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Epifriedelinol Ameliorates DMBA-Induced Breast Cancer in Albino Rats by Regulating the PI3K/AKT Pathway

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We evaluated the protective effect of epifriedelinol against breast cancer and postulated an underlying mechanism. Breast cancer was induced by a single dose of 50 mg/kg 7,12-Dimethylbenanthracene (DMBA), and rats were treated with 100 or 200 mg/kg (i.p.) epifriedelinol for 4 weeks. We then evaluated the effect of epifriedelinol on tumor growth, oxidative stress and serum inflammatory cytokine levels in DMBA-induced breast cancer. Protein and mRNA levels were determined using western blotting and quantitative reverse transcription polymerase chain reaction, respectively. The tumor volume and weight were significantly ($p < 0.01$) decreased in the epifriedelinol-treated group compared to the negative control group. Epifriedelinol decreased the altered levels of oxidative stress and serum inflammatory cytokines in rats with DMBA-induced breast cancer. Protein levels of PI3K, AKT and mTOR and mRNA levels of PI3K, AKT, Map3k1, Erbb2 and Pdk1 were decreased in the mammary tissue of epifriedelinol-treated rats with DMBA-induced breast cancer. Apoptosis was significantly induced in the epifriedelinol-treated group compared to the negative control group. In conclusion, epifriedelinol ameliorates DMBA-induced breast cancer by regulating the PI3K/AKT pathway.

Keywords: apoptosis; breast cancer; epifriedelinol; inflammation; oxidative stress
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Introduction

Breast cancer is the commonest type of cancer in women and has a high mortality rate worldwide. The prevalence of breast cancer is reportedly > 5 lactating women annually, and approximately 12.8% of American women will suffer from breast cancer during their lifetime (Unger-Saldaña 2014). Breast cancer is characterised by cellular differentiation dysregulation, cessation of apoptosis and increased cellular proliferation (Feng et al. 2018). The several pathways involved are related to the development of cancer and resistance to chemotherapy. Phosphatidylinositol 3-kinase (PI3K) is stimulated by binding of a ligand (Wee and Wang 2017). Phosphorylation of PI3K generates phosphatidylinositol 3,4,5-triphosphate (PIP3), which phosphorylates AKT (Hemmings and Restuccia 2012). Phosphorylation of AKT activates mammalian target of rapamycin (mTOR), promoting protein synthesis and cellular growth (Hemmings and Restuccia 2012). Conventional therapies for cancer have important limitations and thus, there is a need for an alternative treatment modality.

Some natural compounds have anticancer and antioxidant activities. Epifriedelinol is a triterpene isolated from the leaves of *Vitex peduncularis* Wall. ex Schauer

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(Verbenaceae) (Kannathasan et al. 2019). Epifriedelinol has antibacterial, antioxidant and anti-inflammatory properties (Ng et al. 2003; Ogunnusi et al. 2010; Kannathasan et al. 2019). Moreover, it protects against traumatic injury (Li et al. 2018) and cervical cancer by inducing cellular apoptosis via the dysregulation of anti- and pro-apoptotic proteins (Yang et al. 2017). We evaluated the effect of epifriedelinol on breast cancer.

Materials and Methods

Animals

Adult Wistar rats (female, weight 170-200 g) were housed in a controlled environment (humidity 50 ± 10%, 25 ± 2°C) as per standard guidelines with a 12-h-day/12-h-night cycle. Animal procedures were approved by the Institutional Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (IEC/TMU-CIH/2020/05).

Chemicals

Epifriedelinol was procured from Toronto Research Chemicals, Toronto, Canada. Dimethylbenanthracene (DMBA) was obtained from Sigma Chemicals, St. Louis, MO, USA. TRIzol reagent and enzyme-linked immunosorbent assay (ELISA) kits were purchased from Thermo Fisher Scientific, Beijing, China. TransStart® Tip Green qPCR Supermix was obtained from TransGen, Beijing, China. The antibodies used for western blotting were procured from Thermo Fisher Scientific.

Experimental procedure

DMBA is a carcinogen which causes DNA methylation, genetic abnormalities and DNA damage leads to breast cancer in rat model. The animals were separated into the control group, negative control group (single 50 mg/kg DMBA dose in sesame oil), and 100 and 200 mg/kg epifriedelinol groups (100 and 200 mg/kg epifriedelinol via intraperitoneal injection for 4 months after oral administration of 50 mg/kg DMBA). Rats were examined weekly for tumors by palpation beginning at 4 weeks after DMBA administration. Rats were sacrificed by cervical dislocation at the end of the experiment, and blood and tissue samples were collected. Tumor tissue was weighed and subjected to histopathological analysis.

Determination of mammary tumor volume

A Vernier calliper scale was used to estimate the mammary tumor volume using the following equation:

$$V\ (cm^3) = (L \times B^2) \div 2$$

where L is the largest diameter and B is the smallest diameter (cm).

Analysis of biochemical parameters

Blood was collected via cardiac puncture, and the serum was separated via centrifugation for 10 min at 2,000 rpm. The serum level of cancer antigen 15-3 (CA15-3) was assayed using a chemiluminescence method per the directions of the kit manufacturer. Serum superoxide dismutase (SOD), malonaldehyde (MDA) and C-reactive protein (CRP) levels were determined using kits following the manufacturers' instructions.

Estimation of transforming growth factor (TGF)-β1, B-cell lymphoma (Bcl)-2 and inflammatory cytokine levels

Serum levels of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, Bcl-2 and TGF-β1 were determined using ELISA kits in accordance with the manufacturer's instructions.

Analysis of mRNA levels

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to determine the mRNA levels of PI3K, AKT, Map3k1, Erbb2 and Pdk1 in breast tissue. TRIzol reagent (Thermo Fisher Scientific) was used to isolate total mRNA from breast cells. An oligo (dT) 15 primer and M-MLV reverse transcriptase were used to synthesise cDNA from 1 μg of total RNA. Reverse transcription was conducted for 15 min at 42°C followed by heating for 5 s at 85°C and removal of gDNA. The primer sequences are shown in Table 1. The reaction took place in a total mixture volume of 20 μL comprising forward (0.4 μL) and reverse primers (10 μmol/L), 2 × TransStart® Tip Green qPCR Supermix (10 μL), cDNA template (1 μL), and sufficient H_2O. The PCR conditions were 30 s at 94°C for denaturation, followed by 5 s at 94°C, 15 s at 60°C and 10 s at 72°C for 45 cycles. The CT values of the samples were determined, and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1. The primer sequence for quantitative reverse transcription polymerase chain reaction (qRT-PCR).

	Forward	Reverse
PI3K	5′CCCATGGGACAACATTCCAA3′	5′CATGGCGACAAGCTCGGTA3′
AKT	5′TCTATGGCGCTGAGATTGTG3′	5′CTTAATGTGCCCGTCCTTGT3′
Map3k1	5′CCAGCCAGTTGTAGACACC3′	5′TGTCCTGTTGACCATCCAAA3′
Erbb2	5′GGAAGTACACGATGCGGAGACT3′	5′ACCTTCCTCAGCTCCGTCTCTT3′
Pdk1	5′CATGTCACGCTGGGTAATGAGG3′	5′CTCAACACGAGGTCTTGGTGCA3′

Western blotting

Breast tissue was treated with lysis buffer and total protein was extracted and its concentration was estimated via a DC protein assay. Proteins were electrophoresed in a sodium dodecyl sulphate-polyacrylamide gel (10%) and transferred to a polyvinylidene difluoride membrane. The membrane was treated with 5% fresh non-fat dry milk, incubated at 4°C overnight with primary antibodies against Bcl-2 (1:1,000 dilution), Bax (1:1,000), caspase-3 (1:1,000), PI3K (1:500), mTOR (1:1,000), AKT (1:1,000) and GAPDH (1:1,000) and thereafter incubated with the corresponding secondary antibodies. Densitometric analysis of the blots was performed using Image Lab software.

Histopathological evaluation

Breast tissue was fixed by soaking in formalin solution (10%) for 1 day, dehydrated in ethanol and mounted in paraffin wax. Mounted breast tissue was sectioned at 5-μm thickness, stained with hematoxylin and eosin, and observed under a trinocular microscope.

Statistical analysis

Data are presented as the means ± standard errors of the mean (SEM). Statistical analysis was performed in Prism software (ver. 6.1; GraphPad) and comprised one-way analysis of variance followed by a *post hoc* Dunnett test. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Epifriedelinol reduces the tumor volume and weight

The tumor volume and weight were greater in the negative control group than in the control group. The tumor volume and weight were significantly ($p < 0.01$) decreased in the epifriedelinol-treated groups in a dose-dependent manner (Fig. 1).

Epifriedelinol ameliorated alterations in CA15-3, CRP and oxidative parameters

Serum levels of CA15-3, CRP and MDA were significantly increased, and that of SOD was significantly decreased, in the negative control group compared to the control group. Epifriedelinol ameliorated the alterations in serum levels of CA15-3, CRP, SOD and MDA in epifriedelinol-treated rats with DMBA-induced breast cancer (Fig. 2).

Epifriedelinol reduced the levels of TGF-β1, Bcl-2 and inflammatory cytokines

Serum levels of TGF-β1, Bcl-2, and inflammatory cytokines (IL-1β, IL-6 and TNF-α) were significantly higher in the negative control group than in the control group. Epifriedelinol ameliorated the alterations in serum levels of TGF-β1, Bcl-2 and inflammatory cytokines in rats with DMBA-induced breast cancer (Fig. 3).

Epifriedelinol reduced apoptosis

The protein levels of caspase-3 and Bax decreased, and that of Bcl-2 increased significantly, in mammary tissue homogenate from the negative control group compared to that from the control group. Bcl-2, Bax and caspase-3 protein levels in the mammary tissue homogenate from the epifriedelinol-treated groups also differed from those of the negative control group (Fig. 4).

Epifriedelinol ameliorated alterations in the PI3K/AKT/mTOR pathway

PI3K, AKT, Erbb2, Map3k1 and Pdk1 mRNA levels in mammary tissue were higher in the negative control group than in the control group. Further, PI3K, AKT, Erbb2 and Pdk1 mRNA levels were lower, and that of Map3k1 higher, in tissue homogenate from the epifriedelinol-treated groups compared to the negative control group (Fig. 5A). PI3K, AKT and mTOR protein levels in tissue homogenate were higher in the negative control group than in the control group, whereas PI3K, AKT and mTOR protein levels in

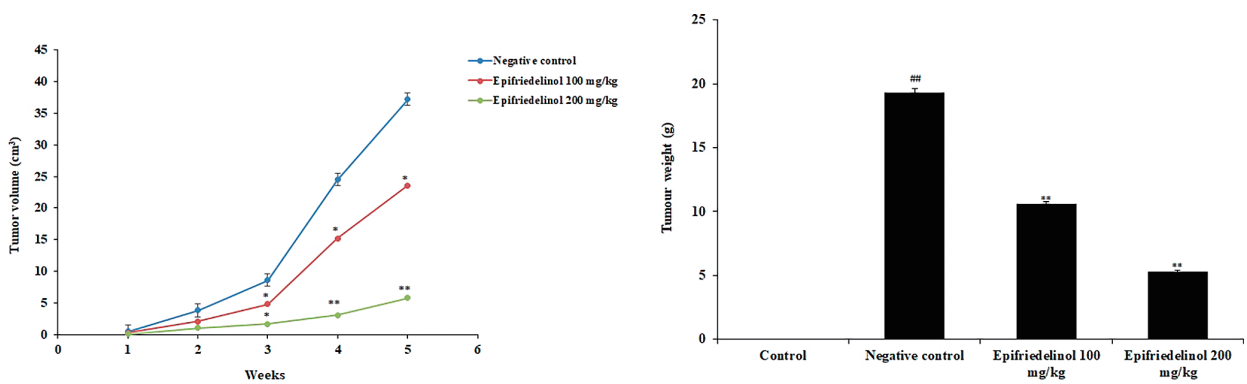

Fig. 1. Effect of epifriedelinol on tumor volume and weight in rats with dimethylbenanthracene (DMBA)-induced breast cancer.
Data are presented as means ± SEM (n = 10). ##$p < 0.01$ compared to the control group; **$p < 0.01$ compared to the negative control group.

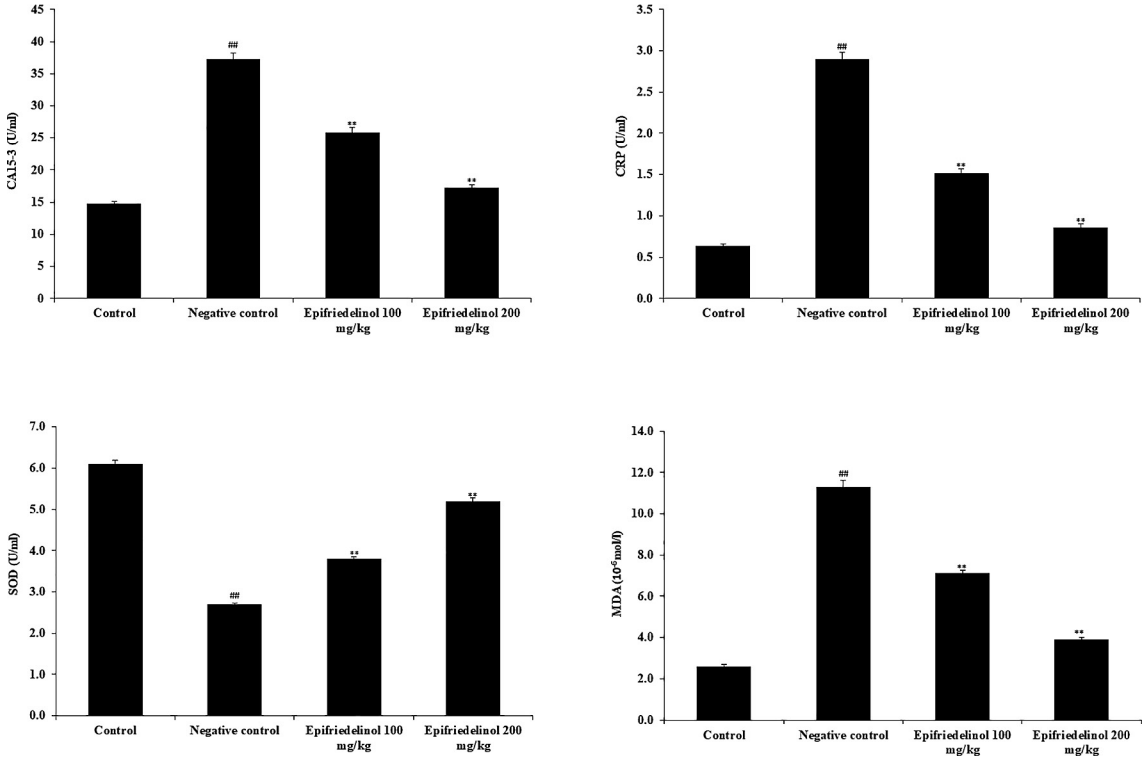

Fig. 2. Effect of epifriedelinol on serum CA15-3, C-reactive protein (CRP), superoxide dismutase (SOD) and malonaldehyde (MDA) levels in rats with DMBA-induced breast cancer.
Data presented as means ± SEM (n = 10); $^{\#\#}p < 0.01$ compared to the control group; $^{**}p < 0.01$ compared to the negative control group.

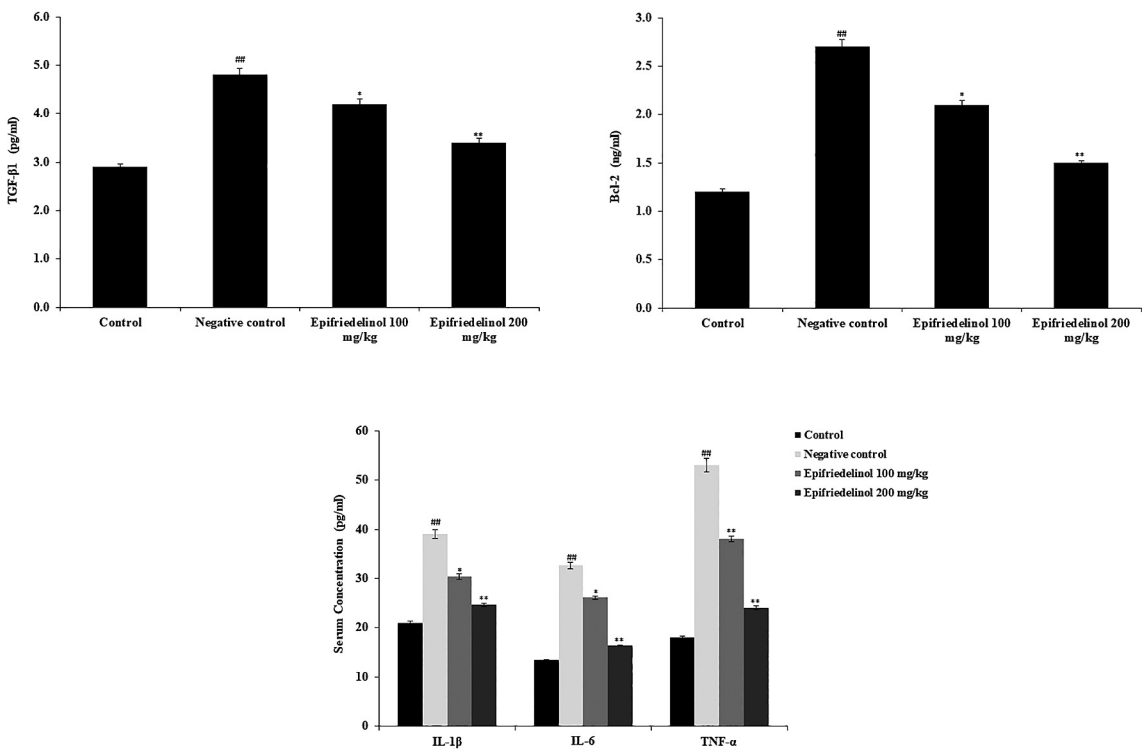

Fig. 3. Effect of epifriedelinol on serum TGF-β1, Bcl-2 and inflammatory cytokine levels in rats with DMBA-induced breast cancer.
Data presented as means ± SEM (n = 10); $^{\#\#}p < 0.01$ compared to the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the negative control group.

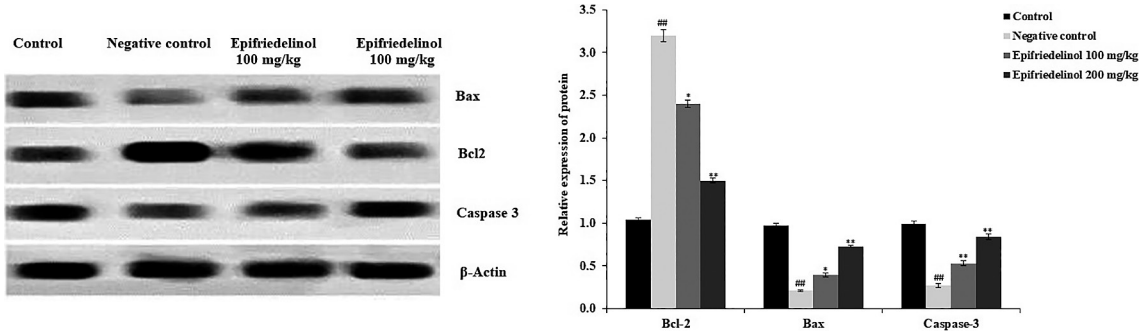

Fig. 4. Effect of epifriedelinol on Bcl-2, Bax and caspase-3 protein levels in mammary tissue homogenate from rats with DMBA-induced breast cancer.
Data presented as means ± SEM (n = 10); $^{\#\#}p < 0.01$ compared to the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the negative control group.

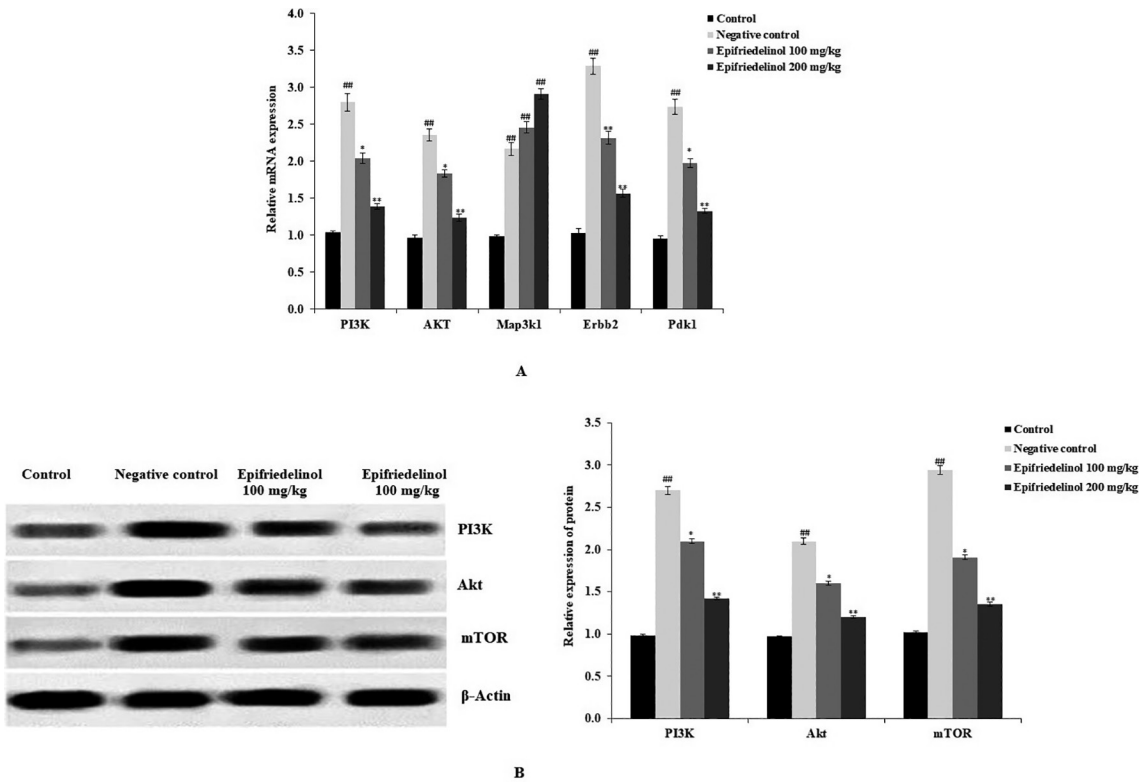

Fig. 5. Effect of epifriedelinol on the PI3K/AKT pathway in mammary tissue homogenate from rats with DMBA-induced breast cancer.
(A) PI3K, AKT, Map3k1, Erbb2 and Pdk1 mRNA levels as analysed using qRT-PCR. (B) PI3K, AKT and mTOR protein levels as analysed using western blotting. Data presented as means ± SEM (n = 10); $^{\#\#}p < 0.01$ compared to the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the negative control group.

mammary tissue were significantly ($p < 0.01$) lower in the epifriedelinol-treated groups compared to the negative control group (Fig. 5B).

Epifriedelinol ameliorated histopathological changes

The structure of mammary tissue was normal in the control group (Fig. 6, upper left). Cells with granulated cytoplasm in the ductal lumen and malignant cells with papillary outgrowth at the basement membrane were observed in cancerous tissue (Fig. 6, upper right). Epifriedelinol attenuated those structural alterations in mammary tissue (Fig. 6, lower panels).

Discussion

Breast cancer is the leading cause of mortality in fertile women worldwide, and malignant breast cancer accounts for approximately 29% of new carcinoma cases (Momenimovahed and Salehiniya 2019). The major issues

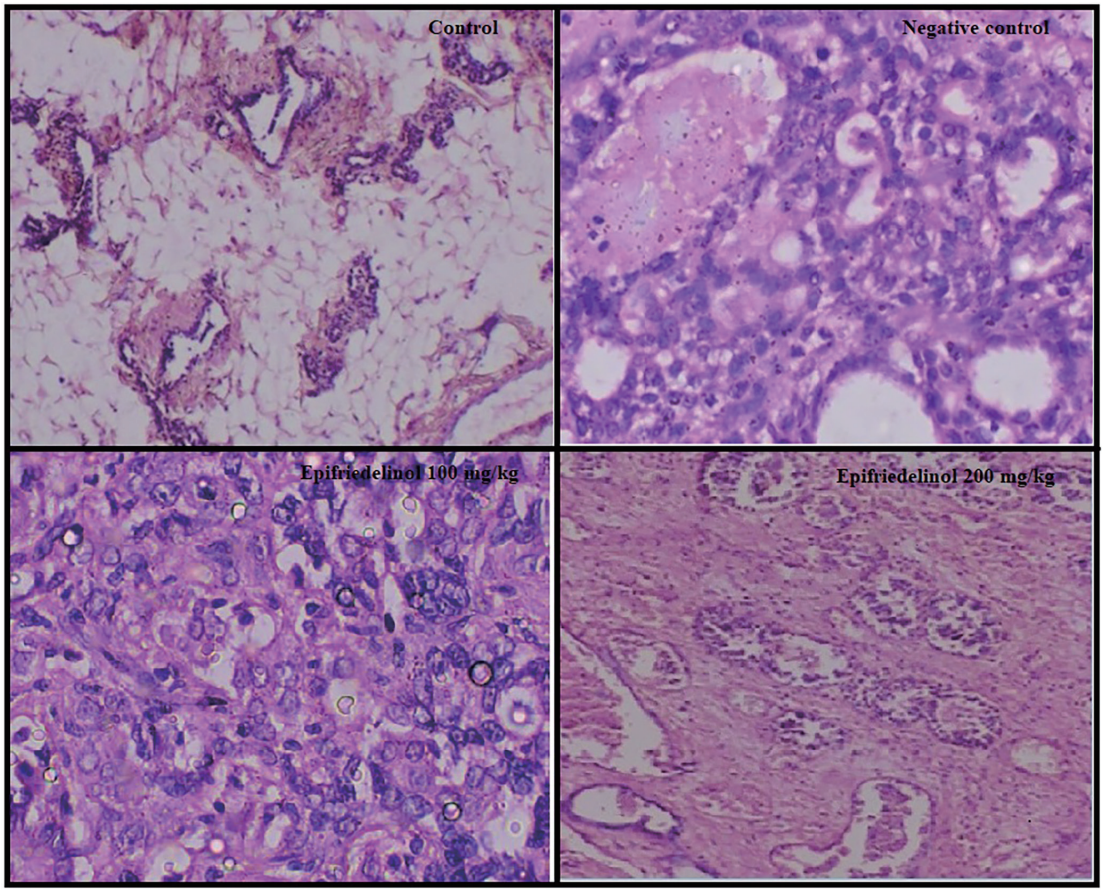

Fig. 6. Effect of epifriedelinol on histopathology of mammary tissue from rats with DMBA-induced breast cancer. Data presented as means ± SEM (n = 10).

in the management of breast carcinoma are the proliferation of mammary cells and drug resistance; thus, there is a need for alternative therapies. We evaluated the effect of epifriedelinol on breast cancer cells.

Carcinoma is mediated by aberrantly increased cell proliferation, resulting in increasing tumor volume and weight (Quail and Joyce 2013). In this study, the tumor volume and weight were increased in the negative control group, but those increases were significantly ($p < 0.01$) reduced by epifriedelinol. Oxidative stress is involved in the development of several chronic disorders including cancer; it promotes the migration and proliferation of cancer cells (Reuter et al. 2010). Reactive oxygen species modulate cell membrane integrity and increase the superoxide anion level (Nita and Grzybowski 2016). The serum SOD level was significantly increased, and that of MDA significantly decreased, in the epifriedelinol-treated groups compared to the negative control group. Inflammatory cytokines activate CRP and TGF-β1, inhibiting apoptosis of cancer cells. Cytokine levels also promote breast cancer (Lyon et al. 2008). Epifriedelinol ameliorated the altered levels of cytokines, TGF-β1 and CRP in this animal model of breast cancer.

The inhibition of apoptosis promotes cell proliferation. Pre- and pro-apoptotic proteins, such as Bax, Bcl-2 and caspase-3, regulate apoptosis (Shamas-Din et al. 2013). Caspase-3 triggers apoptosis and is inhibited in cancer cells (Boudreau et al. 2019). Epifriedelinol ameliorated the alterations in Bax, Bcl-2 and caspase-3 protein levels in our animal model of breast cancer.

The metabolism, membrane, cytoskeleton and survival of cells are influenced by the PI3K/AKT signalling pathway (Hassan et al. 2013). Activation of this pathway is linked to cancer, autoimmune disorders and diabetes. Map3k1 regulates the mitogen-activated protein kinase cascade, thus modulating cell proliferation and apoptosis (Cargnello and Roux 2011). In this study, Map3k1 expression was significantly higher in the epifriedelinol-treated groups compared to the negative control group. Breast cancer cells acquire drug resistance by activating the expression of Erbb2 (Hsu and Hung 2016). Epifriedelinol ameliorated the increased protein level of Erbb2, thereby regulating the activation of the PI3K/AKT pathway.

In conclusion, epifriedelinol prevents breast cancer by regulating the PI3K/AKT pathway, suggesting that it has a therapeutic potential for breast cancer. The effects of epifriedelinol on other molecular pathways related to breast cancer warrant investigation and a pharmacokinetic study is needed.

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Author Contributions

Jing Zhang and Yang He performed experimental work and drafted the manuscript; Ying Zhou and Liping Hong analyzed the sample and contributed to the histopathological analysis; Zhansheng Jiang and Ying Zhao performed statistical analysis and review the drafted manuscript; Zhanyu Pan designed and supervised the experimental work and reviewed the drafted manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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