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Original Research Article

Leonurine inhibits breast cancer cell growth and angiogenesis via PI3K/AKT/mTOR pathway

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Abstract

Purpose: *To elucidate the effect of leonurine on the proliferation, invasion, migration, and angiogenetic potential of breast cancer cells.*
Methods: *Human breast cancer cell line (MDA-MB-231) and normal breast cell line, (SK-BR-3) were cultured. Both cell lines were treated with 200, 400, or 800 μM leonurine and cultured for 0 (control), 24, 48, or 72 h. Cell counting kit-8 (CCK8) and colony formation assays were performed to measure cell viability and proliferation. Invasion and migration were evaluated using in vitro invasion and wound healing assays, respectively, while angiogenesis was evaluated by the formation of branching point structures. Furthermore, phosphorylation of PI3K, AKT, and mTOR were assessed by Western blot. Cell viability, invasion, migration, and angiogenesis were further investigated in media including 740Y-P, 800 μM leonurine, and 800 μM leonurine plus 740Y-P.*
Results: *Leonurine inhibited the proliferation of breast cancer cells and weakened breast cancer cell invasion, migration, and angiogenetic potential in a dose-dependent manner ($p < 0.05$). Furthermore, leonurine repressed PI3K/AKT/mTOR pathway by reducing the phosphorylation of PI3K, AKT, and mTOR. Leonurine also inhibited breast cancer progression ($p < 0.05$).*
Conclusion: *Leonurine inhibits breast cancer progression by inhibiting PI3K/AKT/mTOR pathway, and is thus, a potential agent for the management of breast cancer.*

Keywords: *Angiogenesis, Breast cancer, Cell invasion, Cell migration, Leonurine, PI3K/AKT/mTOR pathway*

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INTRODUCTION

Breast cancer has been seriously threatening women's health, with approximately 1.7 million new cases each year. Recently, its survival rate has improved, but the median survival rate remains very low. Most metastatic or recurrent breast tumors are incurable, and it remains difficult to improve survival rates [1]. Breast cancer carcinogenesis is complicated, and further exploration of the mechanisms of its occurrence and treatment is necessary [2]. The phosphatidylinositol-3-kinase (PI3K)/AKT/ mammalian target of rapamycin (mTOR) pathway exerts critical functions in cell proliferation, cell survival, neovascularization, and tumor growth. Previous studies have identified a crucial role for PI3K/AKT/mTOR

signaling in the progression and maintenance of tumor cells. For example, knockdown of FAM83D induces autophagy and suppresses endometrial cancer cell viability [3]. Similarly, keratin 17 regulates AKT/mTOR pathway to enhance carboplatin resistance in ovarian cancer [4]. Moreover, the oncogene DEK promotes epithelial-to-mesenchymal transition and angiogenesis in breast cancer via the PI3K/AKT/mTOR pathway [5]. Taken together, the PI3K/AKT/mTOR pathway is of immense importance for cancer progression.

Numerous studies have confirmed that leonurine functions in various physiological processes, including inflammation, energy metabolism, and apoptosis. In addition, leonurine exhibits potential anticancer activity as it suppresses the proliferation of lung cancer cells and induces apoptosis by inhibiting the mitochondrial pathway [6]. Moreover, leonurine suppresses colony formation and proliferation and enhances apoptosis of chronic myeloid leukemia cells [7]. Leonurine also inhibits the activity of PI3K/Akt pathway by reducing PI3K and Akt phosphorylation [8]. However, the effects of leonurine on breast cancer and its related mechanisms are still unclear.

This study focuses on investigating the cell growth, invasion, migration, and angiogenic capability of breast cancer cells in response to various doses of leonurine. Regulation of PI3K/AKT/mTOR pathway was also evaluated in response to leonurine treatment.

EXPERIMENTAL

Cell lines and cell culture

The human breast cancer cell line MDA-MB-231 (ATCC Cat No. HTB-26) was cultured in Leibovitz's L-15 Medium (ATCC Cat No. 30-2008) supplemented with 10 % fetal bovine serum (FBS). Another breast cell line, SK-BR-3 (ATCC No. HTB-30), was cultured in McCoy's 5a Medium (ATCC Cat No. 30-2007) supplemented with 10 % FBS. Both cell lines were cultured in a 37 °C incubator supplied with constant 5 % CO_2.

Cell proliferation assay

MDA-MB-231 or SK-BR-3 cells were treated with 200, 400, or 800 µM leonurine and cultured for 0, 24, 48, or 72 h. Viability was assessed using a cell counting kit-8 (CCK8) (Cat No. CA1210; Solarbio, Beijing, China) as described previously[9]. Absorbance was determined at 450 nm, and cell viability curves were calculated.

Colony formation assay

MDA-MB-231 or SK-BR-3 single cells were suspended and seeded in separate 6-well plates. Each well contained medium with 200, 400, or 800 µM leonurine. When visible cell clusters were formed, cells were fixed with 4 % paraformaldehyde followed by 0.1 % crystal violet staining (Cat No. 548-62-9, Sigma-Aldrich, Shanghai, China). Visible colonies were counted and survival curves were calculated using GraphPad Prism 8 software.

In vitro invasion assay

MDA-MB-231 and SK-BR-3 cells were cultured with 200, 400, or 800 µM leonurine for 24 h. The cells were then seeded into an invasion chamber, in which the lower chamber contained media supplemented with 10 % FBS and the upper space was filled with serum-free medium. Cells that passed through the polycarbonate membrane were stained with 0.1 % crystal violet. For each sample, the mean number of stained cells in 10 random fields was assessed.

Wound healing migration assay

A monolayer of MDA-MB-231 or SK-BR-3 cells was scratched with a pipette tip. The wound gaps were photographed at 0 and 24 h, and the relative distance migrated was calculated.

Formation of branch point structures

Branch point formation assays were conducted to assess the angiogenic ability of breast cancer cells as described previously [10]. Briefly, MB-231 or SK-BR-3 cells were detached from dishes and spread on type-I collagen matrix. Medium supplemented with 200, 400, or 800 µM leonurine was added and cells were incubated for 16 h. The cells were transferred to polymerized type-I collagen gel for 12 h. The attached cells were overlaid with a second layer of type-I collagen gel. After 36 h, the cells were washed and photographed. At least 10 fields were randomly selected for branch point quantification, and the branches of each node were determined.

Western blot

Total protein from MDA-MB-231 or SK-BR-3 was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific, Cat No. 89901, Carlsbad, CA, USA) as described previously [11]. Lysates were processed for immunoblots using the primary antibodies anti-phosphor(p)-PI3K (Cat No. 13857; Cell Signaling

Technology (CST), MA, USA; 1:1000), anti-PI3K (Cat No. 4255; CST; 1:2000), anti-p-AKT (Cat No. 4060; CST; 1:1000), anti-AKT (Cat No. 4691; CST; 1:2000), anti-p-mTOR (Cat No. 5536; CST; 1:1000), anti-mTOR (Cat No. 2983; CST; 1:2000), and anti-GAPDH antibody (Cat No. 10494-1-AP, ProteinTech Group, Chicago, IL, USA; 1:8000). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat No. B900210, ProteinTech Group; 1:5000) was used as the secondary antibody. Target bands were visualized using an ECL Western Blotting Detection Kit (Solarbio Life Sciences, Beijing, China). For quantification of western blot signals, the relative intensity of each band was measured using mageJ software, and the relative expression levels were normalized to the relative GAPDH levels.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 as previously described [12]. Data are presented as mean ± standard error of the mean (SEM) from three biological replicates. The differences between any two groups were compared by unpaired *t*-test. Multiple group comparisons were analyzed with ANOVA. *$P < 0.05$, **$p < 0.01$, and ***$p < 0.001$ were considered statistically significant, as applicable.

RESULTS

Leonurine inhibited breast cancer cell growth

The CCK8 assay illustrates that cell viability decreased significantly in MDA-MB-231 cells cocultured with 400 and 800 μM leonurine. Leonurine (> 200 μM) decreased SK-BR-3 cell viability significantly (Figure 1 A). Colony formation assays showed a significant reduction in colony formation in wells supplemented with 400 or 800 μM leonurine when compared to the control group; 200 μM leonurine did not reduce colony formation (Figure 1 B). Therefore, leonurine dose-dependently inhibited proliferation of breast cancer cells.

Leonurine decreased breast cancer cell invasion and migration

The data indicate that there were nearly 80 and 90 % reductions in the number of invaded cells treated with 400 and 800 μM leonurine, respectively, compared to control cells. Similar experiments on SK-BR-3 cells demonstrated approximately 70 and 80 % decreases in invasion ability when treated with 400 and 800 μM leonurine, respectively (Figure 2 A). Thus, leonurine suppressed breast cancer cell invasion in a dose-dependent manner. In MDA-MB-231 cells, the remaining wound areas following treatment with 400 or 800 μM leonurine were 2 to 3 times greater than that of the control group. In SK-BR-3 cells, the wound areas following 400 or 800 μM leonurine treatment were significantly greater than the control cells (Figure 2 B). These data indicate that leonurine, at adequate concentrations, weakened breast cancer cell migration.

Leonurine suppressed angiogenic capability in breast cancer cells

To study the potential mechanism of leonurine-induced inhibition of breast cancer cell angiogenesis, MDA-MB-231, and SK-BR-3 cells were treated with 200, 400, or 800 μM leonurine. Branching point formation assays revealed that 400 μM leonurine reduced the number of branching points by approximately 50 % in MDA-MB-231 and SK-BR-3 cells. Furthermore, 800 μM leonurine reduced the number of branching points by nearly 75 % in both cell types, indicating leonurine suppressed the angiogenetic capability of breast cancer cells (Figure 3).

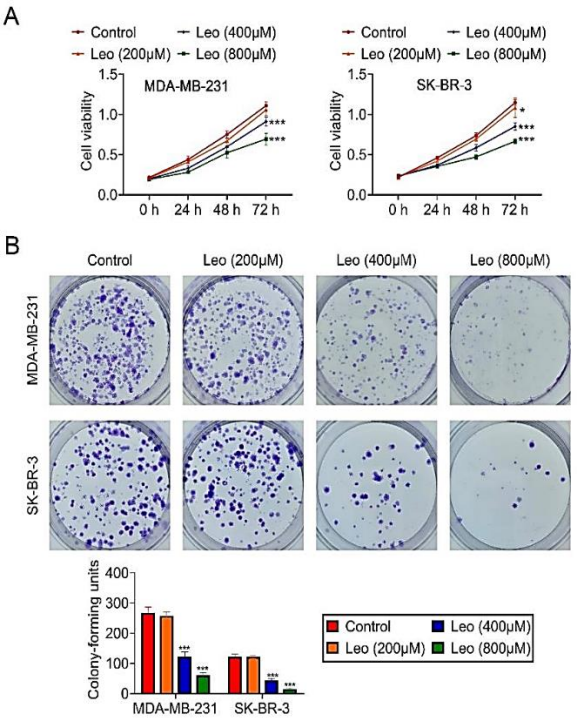

Figure 1: Leonurine inhibited breast cancer cell growth. (A) CCK8 assay showing the viability of MDA-MB-231 and SK-BR-3 cells cultured with 200, 400, or 800 μM leonurine. (B) Colony formation counts of MDA-MB-231 and SK-BR-3 cells cultured with 200, 400, or 800 μM leonurine. *$P < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus control

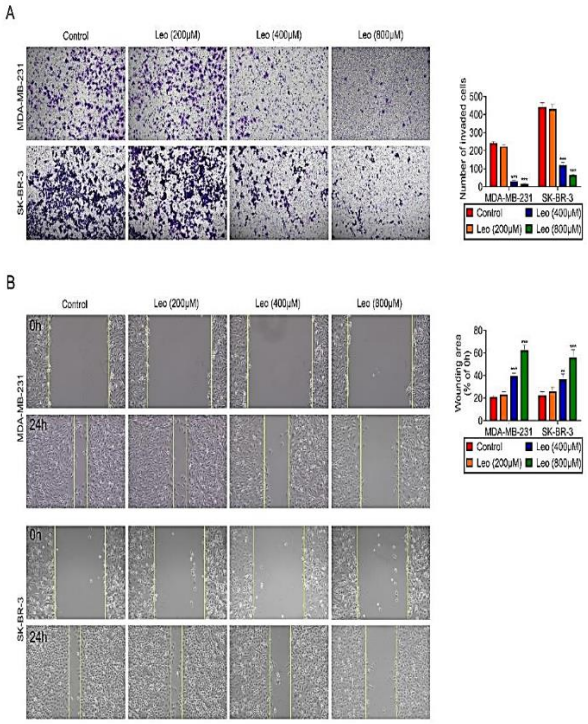

Figure 2: Leonurine reduced breast cancer cell invasion and migration. (A) *In vitro* assays showing the effect of 0, 200, 400, and 800 μM leonurine on the invasion ability of MDA-MB-231 and SK-BR-3 cells. (B) Wound healing results showing migration of MDA-MB-231 and SK-BR-3 cells treated with 200, 400, or 800 μM leonurine for 24 h. *$P < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus control

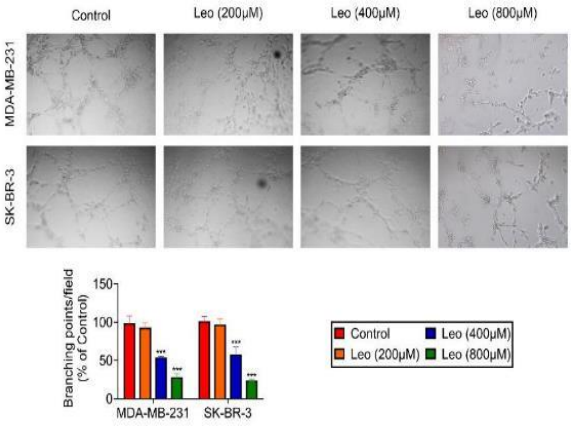

Figure 3: Leonurine suppressed angiogenesis in breast cancer cells. Branch point formation assays showing the effect of leonurine on the angiogenic capability of breast cancer cells. *$P < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus control

Leonurine repressed PI3K/AKT/mTOR pathway

The data demonstrated that PI3K, AKT, and mTOR phosphorylation was reduced significantly in samples cultured with 400 or 800 μM leonurine. There were no significant variations between cells cultured in 200 μM leonurine or without leonurine (Figure 4). Thus, leonurine may repress PI3K/AKT/mTOR pathway by reducing phosphorylation of PI3K, AKT, and mTOR.

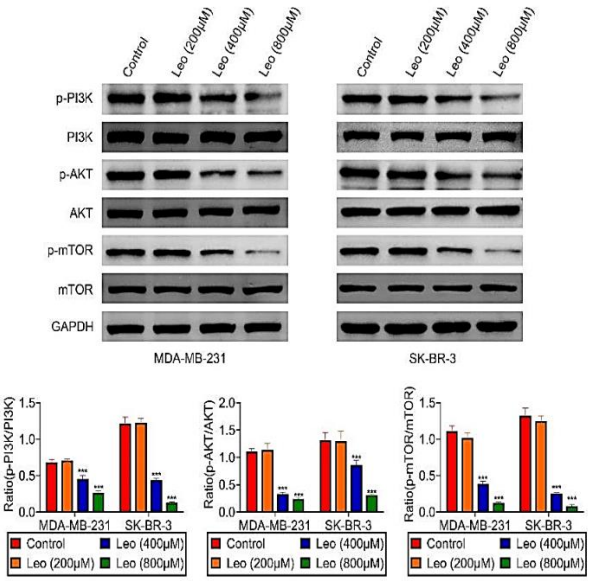

Figure 4: Leonurine repressed the PI3K/AKT/mTOR pathway. Western blot result showing the phosphorylation of PI3K, AKT, and mTOR in MDA-MB-231 and SK-BR-3 cells cultured with 200, 400, and 800 μM leonurine. PI3K, AKT, and mTOR phosphorylation were significantly reduced when cultured with 400 or 800 μM leonurine. *$P < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus control

Leonurine reduced breast cancer progression

As shown in Figure 5 A, MDA-MB-231 cells cultured in medium containing only 740Y-P demonstrated enhanced viability compared to the control group. However, MDA-MB-231 cell viability in 800 μM leonurine plus 740Y-P was significantly reduced compared to cells treated with 740Y-P alone. These data indicate that leonurine repressed breast cancer progression via the PI3K/AKT/mTOR pathway.

Furthermore, 740Y-P increased the invasion and migration capability of MDA-MB-231 cells. In MDA-MB-231 cells treated with 800 μM leonurine plus 740Y-P, invasion and migration were similar to that of the control cells, which were decreased significantly compared to that of 740Y-P-only treated cells (Figures 5 B and C). Therefore, leonurine attenuated the activation of the PI3K/AKT/mTOR pathway, resulting in inhibition of breast cancer progression.

Under the same conditions, the angiogenic capability of MDA-MB-231 cells was also studied. Similarly, there were no significant increases in branching points in cells treated with 740Y-P

alone, but increases in angiogenesis were significantly weakened by the addition of 800 μM leonurine (Figure 5 D). Therefore, leonurine repressed breast cancer progression by inhibiting the activity of the PI3K/AKT/mTOR pathway.

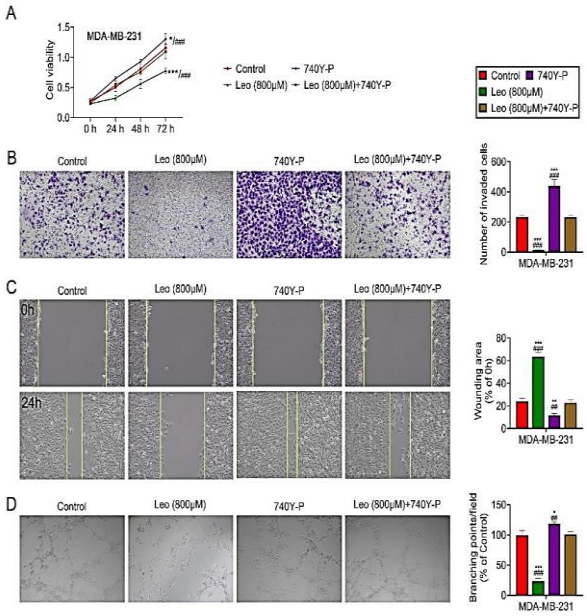

Figure 5: Leonurine inhibited breast cancer progression via the PI3K/AKT/mTOR pathway. (A) Cell viability of MDA-MB-231 cells observed in medium supplemented with 740Y-P alone, 800 μM leonurine alone, or 800 μM leonurine plus 740Y-P. (B) 740Y-P alone enhanced the invasion of MDA-MB-231 cells, which was weakened significantly by the addition of 800 μM leonurine. (C) The migration of MDA-MB-231 cells enhanced in 740Y-P treatment and attenuated significantly by the addition of 800 μM leonurine. (D) Angiogenesis increased in MDA-MB-231 cells cocultured with 740Y-P, and significantly decreased by the addition of 800 μM leonurine. *$P < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus control

DISCUSSION

Breast cancer incidence has been increasing rapidly worldwide, with nearly 1.7 million new cases per year. Although survival rates have improved, the median survival rate is still very low. Recently, leonurine was reported as a promising anticancer drug-like molecule as it repressed proliferation and promoted apoptosis of some cancer cell types [13]. One report showed that leonurine repressed the activity of miR-18a-5p/SOCS5/JAK2/STAT3 axis to disrupt chronic myeloid leukemia malignancy [7]. Three novel analogs of leonurine inhibit melanoma growth and survival through STAT3 signaling pathways [14]. In addition, leonurine promotes apoptosis of lung cancer cells via a mitochondria-dependent pathway [6]. Therefore, it is reasonable to make a hypothesis that leonurine regulates proliferation of breast cancer cells. In this study, the cell viability and colony formation assays demonstrated that leonurine inhibited the growth of breast cancer cells, while it might be meaningful to elucidate whether leonurine regulates apoptosis of breast cancer cells.

Migration of cancer cells plays a key role in rapid proliferation within the host. Regulation of cancer cell migration may be a novel therapeutic strategy for treating various cancers. Thus, this study aims to elucidate the effect of leonurine on migration and invasion of breast cancer cells, and the data revealed that leonurine reduced their migration and invasion in a dose-dependent manner. Consistent with the findings in this study, leonurine also inhibited melanoma proliferation, disrupts chronic myeloid leukemia malignancy, and reduced the proliferation of lung cancer cells [6,7,14]. These findings suggest that leonurine may be beneficial for the prevention of tumor replication in breast cancer patients.

Leonurine affects the regulation of angiogenesis. Leonurine regulates angiogenesis in hindlimb ischemia. It efficiently increased the number of capillaries in young patients and restored this function in elderly patients [15]. In a full-thickness cutaneous wound model, leonurine promotes angiogenesis and tissue regeneration via activating mTOR/ERK pathway [16]. In addition, leonurine ameliorates oxidative stress and insufficient angiogenesis by activating the PI3K/Akt-eNOS pathway [17]. This study focused on elucidating the effect of leonurine on angiogenesis in breast cancer cells. The results revealed that leonurine supplementation suppressed angiogenesis in breast cancer cells.

Multiple signal pathways are involved in the regulation of cancer cell progression, such as NF-κB and PI3K/Akt, which are key signaling factors in the control of cell proliferation, survival, neovascularization, and tumor growth [5]. Yuan *et al* reported that leonurine hydrochloride inhibited the NF-κB and PI3K/Akt signaling pathways, preventing estrogen-efficiency-associated osteoporosis [18]. Moreover, leonurine inhibits the activation of PI3K/Akt pathway by reducing the phosphorylation levels of PI3K and Akt [8]. To study the signaling mechanisms affected by leonurine in regulation of human breast cancer cells, the effect of leonurine on PI3K/AKT/mTOR pathway was evaluated. The data in this work indicate that the proliferation, invasion, migration, and angiogenic capability of breast cancer cells was positively regulated by phosphorylation of the PI3K/AKT/mTOR pathway. This activation was weakened by leonurine in a dose-dependent manner. Consistent with previous data, this study

has identified a key role for PI3K/AKT/mTOR signaling in breast cancer progression and maintenance [5].

CONCLUSION

Leonurine suppresses the proliferation, invasion, migration, and angiogenetic capability of breast cancer cells *in vitro via* repression of PI3K/AKT/mTOR pathway by reducing phosphorylation of PI3K, AKT, and mTOR. Thus, leonurine is a potential novel agent for the treatment of breast cancer treatment. However, *in vivo* studies are required in this regard ascertain its full potential.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Junjun Tian, Lizhong Peng, and Dongjie Wang designed the study, performed the experiments, supervised the data collection, analyzed the data, interpreted the data, prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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