autophagy induced by antitumor therapy may serve a protective role. Wang et al. [10] reported that inhibition of autophagy promoted apoptosis induced by adriamycin. Lu et al. [11] discovered that inhibiting autophagy could enhance apoptosis induced by parthenolide against breast cancer. Therefore, learning more about the mechanism of autophagy induced by antitumor therapy will be crucial for the development of breast cancer therapy.

The first regulatory step of autophagy comprises inhibition of mammalian target of rapamycin (mTOR) [12]. When the activity of mTOR is suppressed, the complex of autophagy proteins may be activated [1315]. The role exerted by mTOR is important for the induction of autophagy. mTOR is a member of the phosphoinositide 3kinase (PI3K) family, which is essential for the mechanism of autophagy. Under normal conditions, phosphorylated PI3K can phosphorylate AKT, which subsequently inhibits tuberous sclerosis complex 1/2 (TSC1/2), allowing activated mTOR may suppress autophagy [16]. A related study reported that ATG14 [also known as beclin1 associated autophagyrelated key regulator (Barkor), or ATG14L] may promote membrane tethering of proteinfree liposomes, a process that enhances the hemifusion and full fusion of proteoliposomes reconstituted with the target (t)SNAREs, STX17, and SNA29, which are able to promote autophagosomeendolysosome fusion [17]. Autophagyrelated protein 13 (ATG13) is necessary for the induction of autophagy. The Nterminus of the protein folds into a HORMA domain, which can directly interact with ATG14. TORC1 (target of rapamycin complex 1) phosphorylates ATG13 and represses autophagy. However, when TORC1 is inactive, ATG13 dephosphorylation occurs rapidly, thereby inducing autophagy [18]. Therefore, the PI3K/AKT/mTOR pathway serves an important role in autophagy. The present study was performed to investigate the mechanism of apoptosis and autophagy induced by parthenolide.

2. Materials And Methods

2.1 Cell culture

MCF7 and MDAMB231 breast cancer cells were provided by the Pathology Department of Dalian Medical University (Dalian, China). MCF7 cells were maintained in minimal essential medium (MEM) (HyClone™; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone™), and MDAMB231 cells were cultured in Dulbecco's modified Eagle's medium DMEM/F12 (HyClone™, Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone™, Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

2.2 Cell Viability Assay

A Cell Counting Kit8 (CCK8) (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China) assay was used to examine cell viability. CCK8 assays are convenient to use because of Dojindo's highly watersoluble tetrazolium salt. WST8 [2(2methoxy4nitrophenyl)3(4nitrophenyl)5(2,4disulfophenyl)2Htetrazolium, monosodium salt] produces a watersoluble formazan dye upon reduction in the presence of an electron mediator, 1methoxy phenazinium methylsulfate (PMS). WST8 is bio-reduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. The amount of formazan produced is directly proportional to the number of living cells. MCF7 and MDAMB231 cells were plated at a density of 5×10^3 cells per well in 96well plates and treated with different concentrations of parthenolide (purchased from SigmaAldrich, Co; now Merck Millipore, Burlington, MA, USA) for 24 and 48 h, respectively. Thereafter, 10 μ l CCK solution and 100 μ l fresh medium was added to each well and incubated in the dark at 37°C for 2 h. After incubation, the absorbance of each well was measured at a wavelength of 452 nm. All experiments were repeated at ≥ 3 times. The halfmaximal lethal concentration (LC₅₀) values were calculated according to the concentration of parthenolide required to inhibit cell viability by 50% compared with untreated cells.

2.3 4',6-Diamidino-2-phenylindole (DAPI) staining

Nuclear fragmentation and chromatin condensation were analyzed through DAPI (eBioscience; Thermo Fisher Scientific, Inc.) staining. MCF7 and MDAMB231 cells were treated with parthenolide at different concentrations (0, 2, 4, and 8 μ M; and 0, 2.5, and 5 μ M) for 24 h. Cells were fixed with 3.7% formaldehyde, and subsequently stained with DAPI. The plates were then washed with PBS three times prior to viewing under a fluorescence microscope (Olympus Corporation, Tokyo Japan).

2.4 Annexin V/propidium Iodide Apoptosis Assay

Apoptosis assay was performed using a commercially available kit, as per the manufacturer's protocol. MCF7 and MDAMB231 cells (0.1×10^6 cells) were plated in each well of 6well plates. After 24 h, parthenolide was added in different concentrations for 24 h. After 24 h treatment, cells were harvested by trypsinization and suspended in PBS. The cells were subsequently suspended in 500 μ l binding buffer (Nanjing KeyGen Biotech. Co., Ltd.). Later, 5 μ l annexinV fluorescein isothiocyanate (FITC) (Nanjing KeyGen Biotech. Co., Ltd.) and 5 μ l propidium iodide (PI) (both purchased from Nanjing KeyGen Biotech. Co., Ltd.) were added, and the cells were incubated for 20 min in the dark. Finally, the samples were analyzed using flow cytometric analysis.

2.5 Western Blot Analysis

Antibodies raised against PI3K, AKT, phosphorylated (p)AKT, PTEN, mTOR, ATG13, ATG14, Beclin1, P62, caspase3 and Bcl2 were purchased from Proteintech Group, Inc. MCF7 and MDAMB231 cells were treated with parthenolide at different concentrations for 24 h. Total protein was extracted using a protein extraction kit (Nanjing KeyGen Biotech. Co., Ltd.), according to the manufacturer's protocol, and then quantified with a bicinchoninic acid (BCA) kit (Nanjing KeyGen Biotech. Co., Ltd.). Protein lysates were

heated at 100°C for 5 min, subsequently separated with sodium dodecyl sulfate (SDS)polyacrylamide gel electrophoresis (PAGE), and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore). Membranes were blocked with 5% nonfat milk in Trisbuffered saline/Tween 20 (TBST) for 1 h. PVDF membranes were incubated with the primary antibodies at 4°C overnight, and then washed with TBST three times, followed by incubation with the secondary antibodies at 37°C for 1 h. Protein bands were detected using an enhanced chemiluminescence (ECL) kit (Advansta Inc., Menlo Park, CA, USA). The density of each band was quantified with ImageJ software, and corrected by reference to the expression value for GAPDH.

2.6 Immunofluorescence Analysis

Cells were seeded in a 24well plate (1×10^4 cells/well) and allowed to attach overnight. After treatment with parthenolide for an additional 24 h, the cells were fixed with methanol for 10 min, permeabilized with 0.1% Triton X100 for 10 min and blocked with 5% BSA (SigmaAldrich; now Thermo Fisher Scientific, Inc.) for 1 h, after which cells were incubated overnight with primary antibody (antiBeclin 1). Cells were washed and incubated with AlexaFluor 596 secondary antibody(Invitrogen; Thermo Fisher Scientific, Inc.) on the following day. After washing, DNA was counterstained with DAPI, and images were captured using a fluorescence microscope.

2.7 Statistical Analysis

Prism 5.0 software (GraphPad Software Inc, San Diego, CA, USA) were used for all statistical analyses. Results are presented as the mean \pm standard deviation (SD) or standard error of the mean. Statistical significance was determined using Student's ttest. $P < 0.05$ was considered to indicate a statistically significant value.

3. Results

3.1 Parthenolide suppresses the growth of human breast cancer cells

Parthenolide was found to exert an effect on the cell viability of human MCF7 and MDAMB231 cells, which could be examined by CCK8 assay. Compared with the control group, other groups treated with different concentrations of parthenolide exhibited reduced survival rates of the cells. The rates of cell viability were 89.1, 80.1, 62.5, 47.3 and 36.6% following treatment of MCF7 cells with parthenolide concentrations of 2, 4, 6, 8, and 10 μ M, respectively, for 24 h (Fig. 1A). Compared with MCF7 cells, the cell viability rates of MDAMB231 cells treated with parthenolide at the same concentrations for 24 h were 96.2, 90.1, 81.3, 65.9, and 50.9% (Fig. 1B). It was also possible to determine the LC_{50} value of parthenolide in the two cell lines. For MCF7 cells, the LC_{50} value was approximately 8 and 5 μ M following 24 and 48 h of parthenolide treatment, respectively (Fig. 1A). By contrast, compared with MCF7 cells, the cell viability results showed that the LC_{50} value of MDAMB231 cells was approximately 10 and 7 μ M under the same experimental conditions (Fig. 1B).

3.2 Induction Of Apoptosis By Parthenolide

The present study further assessed whether parthenolide could induce apoptosis in breast cancer. An annexinV/PI apoptosis assay was performed using flow cytometry. The percentages of apoptotic MCF7 cells were 2.6 and 6.2% following treatment with 4 and 8 μ M parthenolide, respectively (Fig. 2A). Similarly, the percentages in MDAMB231 cells were found to be 16.4 and 26.6% under the same experimental conditions (Fig. 2A).

Subsequently, a DAPI assay was performed to verify the results of the apoptosis experiments. Nuclear fragmentation was clearly observed in cells upon parthenolide treatment (Fig. 2B). Finally, western blot analysis was performed to confirm the apoptosis results. A high expression level of caspase3, and a low expression level of Bcl2, were observed in MDAMB231 and MCF7 cells (Fig. 3A and B).

3.3 Parthenolide Induces Autophagy Of Breast Cancer Sells

The western blotting results also showed higher expression levels of Beclin 1 and lower expression levels of P62/SQSTM1 upon parthenolide treatment compared with the control groups (Fig. 4A and B).

Autophagy induction by parthenolide was further confirmed by microscopy after staining the breast cancer cells with Beclin 1. Immunofluorescence experiments revealed red staining in MCF7 and MDAMB231 cells following 24 h treatment with parthenolide at concentrations of 4 and 8 μ M, and 5 and 10 μ M, respectively (Fig. 5).

3.4 Parthenolide increases the expression of autophagy-related markers.

ATG13 and ATG14 are important proteins for autophagy induction and phagophore biogenesis. We used Western blotting to observe the expression of ATG13 and ATG14, and found parthenolide can increase the expression of ATG13 and ATG14 (Fig. 6)

3.5 Mechanism of parthenolide in breast cancer cells.

Based on the above analysis, it has been demonstrated that parthenolide induced apoptosis and autophagy, but the detailed mechanism required further elucidation. The PI3K/AKT/mTOR signaling pathway has a critical role in regulating apoptosis and autophagy. Consequently, the effect of parthenolide on the expression levels of mTOR, AKT, pAKT, PTEN and PI3K were examined by western blot analysis, which revealed that the expression levels of the proteins were decreased (Fig. 6A and D). When mTOR is inactive, Atg13 dephosphorylation occurs rapidly. The activity of Atg14 enhanced the lipid kinase activity of the core VPS34P150Beclin1 complex. ATG13 and ATG14 expression levels were higher on parthenolide treatment compared with normal groups (Fig. 6A and D).

4. Discussion

Several previously published studies have reported that autophagy, a type of programmed cell death, serves a controversial role in different types of cancer [2022]. Some anticancer agents, such as resveratrol [23, 24], curcumin [25, 26], and genistein [27], are able to induce autophagy. Parthenolide promotes the activity of caspase family members and causes the release of proapoptotic proteins in colorectal cancer [28]. It can also cause apoptosis via the tumor necrosis factor receptor superfamily member 10B (TNFRSF10B) and phorbol12myristate13acetateinduced protein 1 (PMAIP1) signaling pathways in human lung cancer cells [29]. Parthenolide suppresses the I κ B kinase complex, and as a potent inhibitor of NF κ B, can lead to apoptosis in cancer cells [30]. Furthermore, it can also generate reactive oxygen species and autophagy in MDAMB231 cells [31]. Therefore, the available evidence has shown that parthenolide may induce apoptosis in cancer cells, especially in breast cancer. However, in the present study, we explored the anticancer activities of a plant-derived agent, parthenolide, in breast cancer cells, and aimed to further understand its role and molecular mechanism.

The present study has shown that parthenolide markedly inhibited the growth of MCF7 and MDAMB231 cells. A CCK8 assay indicated a dramatic loss of viability of cancer cells following their treatment with parthenolide, which occurred in a dose-dependent fashion. Additionally, a colony formation assay also revealed that parthenolide repressed the growth of cells. Flow cytometric analysis and a DAPI assay demonstrated that parthenolide led to the onset of apoptosis, and an MDC assay clearly demonstrated that parthenolide induced autophagy of cells. Taken together, all these data indicate that treatment with parthenolide could be a novel therapeutic method. The PI3K/AKT/mTOR pathway has been shown to serve a crucial role in apoptosis and autophagy [32]; inhibiting the activation of this pathway can induce apoptosis and autophagy. In the present study, it was shown that parthenolide could suppress the levels of PI3K, AKT, pAKT, and mTOR, proteins that have roles in controlling apoptosis and autophagy. Other studies have demonstrated that autophagy induced by anticancer drugs exerts a negative role for cancer therapy. Therefore, an improved understanding of the mechanisms associated with autophagy and apoptosis induced by parthenolide is of importance to breast cancer therapy.

The antiapoptotic gene, B-cell lymphoma 2 (Bcl2), serves a significant role in cancer, and its activity has been reported in several types of cancer [33,34]. Caspase3 is important for proteolytic cleavage of numerous proteins, and controls several upstream genes involved in cell death and the induction of apoptosis. Bcl2 and caspase3 proteins are effective regulators of mitochondrial-mediated apoptosis [35]. In the present study, caspase3 was upregulated and Bcl2 protein was downregulated. Autophagy also plays an essential role in tumor repression due to its involvement with metabolic stress, damage, and tumorigenesis [36]. The present study demonstrated that parthenolide clearly increases the percentage of autophagic cells in breast cancer cells in a concentration-dependent manner. Autophagy induction by parthenolide treatment was further demonstrated by western blot analysis. The present study revealed that parthenolide treatment enhanced the expression of Beclin 1 compared with the control group, a process that is important for autophagy induction. Other published studies have also indicated that parthenolide could cause autophagy in breast cancer. However, the detailed role of autophagy is yet to be fully elucidated. The present study has shifted the focus on to delineating the different mechanisms associated with autophagy, with the aim of identifying novel therapeutic

treatments based on parthenolide. ATG13/ATG14 is another marker of autophagy. ATG13 is essential for autophagy induction. When TORC1 is inactive, ATG13 dephosphorylation occurs rapidly. ATG14 (a Beclin 1 associated, autophagy-related key regulator) can promote autophagosome-endolysosome fusion. The present study revealed that the expression levels of ATG13 and ATG14 were increased, as determined by western blotting and RT-qPCR. Since the PI3K/AKT/mTOR signaling pathway is the key axis in autophagy regulation mediated by anticancer drugs, the PI3K/AKT signaling pathway is activated, and negatively regulates autophagy by repressing mTOR expression in cancer cells. PTEN is a potent inhibitor of the PI3K/AKT pathway, acting as a tumor suppressor [37, 38]. The results of the present study demonstrated that the expression levels of PI3K, AKT, pAKT and mTOR were lower, whereas that of PTEN was higher.

5. Conclusion

Parthenolide can suppress the growth of breast cancer cells. It also induces autophagy and apoptosis by inhibiting the PI3K/AKT/mTOR signal pathway through the activation of PTEN expression. Overall, our study has revealed new information concerning the apoptosis and autophagy-mediated mechanisms of parthenolide in breast cancer. Based on prior studies, autophagy may exert a negative effect on anticancer drugs. Gaining a deeper understanding of the mechanism of autophagy is essential for the development of a new treatment method with parthenolide, which would involve enhancing apoptosis induced by parthenolide.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Acknowledgements

The authors would like to apologize to the many authors whose studies are important, but could not be cited due to space limitations. We are grateful for the members of the Department of Pathology of Dalian Medical University for their helpful discussions and suggestions during the course of this study.

Competing interests

The authors declare that they have no competing interests.

Funding

Not funding

Author Contributions

Yanyan Han contributed to the design and writing of the study. Jinfeng Yang and Yan Sun acquired and analyzed the data. ShuJun Fan and Yanyan Han performed the experiments. Ying Lu read the manuscript, and made substantial contributions to the interpretation and analysis of the data. Yanyan Han and Lianhong Li contributed to drafting the manuscript, critically modifying important content, and approving the version to be published. All authors read and approved the final manuscript.

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Figures

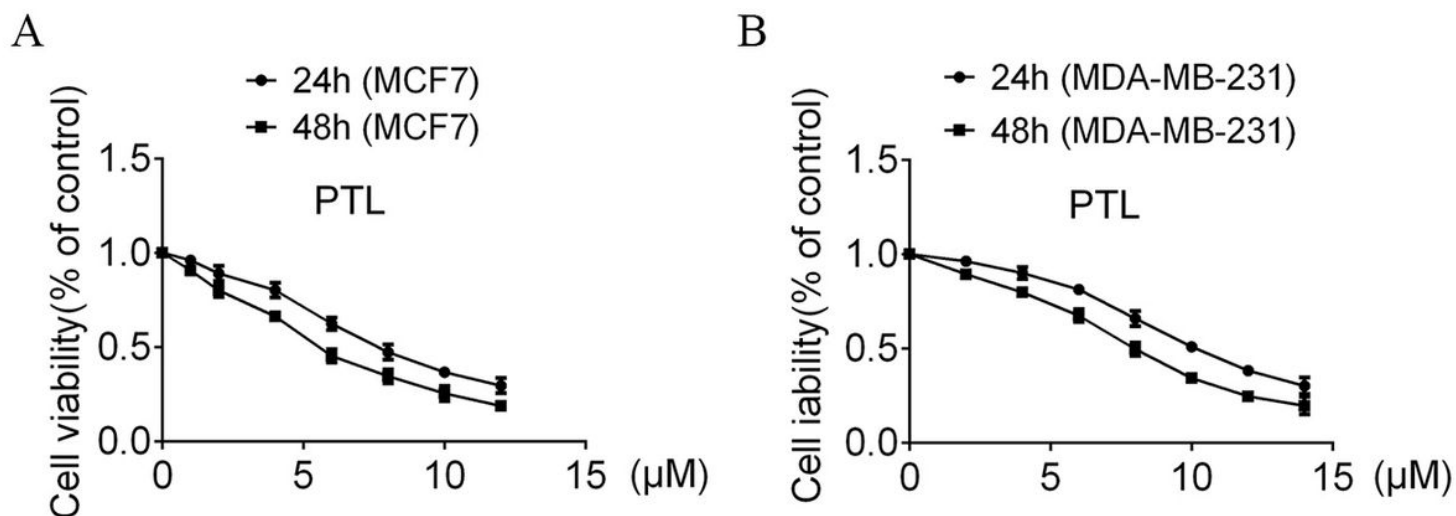


Figure 1

Effects of parthenolide on breast cancer cells. (A and B) MCF 7 and MDA MB 231 cells were treated with different concentrations of parthenolide for 24 and 48 h. Parthenolide suppressed the proliferation of both cell lines in a dose dependent manner. These experiments were performed in triplicate, and data were analyzed using GraphPad Prism 5 software. * $P < 0.05$ vs. control group. Abbreviations: PTL, parthenolide.

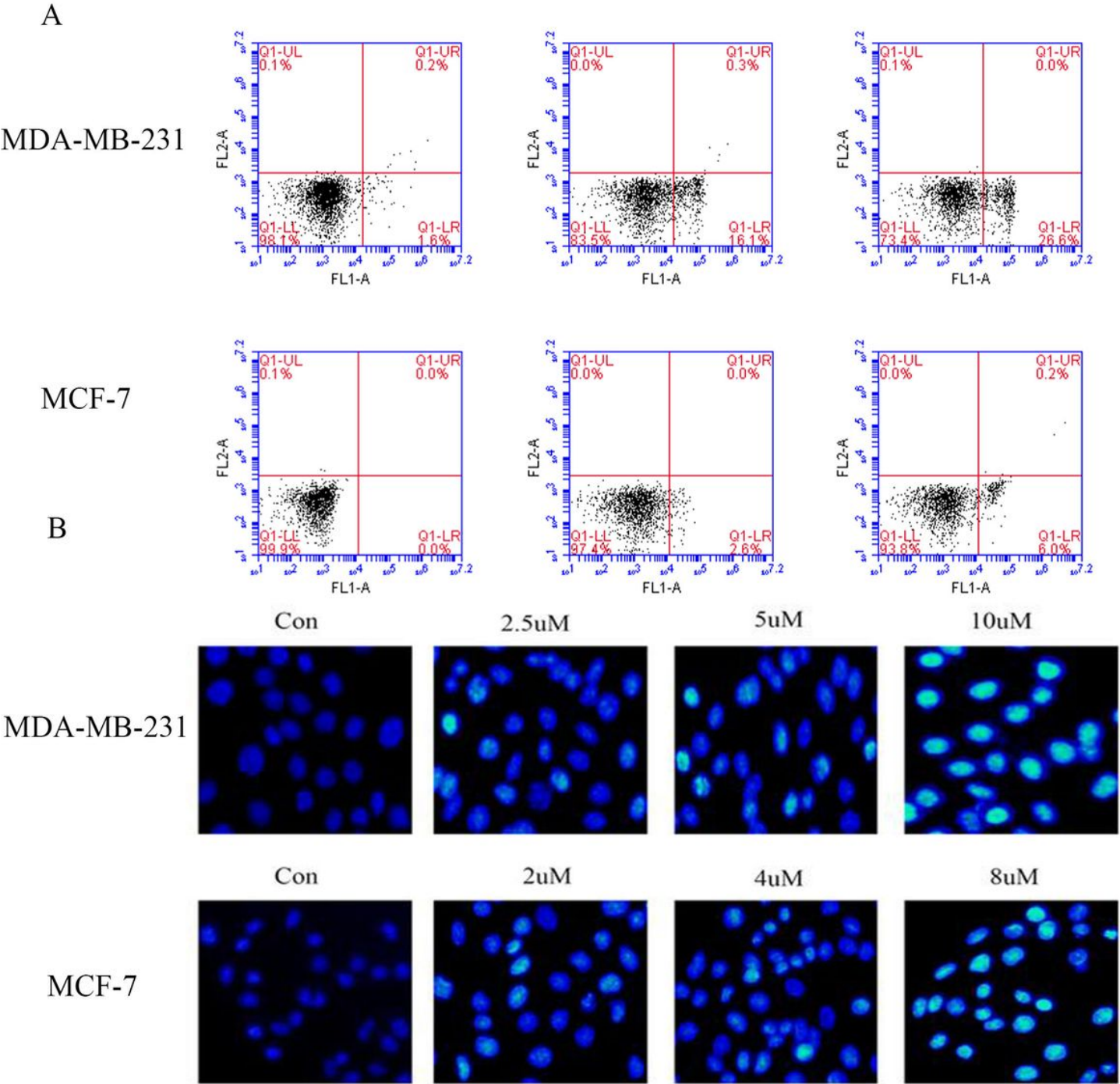


Figure 2

Parthenolide induces apoptosis in human breast cancer cells. (A) MCF 7 and MDA MB 231 cells were treated with different concentrations of parthenolide for 24 h, and annexin V/PI apoptosis assay was performed using flow cytometry. The proportion of apoptotic cells was expressed as a percentage. (B) Nuclear morphology of cell lines pre treated with parthenolide were observed using DAPI staining. Cells of both lines underwent apoptosis, exhibiting an apoptotic nuclear morphology, with features such as

karyopyknosis, chromatic agglutination, and apoptotic bodies, which became increasingly evident with increasing parthenolide concentrations. Abbreviation: DAPI, 4',6 diamidino 2 phenylindole.

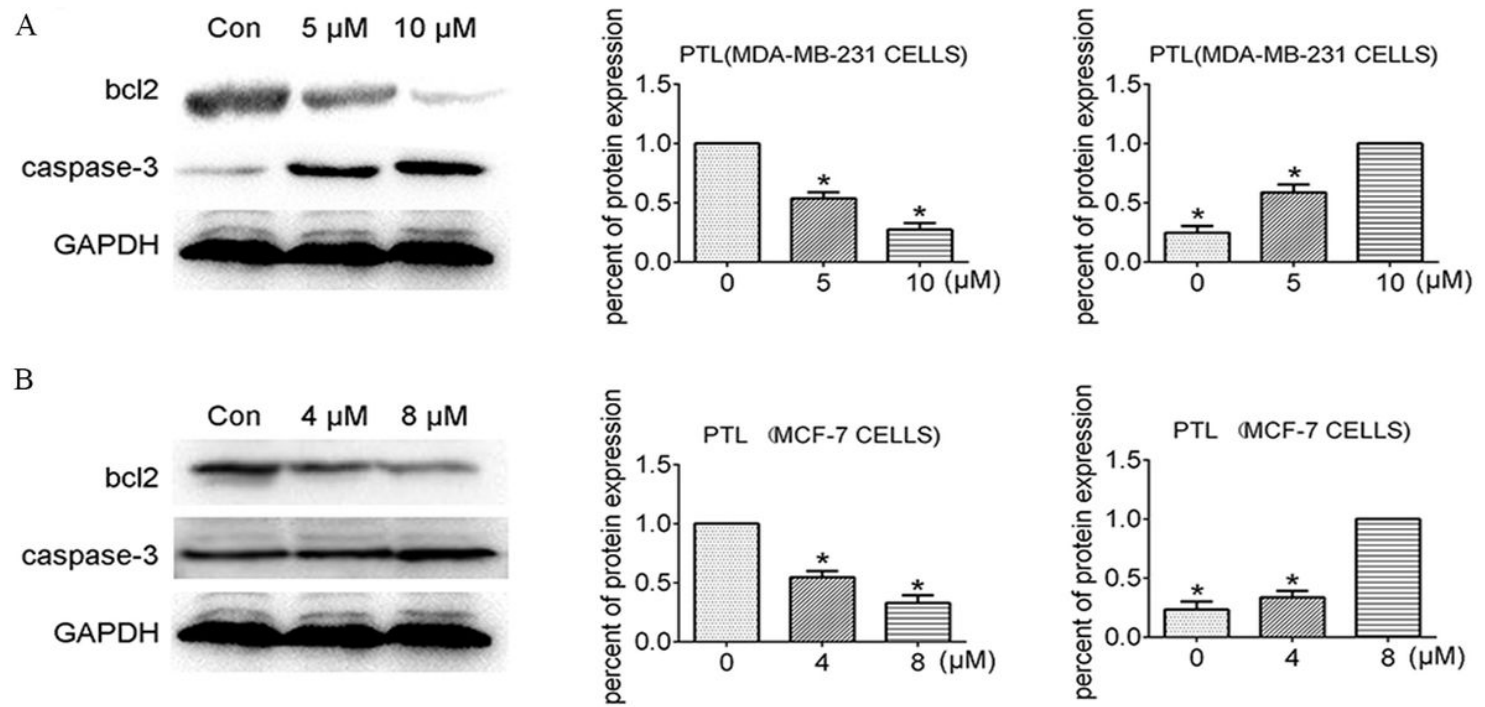


Figure 3

Parthenolide induces the expression of apoptotic proteins. (A and B) MCF 7 and MDA MB 231 cells were treated with various concentrations of parthenolide for 24 h and analyzed by western blotting. The expression levels of pro apoptotic proteins (cleaved caspase 3) and anti apoptotic proteins (Bcl 2) were measured. Blots were quantified using ImageJ software, and data are expressed as the mean±S.D. from three independent experiments. *P<0.05 vs. control group.

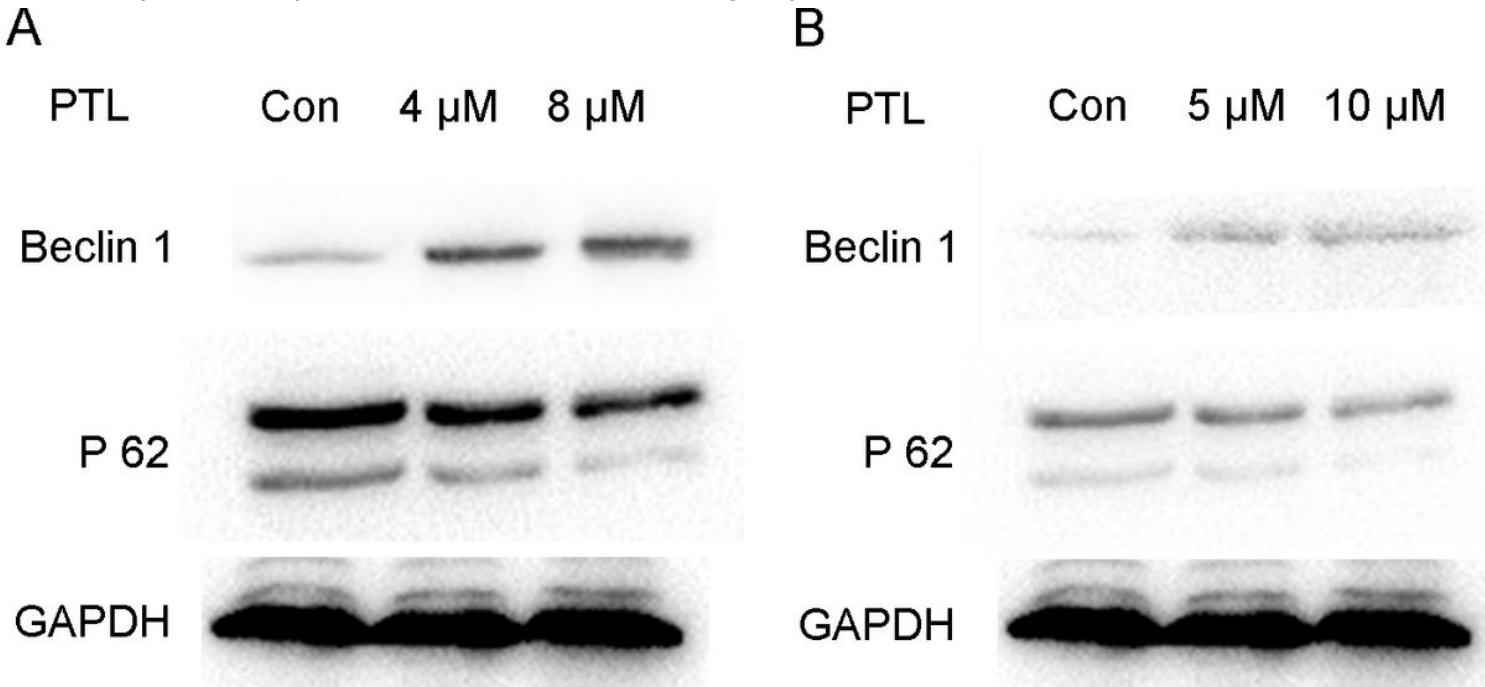


Figure 4

Parthenolide promotes the formation of autophagy. (A and B) Cells were treated with parthenolide for 24 h. The levels of Beclin 1 and P62 were evaluated by western blotting. Representative blots are featured, showing the concentration dependent effect of parthenolide on Beclin1 and P62 expression. Parthenolide treatment led to increased expression of Beclin1 and decreased expression of P62.

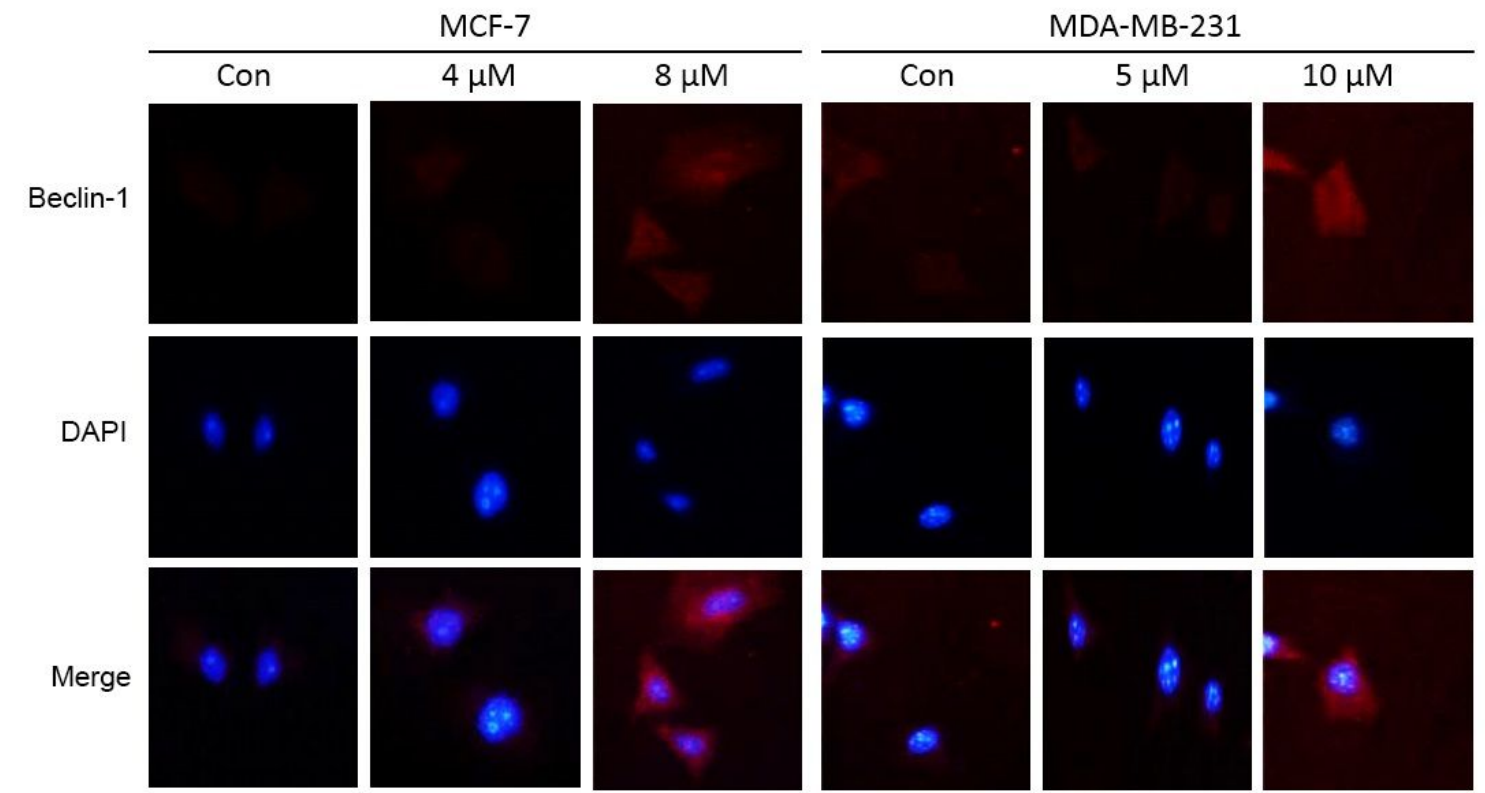


Figure 5

Parthenolide promotes the formation of autophagy. Cells were treated with parthenolide for 24 h, and images were captured using fluorescence microscopy. Red fluorescence indicates the presence of Beclin 1; blue fluorescence represents DAPI. Parthenolide treatment upregulated the expression of Beclin 1. Abbreviations: DAPI, 4',6 diamidino 2 phenylindole.

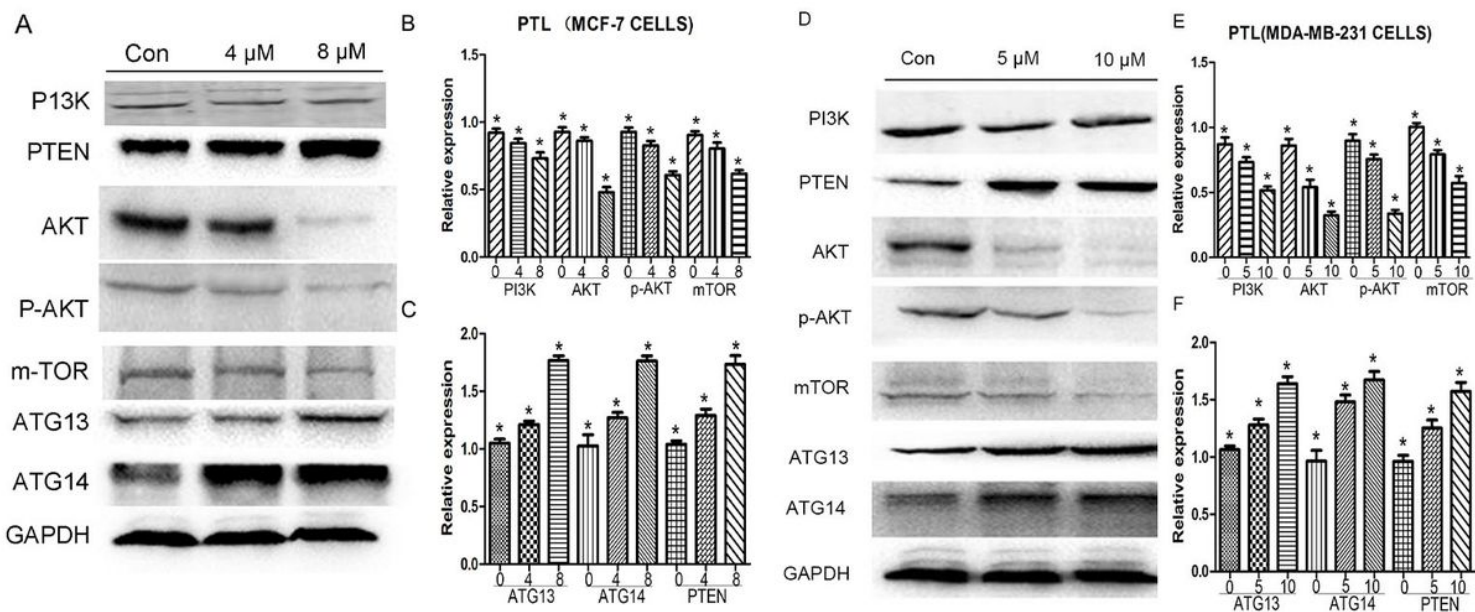


Figure 6

The expression levels of ATG13, ATG14, mTOR, AKT, p AKT, PTEN and PI3K in MCF 7 and MDA MB 231 cells. The cells were treated with parthenolide for 24 h. Western blot analysis was performed to detect protein expression using the indicated antibodies. Parthenolide treatment led to dose dependent decreases in the expression levels of mTOR, AKT, P AKT and PI3K, and increases in the expression of ATG13, ATG14 and PTEN. Data are expressed as the mean \pm S.D. from three independent experiments. *P < 0.05 vs. the control group. Abbreviations: ATG13, autophagy related protein 13; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3 kinase; PTEN, phosphatase and tensin homolog.