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ORIGINAL RESEARCH

# Taraxasterol stimulates apoptosis of BCa cells *via* restraining PI3K/AKT and epidermal growth factor receptor (EGFR)

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**Abstract**

Breast cancer (BCa) is a prevalent form of tumor, necessitating the development of more effective therapeutic drugs to improve patient prognosis. Taraxasterol (TAX), derived from the dandelion plant, has demonstrated significant physiological and pharmacological activity, but its role in BCa and underlying mechanism remains unexplored. In this study, we investigate the effects of TAX on the growth, motility and apoptosis of BCa cells *in vitro* to determine its mechanistic actions. Our results demonstrate that TAX significantly reduces both the proliferation and motility of BCa cells and induces apoptosis in BCa cells, as confirmed through flow cytometry (FCM). This apoptotic effect was further supported by JC-1 staining, which showed alterations in mitochondrial membrane potential in BCa cells after TAX treatment. Mechanistically, TAX suppresses the phosphatidylinositide 3-kinases/protein kinase B (PI3K/AKT) and EGFR pathways and impedes BCa progression. Collectively, TAX stimulates BCa cell apoptosis by inhibiting the PI3K/AKT and EGFR pathways.

**Keywords**

BCa; Taraxasterol (TAX); Apoptosis; Mitochondrial membrane potential; PI3K/AKT

## 1. Introduction

Breast cancer (BCa) is a prevalent and clinically challenging tumor type [1]. In recent decades, our understanding on BCa has gradually evolved, revealing its complexity, based on which it is currently classified into various subtypes according to patient immunohistochemical markers, clinicopathological features, genomic alterations and gene expression profiles [2]. Abnormal expression of the EGFR has been implicated in driving growth stimulation and tumorigenesis across multiple cancer types [3, 4]. To combat BCa, EGFR inhibitors such as gefitinib, lenatinib and erlotinib have been investigated in clinical trials and have shown promise in modulating BCa cell proliferation and improving treatment outcomes [5]. Nevertheless, despite these advancements, there remains an urgent need for the development of more effective therapeutic drugs to further enhance the survival rates of patients with advanced BCa.

Taraxasterol (TAX) is a triterpenoid compound derived from the dandelion plant, known for its robust physiological and pharmacological properties [6]. TAX has been found to possess anti-inflammatory, antioxidant and anticancer activities. It has also been found to ameliorate liver damage in broiler chickens by modulating oxidative stress, apoptosis and autophagy [7, 8] and alleviate fatty acid-induced lipid deposition [9]. Moreover, a previous study indicated that TAX suppresses inflammation in an osteoarthritis rat model by targeting the nuclear factor kappa-B (NF-$\kappa$B) pathway.

In tumors, TAX has been found to exhibit various inhibitory effects. For instance, it can hinder Transforming growth factor (TGF)-$\beta$1-induced epithelial-mesenchymal transformation in thyroid papillary cancer cells through the Wnt/$\beta$-catenin pathway [10], restrain the proliferation of prostate cancer cells [11], inhibit the growth of gastric cancer by suppressing EGFR/AKT1, and hinder the growth of lung cancer by inducing early apoptosis and changes in mitochondrial membrane potential [12]. However, its role and potential mechanism in BCa remain unclear.

Herein, we investigated the effects of TAX on the growth, motility and apoptosis of BCa cells, as well as uncovering its underlying mechanisms.

## 2. Materials and methods

### 2.1 Cell culture and treatment

Human tripple-negative breast cancer (TNBC) cell lines MDA-MB-231 and MDA-MB-468 were purchased from the American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), then exposed to varying concentrations (0, 5, 15 and 30 $\mu$M) of TAX (Sigma) for 24 hours.


  

## 2.2 Western blotting

The samples were initially separated through Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and subsequently transferred onto a Polyvinylidene Fluoride (PVDF) membrane. Next, they were blocked using a 5% milk solution for 1 hour, incubated with primary antibodies, including Bax (Abcam, ab32503; 1:1000), Bcl-2 (Abcam, ab182858; 1:1000), Cleaved caspase-3 (Abcam, ab32042; 1:1000), Cleaved caspase-9 (Abcam, ab202068; 1:1000), Akt (Abcam, ab8805; 1:1000), p-Akt (the phosphorylation is T308, ab38449; 1:500), PI3K (Abcam, ab302958; 1:1000), p-PI3K (Abcam, ab278545; 1:500), EGFR (Abcam, ab52894; 1:1000), p-EGFR (Abcam, the phosphorylation is Y1068, ab40815; 1:500) and $\beta$-actin (Abcam, ab8226; 1:3000) overnight at 4 °C , incubated with secondary antibodies for 1 hour, and underwent chemiluminescence before being photographed and assessed.

## 2.3 CCK-8 assay

MDA-MB-231 cells (1000 cells per well) were plated and cultured for 1, 2, 3 and 4 days. The cells were incubated with cell counting kit-8 (CCK-8, Beyotime, China) for 3 hours every day, following which their optical density (OD) 450 values were measured.

## 2.4 Colony formation assay

MDA-MB-231 cells (1000 cells per well in a 6-well plate) were cultured for 14 days. Following incubation, the cells were fixed with 4% paraformaldehyde, stained with a 0.1% crystal violet solution for 15 minutes, and subsequently, the number of colonies formed was quantified.

## 2.5 Transwell assay

For the invasion and migration assays, BD Falcon inserts from BD Biosciences, Inc. (NJ, USA) were used as the upper chambers and 24-well plates as the lower chambers. In each well of the 24-well plate, $10^5$ cells were plated into the upper Transwell chambers. For the invasion assay, 20% matrigel was added to the upper chamber, while for the migration assay, no matrigel was added. Both assays were conducted in a culture medium devoid of serum. Subsequently, the cells that had invaded the lower chamber were fixed using 4% paraformaldehyde for 25 minutes and stained with a 2% crystal violet solution for an additional 25 minutes.

## 2.6 Cell apoptosis assay

MDA-MB-231 cells, plated in a 6-well plate at a density of $1 \times 10^6$ cells per well, were fixed by incubation in 70% ethanol at −20 °C for 2 hours. Then, the cells were stained with Propidium Iodide (PI, 10 $\mu$L) and fluorescein isothiocyanate isomer I (FITC)- Annexin V (10 $\mu$L, C1062, Beyotime, Shanghai, China) at 4 °C , and their apoptosis levels were assessed using a flow cytometer (FACSCalibur, BD Biosciences, Inc., Franklin Lake, NJ, USA).

## 2.7 JC-1 staining

MDA-MB-231 cells were seeded at a density of $10^5$ cells per well in a 24-well plate and allowed to adhere for 24 hours. Following two washes with phosphate-buffered saline (PBS), the cells were incubated with a 2 $\mu$M solution of JC-1 (Beyotime, Shanghai, China) for 15 minutes at 37 °C to assess mitochondrial damage.

## 2.8 Statistics

Statistical analysis was performed using GraphPad v8.0 (GraphPad, La Jolla, CA, USA). Each experiment was repeated thrice, and the data are shown as mean $\pm$ standard deviation (SD). The unpaired Student's *t*-test was used to determine the statistical significance between two groups, which was set at $p < 0.05$.

# 3. Results

## 3.1 TAX reduces BCa cell proliferation and migration

To assess the impact of TAX on BCa cell growth, we initially examined its effects on the MDA-MB-231 and MDA-MB-468 BCa cell lines at various concentrations (0, 5, 15, 30 $\mu$M) and time intervals (1, 2, 3, 4 days). The results showed that TAX treatment led to a reduction in the optical density (OD) of both MDA-MB-231 and MDA-MB-468 cells, particularly at higher concentrations (Fig. 1A). Additionally, our data revealed that TAX treatment significantly diminished the colony counts of BCa cells, indicating inhibition of cell proliferation (Fig. 1B). Further investigations using transwell assays using MDA-MB-231 cells treated with TAX at 0, 5, 15 and 30 $\mu$M for 24 hours showed that TAX treatment effectively suppressed cell migration (Fig. 1C). Similarly, the transwell assay results confirmed that TAX treatment restrained the invasion capability (Fig. 1D). Overall, these findings demonstrate that TAX reduces both the proliferation and motility of BCa cells.

## 3.2 TAX induces apoptosis of BCa cells

FCM assays were performed to investigate the impact of TAX on the apoptosis, and the results revealed that TAX treatment induced apoptosis in these cells (Fig. 2A,B). Additionally, we observed an upregulation of Bax, cleaved caspase-3 and cleaved caspase-9, along with a concurrent downregulation of Bcl-2 expression, in MDA-MB-231 cells following TAX treatment (Fig. 2C,D), providing further evidence supporting that TAX stimulates apoptosis in BCa cells.

## 3.3 TAX affected the potential of mitochondrial membrane in BCa cells

The aggregate dye JC-1 is a commonly used probe for assessing changes in mitochondrial potential. Our observations in TAX-treated MDA-MB-231 cells revealed an increase in JC-1 monomers and a decrease in JC-1 aggregates, signifying a reduction in mitochondrial membrane potential (Fig. 3), suggesting that TAX influences mitochondrial membrane potential in BCa cells.

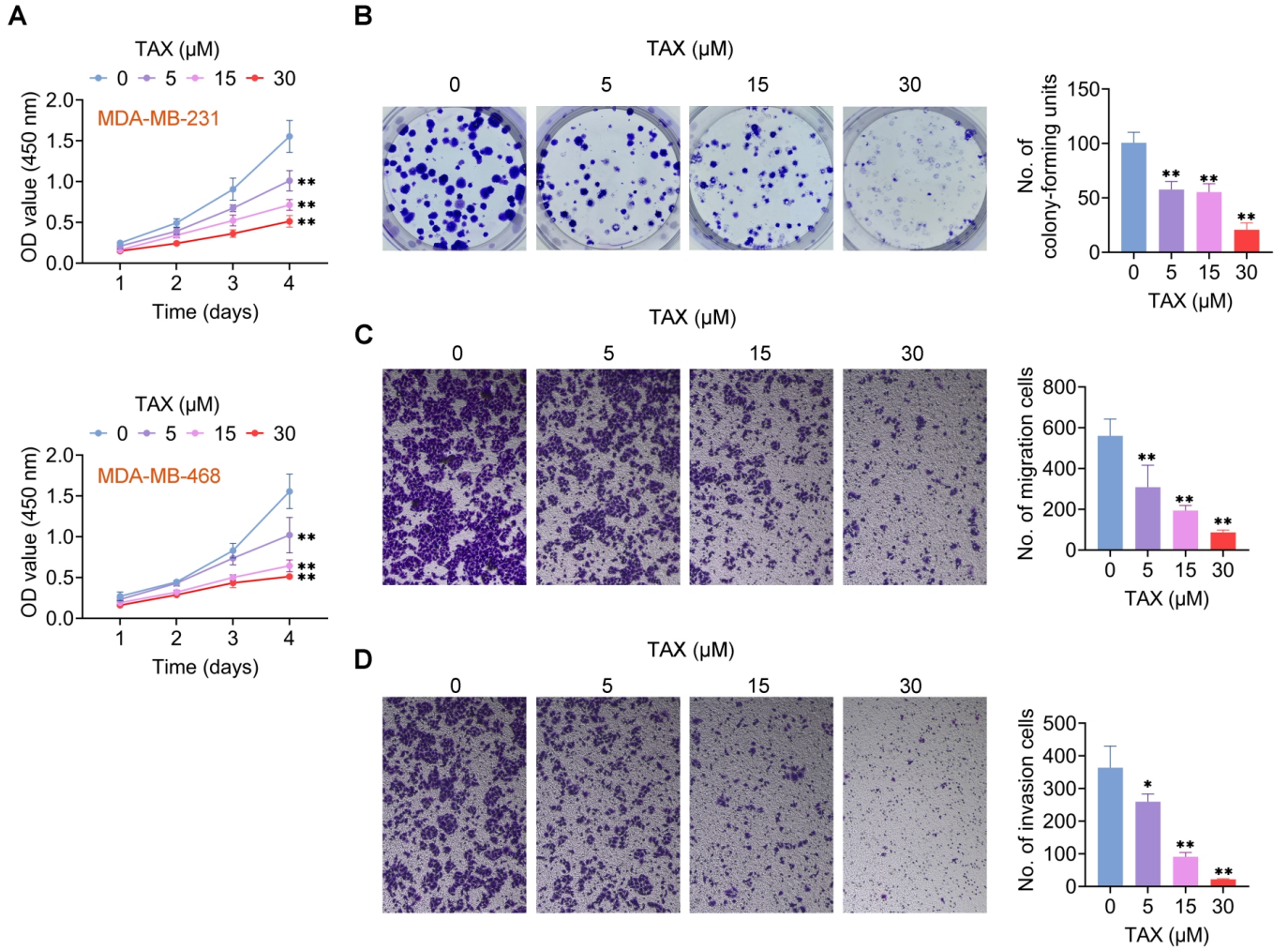


**FIGURE 1. TAX reduces the proliferation and migration of BCa cells.** (A) CCK-8 assays showing the impact of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the cell viability, with measurements of the corresponding OD450 values shown. (B) Colony formation assays illustrating the effects of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the viability of MDA-MB-231 cells and quantification of the colony numbers. (C) Transwell assays demonstrating the influence of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the migration of MDA-MB-231 cells and counting of the number of migrating cells. (D) Transwell assays showing the impact of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the invasion of MDA-MB-231 cells and counting the number of invading cells. Data are presented as mean $\pm$ SD. *$p < 0.05$, **$p < 0.01$. OD: optical density; TAX: Taraxasterol.

### 3.4 TAX suppressed PI3K/AKT and EGFR pathways in BCa cells

Lastly, we examined the impact of TAX on the PI3K/Akt pathway and observed an increase in phosphatase and tensin homolog deleted on chromosome ten (PTEN) levels (Fig. 4A) and a reduction in the phosphorylation levels of Akt and PI3K in both cells following TAX treatment (Fig. 4B), as well as a decrease in EGFR phosphorylation in response to TAX treatment (Fig. 4C), thereby indicating that TAX can effectively suppress the PI3K/Akt and EGFR pathways in BCa cells.

## 4. Discussion

BCa constitutes approximately 23% of cancer cases and accounts for 14% of global cancer-related deaths [13]. Over the past two decades, China has witnessed an alarming increase in BCa incidence and mortality, with a noticeable shift towards younger age groups. The development of BCa is a multifactorial process that unfolds through various stages [14]. Presently, there is a lack of effective therapeutic targets for TNBC, known for its aggressive nature, potentially due to cellular processes, such as proliferation, apoptosis and motility, which are intricately regulated in BCa and impact tumor initiation, distant metastasis and invasion [15]. Additionally, mitochondria, essential for maintaining cellular functions, have been implicated in cancer-related changes [16, 17]. Interestingly, our research findings suggest that TAX has the potential to inhibit BCa progression *in vitro* by influencing key aspects such as cell growth, apoptosis and mitochondrial function, positioning TAX as a candidate for further exploration as a BCa treatment option.

We observed a comprehensive range of effects of TAX on BCa cells, including growth inhibition, motility suppression, apoptosis induction and modulation of mitochondrial function. Our data strongly support TAX's ability to inhibit BCa *in vitro*.

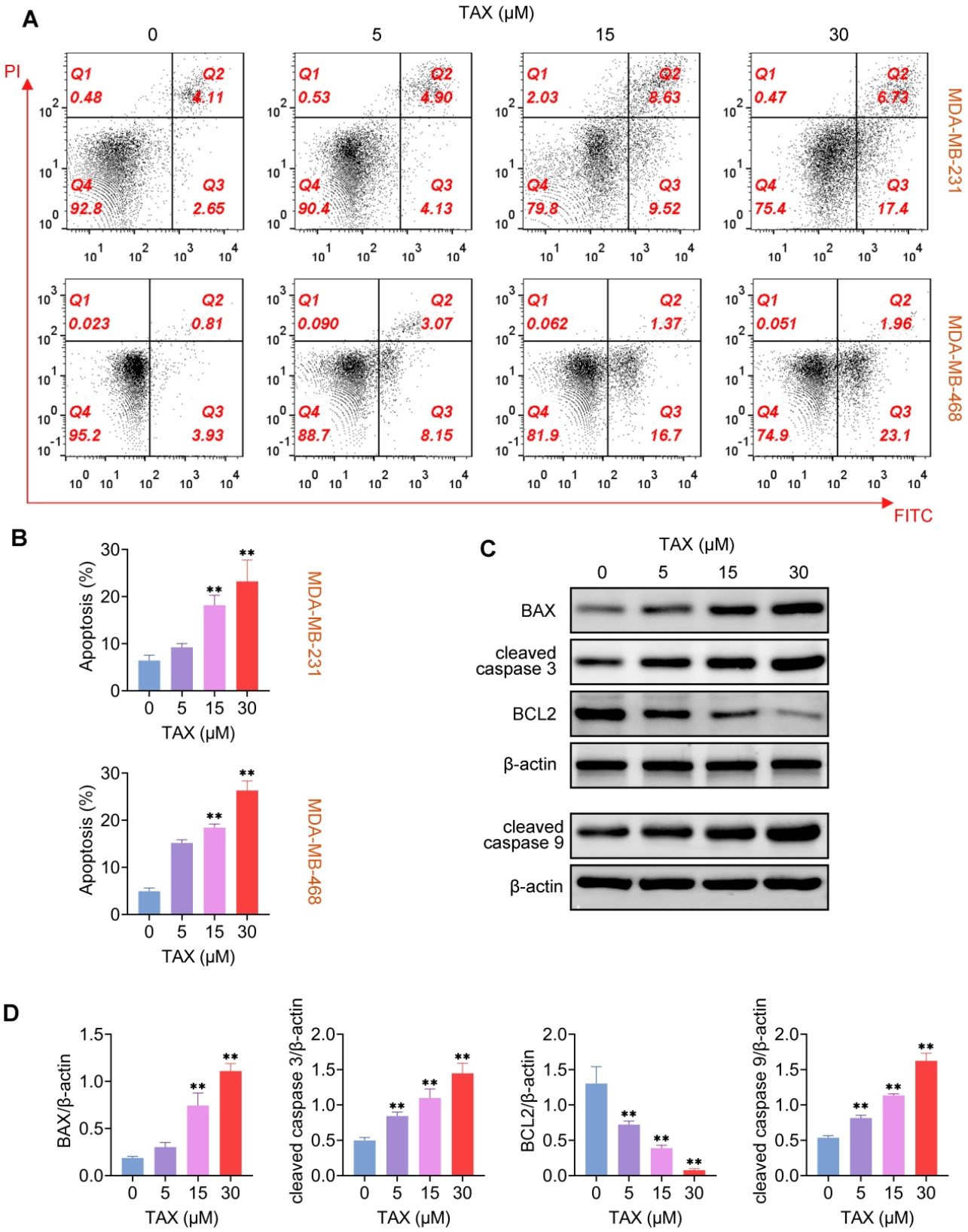


**FIGURE 2. TAX induces apoptosis of BCa cells.** (A) Flow cytometry (FCM) assays illustrating the impact of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the apoptosis of cells. (B) Quantification of the percentage of apoptotic cells from panel A. (C) Immunoblot assays demonstrating the influence of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the expression of Bax, Bcl-2, cleaved caspase-3, and cleaved caspase-9. (D) Measurement of the expression levels of the indicated proteins from panel C. Data are presented as mean $\pm$ SD. $^{**}p < 0.01$. PI: Propidium Iodide; TAX: Taraxasterol; FITC: fluorescein isothiocyanate isomer I; BCL: B-cell lymphoma.

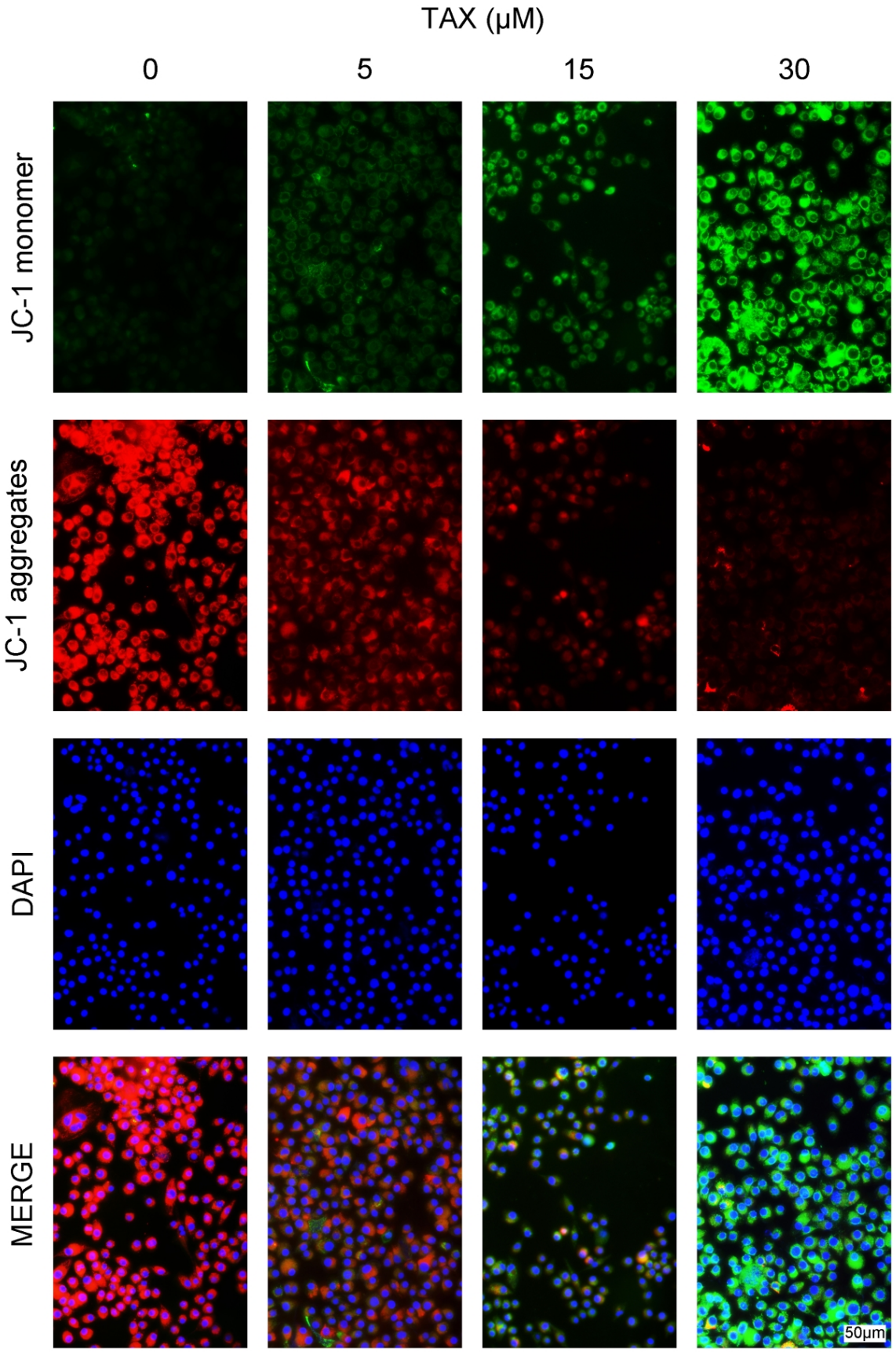


**FIGURE 3. TAX affects the mitochondrial membrane potential in BCa cells.** Immunostaining assays depicting the impact of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the expression of JC-1 monomers and JC-1 aggregates in MDA-MB-231 cells. The green panel represents JC-1 monomers, while the red panel represents JC-1 aggregates. Scale bar, 50 $\mu$m. TAX: Taraxasterol; DAPI: 4′,6-diamidino-2-phenylindole.

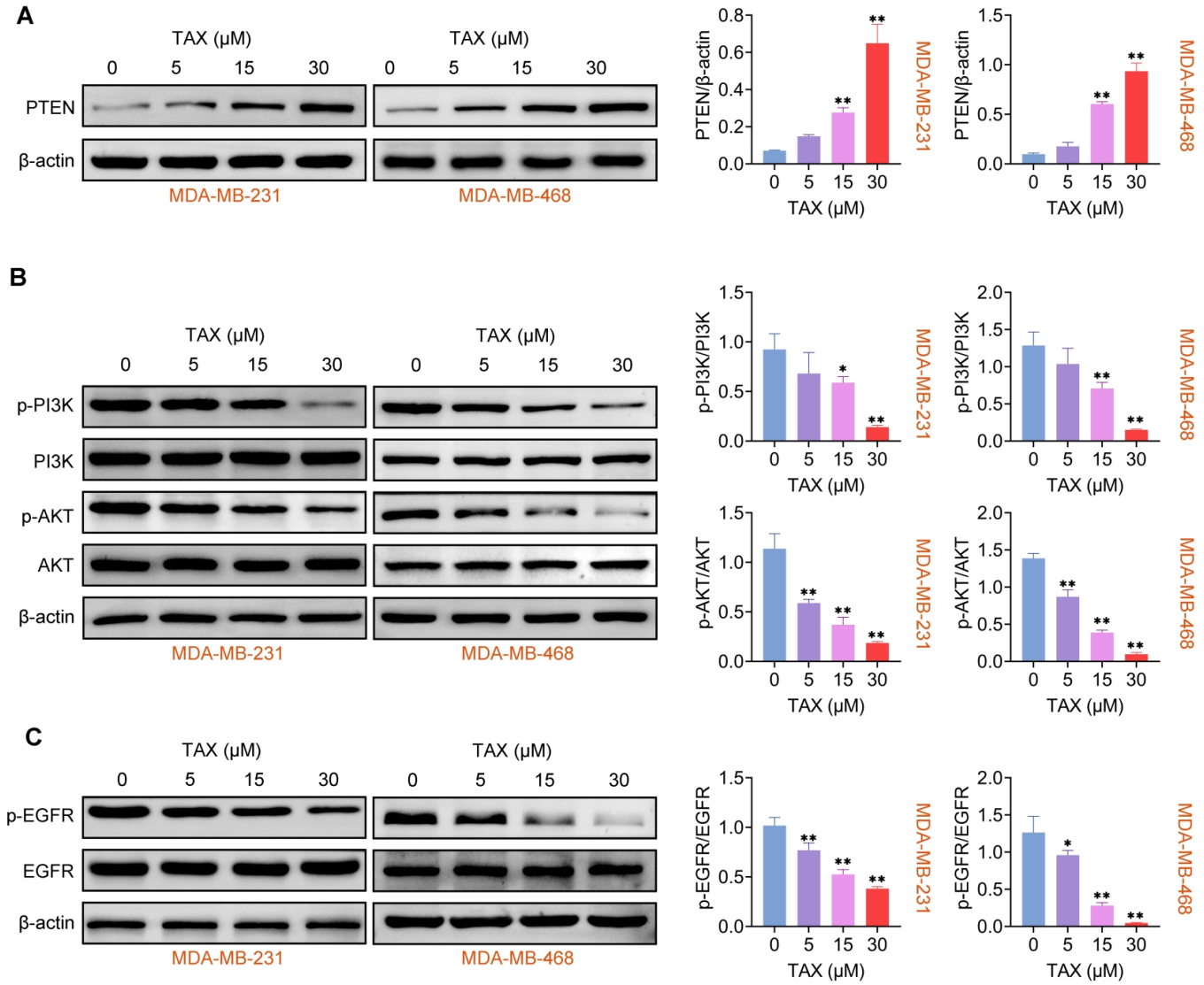


**FIGURE 4. TAX suppresses the PI3K/AKT and EGFR pathways in BCa cells.** (A) Immunoblot assays illustrating the impact of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the expression of PTEN. (B) Immunoblot assays depicting the influence of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the expression and phosphorylation of PI3K and Akt. (C) Immunoblot assays demonstrating the effect of TAX (0, 5, 15, 30 $\mu$M) for 24 hours on the expression and phosphorylation of EGFR. Data are presented as mean $\pm$ SD. $*p < 0.05$, $**p < 0.01$. TAX: Taraxasterol; PI3K/AKT: phosphatidylinositide 3-kinases/protein kinase B; EGFR: epidermal growth factor receptor; PTEN: phosphatase and tensin homolog deleted on chromosome ten.

Prior research has also highlighted TAX's anti-tumor properties, particularly in non-snall cell lung cancer (NSCLC) [7], where it suppressed tumor growth through apoptosis induction and microenvironment modulation [9]. Our study extends these findings by demonstrating TAX's impact on BCa cell apoptosis. Interestingly, TAX exhibited anti-tumor efficacy in a mouse model of hepatocellular carcinoma, involving T lymphocytes [18], prompting further exploration of its effects on the BCa microenvironment. Additionally, TAX has shown promise in attenuating melanoma progression by targeting the ROS-mediated PI3K/Akt pathway [19]. In line with previous findings, our data confirmed TAX's ability to impede the progression of BCa through this pathway, although the precise mechanism warrants further investigation. Notably, our viability and colony formation results indicated that the highest tested concentration (30 $\mu$M) induced a substantial reduction in proliferation, signifying its cytotoxicity to the cells. Consequently, we opted for lower concentrations in subsequent experiments, which did not compromise cell viability. Furthermore, another study demonstrated TAX's capacity to inhibit cell proliferation and enhance apoptosis by targeting glycolysis in gastric cancer. Collectively, these findings reinforce the anti-tumor potential of TAX.

In our study, we have indeed verified TAX's impact on the PI3K/AKT pathway in BCa. This pathway is crucial for cell proliferation and plays a pivotal role in the development, drug resistance, and metastasis of TNBC [6, 7, 20]. Currently, there are several clinical trials exploring inhibitors that target the mutated PI3K/AKT pathway, such as Alpelisib and Ipatasertib, showing promise for enhancing the prognosis of cancer patients [21]. Furthermore, the abnormal activation of PI3K and EGFR pathways is a common occurrence in BCa, making this

pathway a key focus in BCa treatment research [22, 23]. An important limitation of this study is the need for a more detailed understanding of the underlying mechanisms. In our future research, we aim to conduct additional molecular experiments and *in vivo* assays to further validate the effects of TAX on BCa. It is worth noting that the MDA-MB-231 used in this study is highly aggressive and serves as a representative model for TNBC. Identifying substances capable of inducing apoptosis in these cells is of paramount importance and may serve as a starting point for the development of novel pharmaceutical agents.

## 5. Conclusions

In summary, our findings demonstrate that TAX induces apoptosis in BCa cells by inhibiting the PI3K/AKT and EGFR pathways, which suggests the potential of TAX as a promising candidate for BCa treatment.

### AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

### AUTHOR CONTRIBUTIONS

YJH, XPZ and SZ—designed the study and carried them out; supervised the data collection, analyzed the data, interpreted the data; prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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