Verbascoside induces the cell cycle arrest and apoptosis of breast cancer via suppression of the PI3K/AKT signaling pathway

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Research Article

Keywords: Verbascoside, Breast cancer, Cell cycle, Apoptosis, PI3K/AKT signaling pathway

Posted Date: April 13th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1544829/v1

Abstract

Despite the continuous progression in the diagnosis and treatment of breast cancer, the prognosis of breast cancer patients is still not optimistic. Verbascoside (VER), a pharmacologically active extract of Cistanche is proved possessing anti-cancer activities. Our study was aimed to investigate the anti-proliferation activities of VER and to explore the underlying mechanism in breast cancer MCF-7 and MDA-MB-231 cells. Our work evidenced that the anti-proliferation activity of VER in breast cancer cells may associate with cell cycle arrest and apoptosis. VER treatment dose-dependently altered the expression of cell cycle and apoptosis related proteins. CyclinB1, Cdc2, Bcl-2 and surviving were decreased, while cleaved PARP1, BAX, and cleaved caspase3/9 were increased. Mechanistically, VER suppressed the activity of PI3K/AKT signaling pathway. Our experimental data suggests that VER might be an effective and low toxicity drug against breast cancer.

Introduction

Breast cancer (BC) is the most commonly cancer in women worldwide which represented 11.7% of all new cancer cases in 2020 [1]. Traditional treatment strategies for the patients with BC include surgery, chemotherapy, radiotherapy, biological targeted therapy and endocrine therapy [2]. However, due to multi-drug resistance and tumor heterogeneity, the long-term prognosis of BC patients remains poor [3, 4]. Thus, identifying effective and low toxicity drugs for the treatment of BC is urgently required.

Verbascoside (VER), an active extract of a Chinese traditional medical plant genus, Cistanche, possesses multiple pharmacological effects [5–7] including anti-inflammatory [8, 9], antioxidant [10–14], antimicrobial [15], and anti-tumor activities [16]. Recent reports suggest that VER possesses anticancer effects through targeting the process of cell cycle and apoptosis in various solid tumors [17, 18]. Nevertheless, the anticancer effect of VER in BC remains not studied.

Therefore, our study was to examine the anti-tumor activities of VER in BC cells and to explore possible molecular mechanisms. Our data showed that VER possessed anti-proliferation activity in MCF-7 and MDA-MB-231 cells. Moreover, VER might function as anticancer agent via regulating the activity of PI3K/AKT signaling pathway.

Results

VER inhibits the proliferative potential of BC cells.

The chemical structure of VER was showed in Fig. 1A. The cytotoxic effects of VER at 0, 20, 40, 80, 120, 160, 200, or 240 μM for 24 or 48 h were measured in BC (MCF-7, MDA-MB-231, SKBR3) cells, and MCF-10A cells. The viability of BC cells were examined by the CCK-8 assay. The OD values of MCF-7, MDA-MB-231, SKBR3, and MCF-10A cells were shown in Fig. 1B. The results of CCK-8 assay showed that VER inhibited the proliferative potential of BC cells in a concentration- and time-dependent manner. The IC50 values at 24 and 48 h were 169.68 ± 13.21 and 98.45 ± 7.63 μM, respectively, for MCF-7 cells, 193.56 ±

15.45 and 106.73 ± 9.34 μM for MDA-MB-231 cells, and 226.32.32 ± 17.53 and 109.65 ± 9.62 μM for SKBR3 cells (Fig. 1B). Notably, VER was relatively less cytotoxic to MCF-10A than other BC cell lines. And then, colony formation assay further confirmed that VER restrained the proliferation of MCF-7 and MDA-MB-231 cells. As shown in Fig. 1C, incubation with VER (50, 100, or 150 μM) for 48 h suppressed the colony formation abilities of BC cells.

VER induces cell cycle arrest in BC cells.

The proportion of MCF-7 cells in the G2/M phase increased from 17.38% ± 1.23% at 0 μM VER to 25.98% ± 1.54% at 50 μM, 36.41% ± 2.53% at 100 μM, and 45.92% ± 3.64% at 150 μM VER. The proportion of MDA-MB-231 cells in the G2/M phase increased from 15.3% ± 1.06% at 0 μM VER to 34.65% ± 2.87% at 50 μM, 39.63% ± 2.75% at 100 μM, and 49.57% ± 3.54% at 150 μM VER. Accordingly, the proportions of BC cells in the G0/G1 phase were decreased (Fig. 2).

VER induces cell apoptosis in BC cells.

To investigate whether the anti-tumor activity of VER is related to apoptosis, we performed DAPI staining assay, as expected, the morphology of nucleus was changed, including nuclear condensation, nuclear fragmentation in MCF-7 and MDA-MB-231 cells treated with VER (50, 100 or 150 μM), while the untreated control group exhibited intact nuclei (Fig. 3A), indicating that VER might induce apoptosis. To further verify the activity of VER on inducing apoptosis, the BC cells were treated with 0, 50, 100 or 150 μM VER and subjected to a flow cytometry analysis. Compared with untreated control group, the number of apoptotic BC cells in VER treated groups were dramatically increased in a dose-dependent manner. The proportions of apoptotic MCF-7 and MDA-MB-231 cells were: control, 8.3% ± 0.43% and 8.6% ± 0.51%; 50 μM VER, 15.0% ± 1.07% and 19.9% ± 1.42%; 100 μM VER, 30.5% ± 2.64% and 33.2% ± 2.82%; 150 μM VER, 43.0% ± 3.21% and 43.7% ± 3.58%, respectively (Fig. 3B). Our above results suggested that the antiproliferation activity of VER might be associated with cell cycle (G2/M phase) arrest and apoptosis of MCF-7 and MDA-MB-231 cells.

Effects of VER on the cell cycle and apoptosis related proteins of BC cells.

To investigate the possible mechanisms underlying G2/M phase cell cycle arrest in response to 50, 100, or 150 μM VER treatment, the expression of Cdc2 and cyclinB1 in MCF-7 and MDA-MB-231 cells were examined. Our data revealed that the expression of Cdc2 and cyclinB1 significantly decreased after VER treatment in BC cells (Fig. 4A). Our above results showed the proportion of apoptotic BC cells also was also increased by VER treatment. Thus, the expression levels of BAX and Bcl-2 were examined, and the results showed that VER treatment up-regulated BAX while down-regulated Bcl-2 in BC cells. In regard to other apoptosis-related proteins, cleaved PARP1 and cleaved caspase 3/9 were up-regulated in response to VER treatment, while survivin was down-regulated in BC cells (Fig. 4B). Collectively, our data demonstrated that VER regulates the expression of cell cycle and apoptosis related proteins in BC cells.

VER treatment suppresses PI3K/AKT signaling pathway.

The PI3K/AKT signaling pathway has been reported to regulate the differentiation and proliferation of BC cells [19]. Substantial evidence demonstrated that active ingredient of natural plants including VER, possess the activity to regulate the PI3K pathway [20]. Therefore, the expression of PTEN, PI3K, total and phosphorylated AKT were examined after VER treatment. Our data showed that VER treatment down-regulated the expression of phosphorylated AKT and PI3K, while total AKT expression remained constant. PTEN is the upstream suppressor of PI3K/AKT signaling pathway [21]. Treatment with 50, 100, or 150 μM VER for 48 h up-regulated PTEN protein level, thereby inhibited the PI3K/AKT pathway in BC cells (Fig. 5). In sum, our data demonstrated that the anticancer activities of VER might result from suppression of the activity of PI3K/AKT pathway.

Methods

Reagents and kit. VER was purchased from the National Institutes for Food and Drug Control. Antibodies against PTEN (22034-1-AP), PI3K (20584-1-AP), cyclinB1 (55004-1-AP), Cdc2 (catalog no. 10762-1-AP), Bcl-2 (12789-1-AP), survivin (10508-1-AP), PARP1 (13371-1-AP), BAX (50599-2-Ig), and GAPDH (60004-1-Ig) were all purchased from Proteintech Group, Inc. (Rosemont, IL, USA), while antibodies against phosphorylated AKT ser473 (#4060), and total AKT (#4691), cleaved caspase3 (#9664), cleaved caspase9 (#9509) were acquired from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody against cleaved PARP1 (G215) was purchased from ImmunoWay Biotechnology Company (Plano, TX, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan). Cell cycle and apoptosis detection kits were obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China).

Cell culture. BC cell lines MCF-7, MDA-MB-231, SKBR3, and immortalized breast epithelial cells MCF-10A were all obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's minimum essential medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum.

CCK-8 assay. CCK-8 assay was performed to investigate the anti-proliferation effects of VER in BC cells and MCF-10A cells. Briefly, MCF-7, MDA-MB-231, SKBR3 cells and immortalized epithelial cells MCF-10A were seeded in 96-well plates at 1×10^4 cells/well and treated with different concentration (0, 20, 40, 80, 120, 160, 200, or 240 μM) VER for 24 or 48 h. Followingly, 10μL of CCK-8 was added and incubated for 2 h at 37°C. Finally, the optical density at 450 nm (OD 450) was measured by microplate reader.

Colony formation assay. After treatment with 0, 50, 100, or 150 μM VER for 48 h, the BC cells were subcultured for 14 days. Then, viable colonies were fixed, and stained with 0.5% crystal violet.

Cell cycle analysis. The proportions of each cell cycle phase in BC cells were examined through flow cytometry analysis. Briefly, the MCF-7 and MDA-MB-231 cells were cultured overnight, and treated with 0, 50, 100, or 150 μM VER for 48 h. Afterward, viable BC cells were collected, fixed with 70% ethanol

overnight, washed and treated with RNase A and propidium iodide for 30 min in the dark. The DNA contents were determined by a flow cytometer.

DAPI staining assay. MCF-7 and MDA-MB-231 cells were cultured on coverslips overnight. After treatment with 0, 50, 100, or 150 μM VER for 48 h, cells were fixed and stained with DAPI for 30 minutes in room temperature, washed with 1 × PBS, finally observed and photographed by a fluorescence microscopy.

Apoptosis analysis. The proportions of apoptotic BC cells were examined by an Annexin V-fluorescein apoptosis analysis. MCF-7 and MDA-MB-231 cells were treated with 0, 50, 100, or 150 μM VER for 48 h and then incubated with propidium iodide and Annexin V-fluorescein for 30 min in the dark. The apoptotic cells were detected by a flow cytometer.

Western blot analysis. Radioimmunoprecipitation assay buffer containing protease inhibitors was used to extract the total protein of MCF-7 and MDA-MB-231 cells after 0, 50, 100, or 150 μM VER treatment. BC cells protein samples were separated by electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes, blocked for 1 h at room temperature and incubated with primary Antibodies at 4°C overnight. Then, the membranes were washed twice and incubated with the secondary Antibodies at 37°C for 1 h. Target protein bands were detected by the ChemiDoc XRS + System.

Statistical analysis. GraphPad Prism software 6.0 (GraphPad Software Inc., La Jolla, CA, USA) was used to perform Statistical analyses. Data of three independent experiments were presented in bar graphs as the mean ± standard deviation (SD). The probability value < 0.05 was considered statistically significant.

Discussion

Limited options for clinical treatment remain a primary challenge for patients with BC. Growing evidence indicate that active constituents of natural plants may be a new avenue to develop successful cancer treatments [22]. VER, an active agent derived from Cistanche, has been used extensively as an anti-inflammatory, antioxidant, and antimicrobial agent. In vitro and in vivo studies have demonstrated that VER exerts anticancer effect[17, 18, 20]. The present study investigated the effect of VER on BC cells and also explored the underlying mechanisms.

Loss of normal cell cycle control is a crucial hallmark of malignant tumors. Interestingly, VER has been reported to induce cell cycle arrest and apoptosis in various solid malignant tumors, such as CRC, prostate cancer, melanoma, and glioma. As expected, our data showed a significant G2/M phase arrest in MCF-7 and MDA-MB-231 cells after VER treatment for 48 h (Figure. 2). To understand the possible mechanism by which VER induces G2/M phase arrest, Cdc2 and cyclinB1 which are main mediators involved in controlling G2 phase progression and G2/M phase transition were examined. Our results showed that treatment with VER led to down-regulation of Cdc2 and cyclinB1. However, it was reported that VER inhibited the growth of human CRC cancer Caco-2 and HCT-116 cells by inducing G0/G1 phase arrest [20], indicating that the inner molecular mechanism by which VER induces cell cycle arrest may be cancer cell type-dependent.

Inducing apoptosis is a common mechanism of anticancer agents [23]. To investigate the possible mechanisms by which VER induces apoptosis, we examined the protein levels of cleaved PARP1, cleaved caspase3, and cleaved caspase9. Our data showed that treatment with VER increased the expression of cleaved PARP1 and cleaved caspase3/9, indicating that the mitochondrial signaling pathway (intrinsic pathway) may be the possible pattern by which VER induced apoptosis.

Previous reports have showed that the PI3K/AKT pathway regulates cell cycle, apoptosis and thus was involved in the progression of multiple solid tumors [24–26], including BC [19]. As a negative regulator of PI3K/AKT signaling pathway, PTEN exerts inhibitory effect on tumor cells growth. In the present study, we investigated the effect of VER on the PI3K/AKT pathway by examining the expression levels of PTEN, PI3K, phosphorylated and total AKT. Our data showed that VER treatment up-regulated the expression of PTEN and down-regulated PI3K and phosphorylated AKT protein levels in BC cells, while the expression of total AKT remained constant. Thus, regulating the expression of PTEN and suppressing PI3K/AKT signaling pathway may represent a possible molecular mechanism of VER in BC cells.

Conclusion

In conclusion, the present study demonstrated that VER inhibits the proliferation of MCF-7 and MDA-MB-231 cells through inducing cell cycle (G2/M phase) arrest and apoptosis. Furthermore, we have discovered that VER up-regulates PTEN protein level and thus restrains PI3K/AKT pathway, might be an possible inner mechanism by which VER inhibits the proliferation of BC cells. Our study showed that VER could be a candidate for the therapeutic application for BC patients.

Declarations

Acknowledgments. This present study was supported by Natural Science Foundation of Liaoning (20180551104).

The authors declare no competing interests.

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Figures

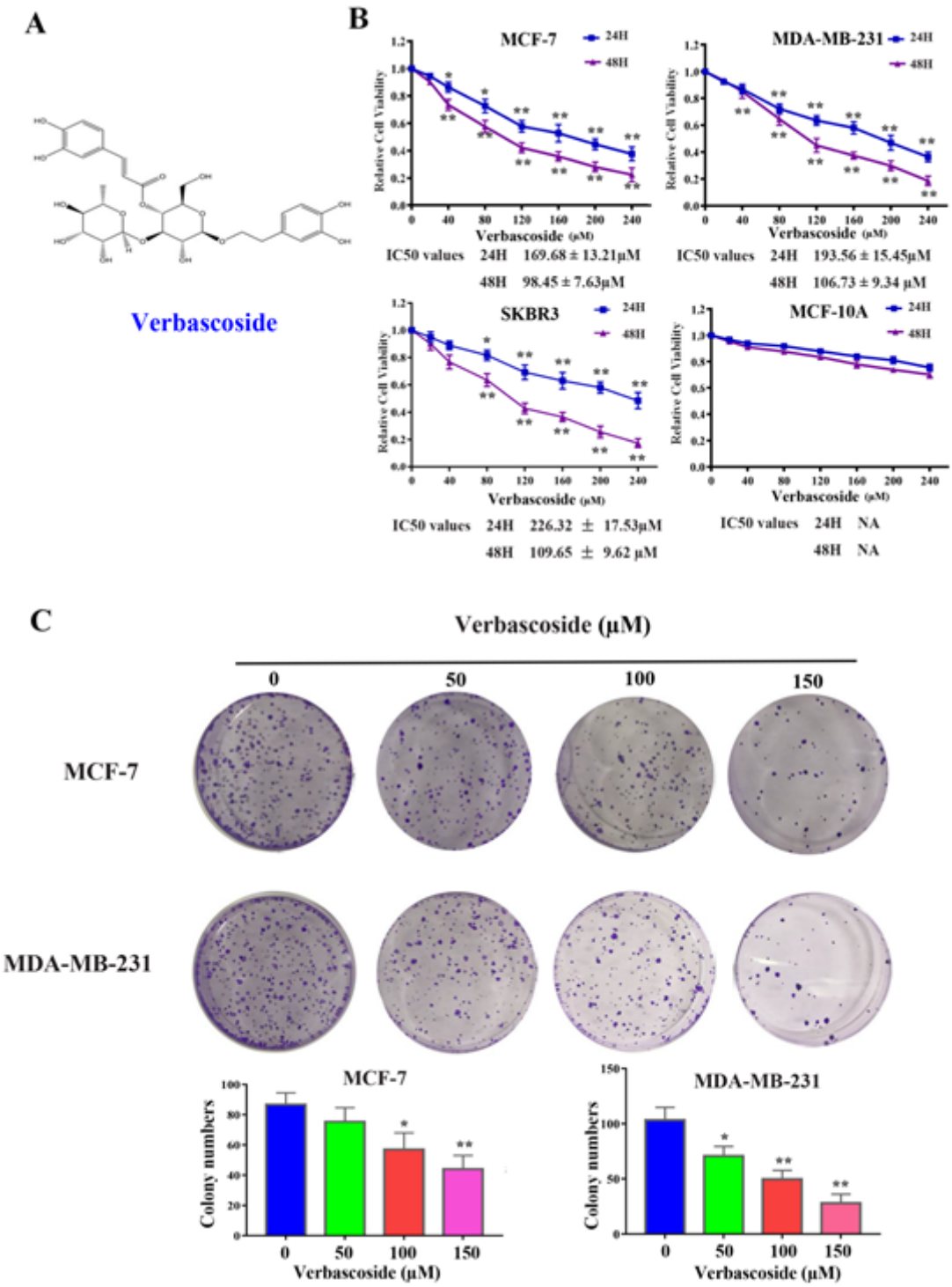

Figure 1

Anti-proliferation effect of Verbascoside (VER) on breast cancer (BC) cells. (A) The chemical structure of VER. (B) MCF-7, MDA-MB-231, SKBR3 and breast MCF-10A cells were incubated with 0, 20, 40, 80, 120, 160, 200, or 240 μM VER for 24 or 48 h. The viabilities of BC cells were measured with the CCK-8 assay. (C) VER inhibited the ability of colony formation in MCF-7 and MDA-MB-231 cells *p < 0.05, **p < 0.01 vs. the control group.

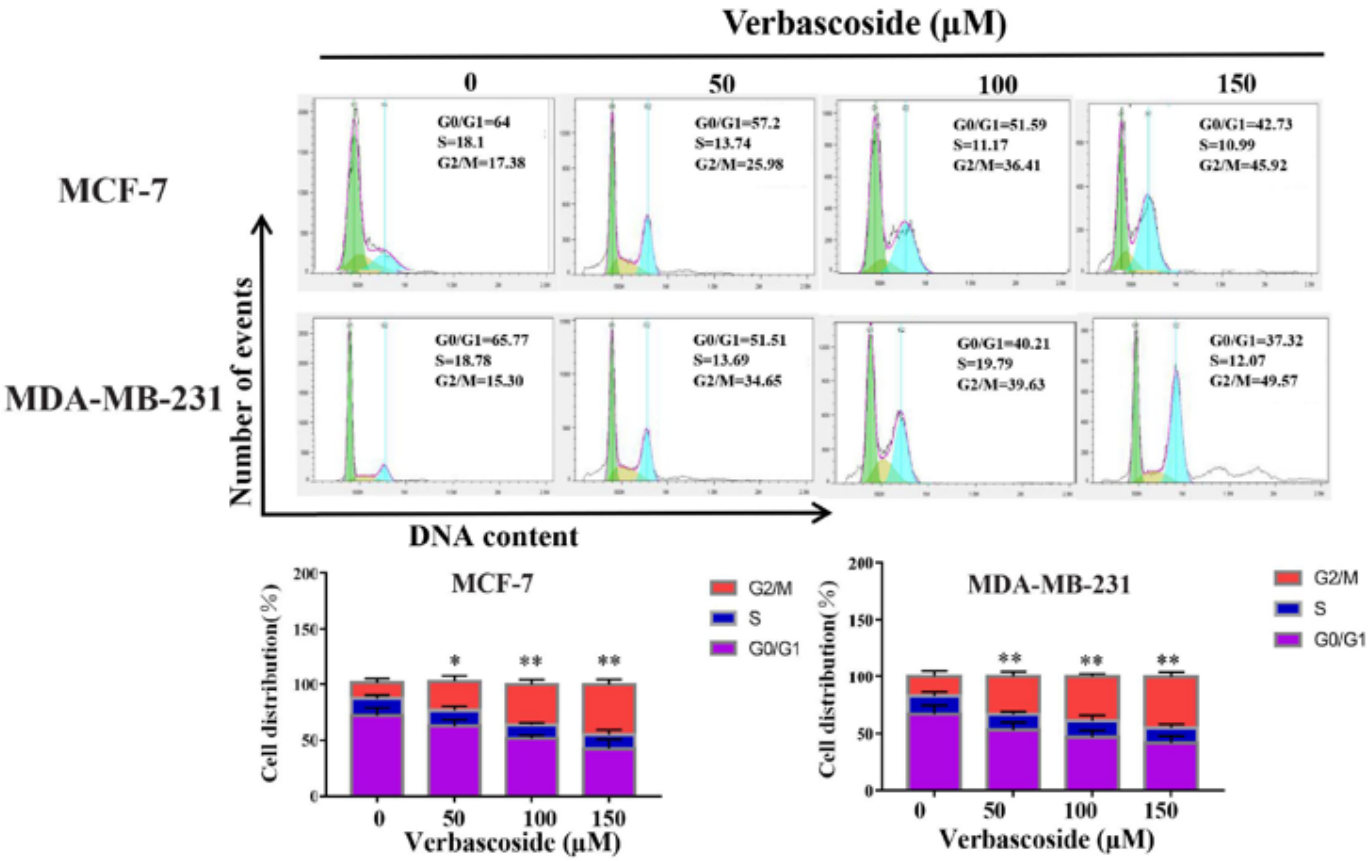

Figure 2

Effect of Verbascoside (VER) on cell cycle distribution in breast cancer (BC) cells. MCF-7 and MDA-MB-231 cells were incubated with VER (0, 50, 100, or 150 μM) for 48 h. Cell cycle distribution of MCF-7 and MDA-MB-231 cells was analyzed by flow cytometry assay. The proportions of MCF-7 and MDA-MB-231 cells in G0/G1 phases, S phases, and G2/M phases are presented in the histograms. *p < 0.05, **p < 0.01 vs. the control group.

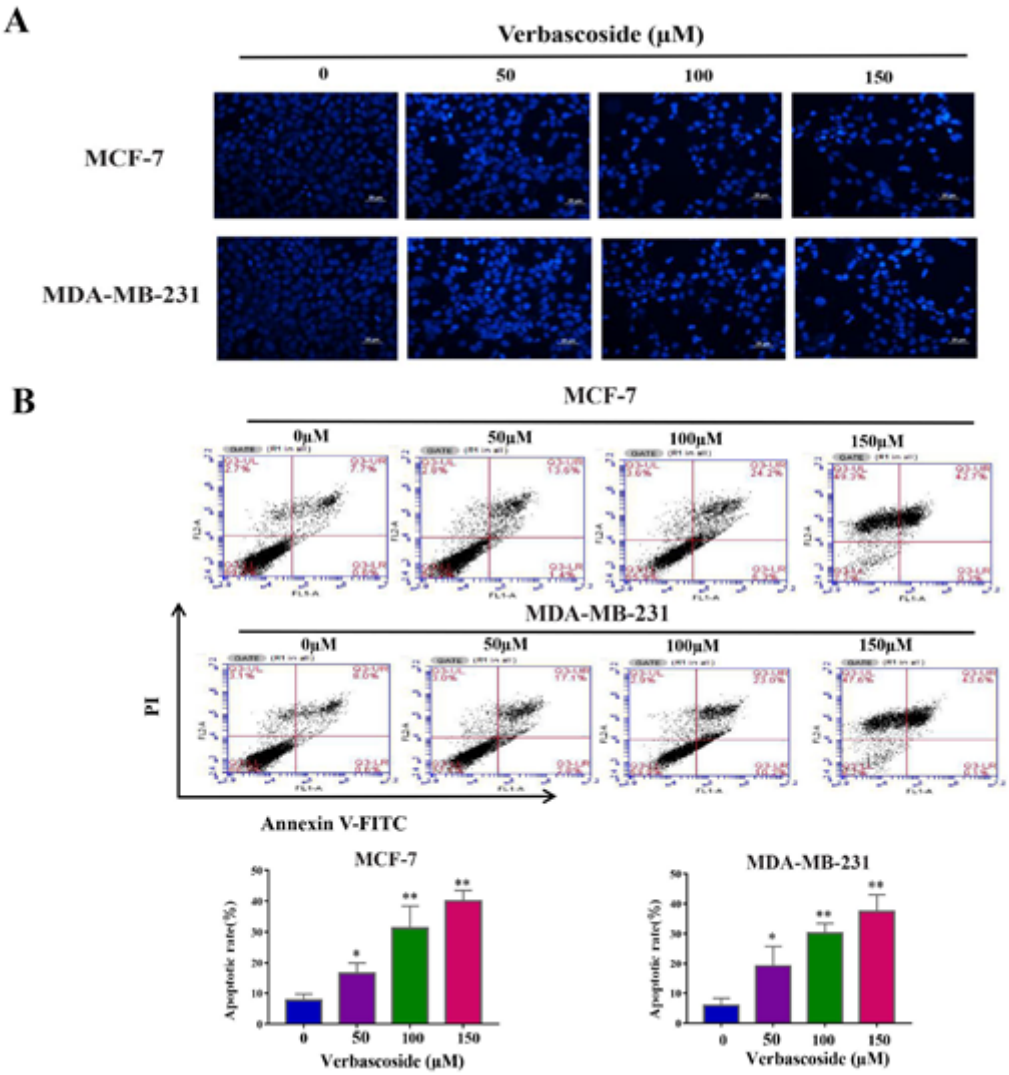

Figure 3

Effect of Verbascoside (VER) on cell apoptosis in breast cancer (BC) cells.

(A) MCF-7 and MDA-MB-231 cells were incubated with VER (0, 50, 100, or 150 μM) for 48 h. The morphology of the cell nucleus was analyzed by DAPI staining (40×). (B) MCF-7 and MDA-MB-231 cells were incubated with 0, 50, 100, or 150 μM VER for 48 h. Annexin V/PI flow cytometry analysis was performed to examine the proportions of apoptotic cells, which are presented in histograms. *$p < 0.05$, **$p < 0.01$ vs. the control group.

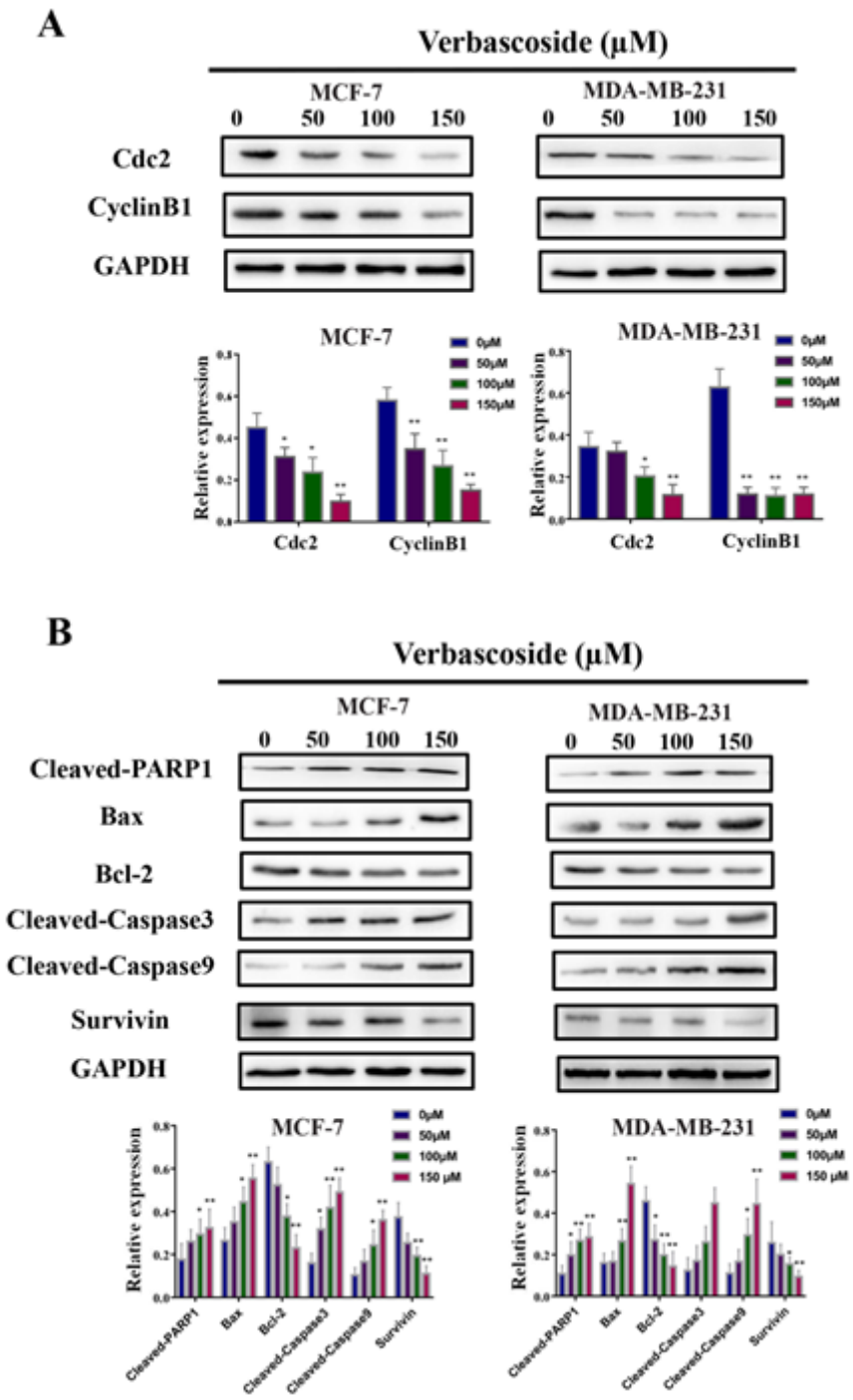

Figure 4

Effects of Verbascoside (VER) on proteins involved in the cell cycle and apoptosis of BC cells. MCF-7 and MDA-MB-231 cells were incubated with 0, 50, 100, or 150 μM VER for 48 h. (A) Expression of cyclinB1 and Cdc2 were examined. (B) Expression of cleaved PARP1, Bax, Bcl-2, cleaved caspase 3, cleaved caspase 9, and survivin were assessed. GAPDH served as an internal control. Bands were quantified using Image J software. Each bar represents the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 vs. the control group.

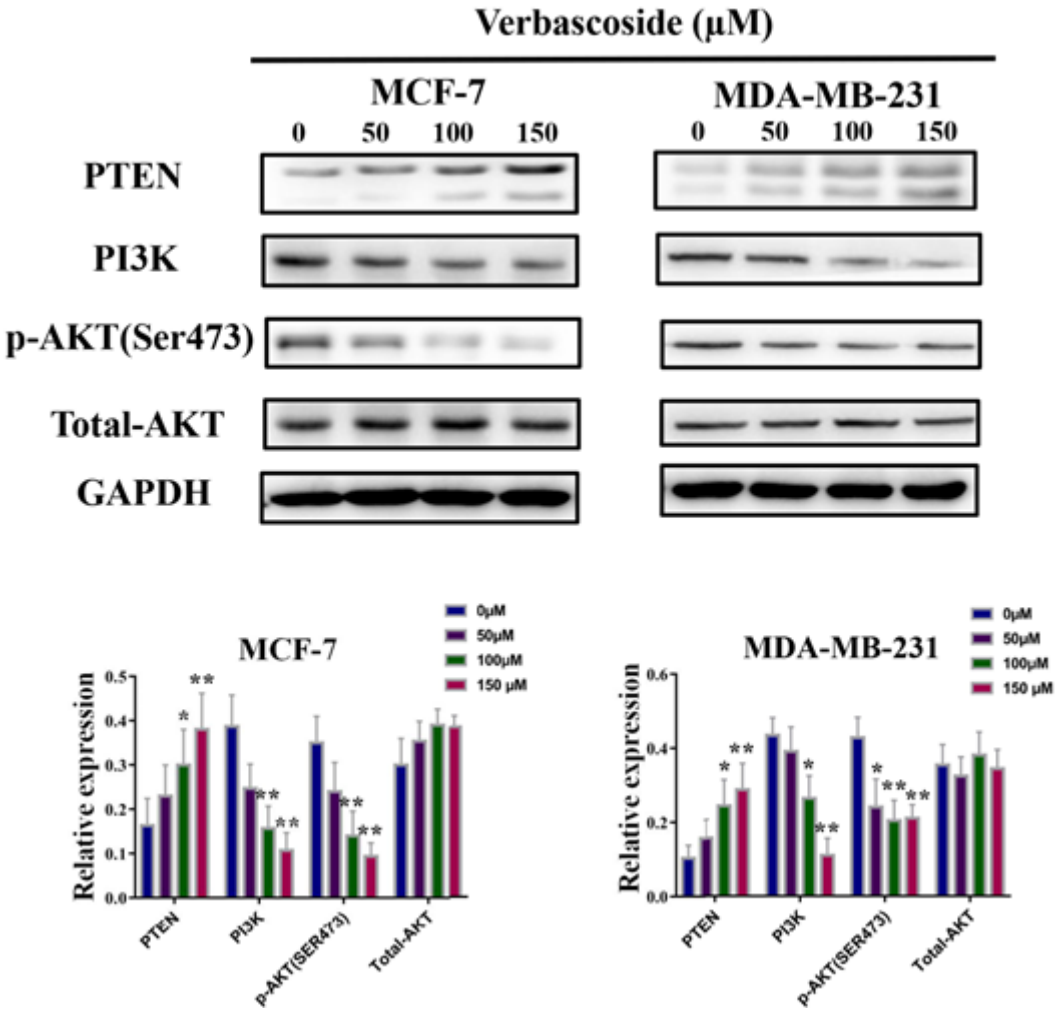

Figure 5

Verbascoside (VER) regulates the PI3K/AKT signaling pathway. MCF-7 and MDA-MB-231 cells were incubated with VER (0, 50, 100, or 150 μM) for 48 h. The expression levels of PTEN, PI3K, phosphorylated and total AKT were measured. GAPDH was used as an internal control. Protein bands were quantified using Image J software. Each bar represents the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 vs. the control group.