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Original Articles

Combined bazedoxifene and paclitaxel treatments inhibit cell viability, cell migration, colony formation, and tumor growth and induce apoptosis in breast cancer

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ABSTRACT

Breast cancer, especially triple-negative breast cancer (TNBC), has limited treatment options. We repurposed the FDA-approved drug bazedoxifene as a novel inhibitor of interleukin 6/glycoprotein 130 signaling. In this study, we investigated the inhibitory effects of bazedoxifene alone or in combination with paclitaxel on several estrogen receptor positive and TNBC cells. Bazedoxifene inhibited the cell viability of these cells, as well as tumor growth of TNBC cells in a xenograft tumor model. Furthermore, bazedoxifene combined with paclitaxel exhibited more potent inhibition of cell viability, colony formation, and cell migration and induced more apoptosis *in vitro*, and generated stronger inhibition of tumor growth of TNBC *in vivo* than either drug alone. Western blotting showed that bazedoxifene inhibited estrogen receptor positive breast cancer cells by suppressing the expression of estrogen receptor, Cyclin D1, p-P70S6K, Survivin, c-Myc, and Bcl-2, and bazedoxifene inhibited TNBC cells by inhibiting the expression of phosphorylated STAT3 $^{(Tyr705)}$, Cyclin D1, p-P70S6K, c-Myc, p-AKT $^{(Ser473)}$ and p-ERK 1/2 $^{(T202/Y\ 204)}$ without changing the expression of total STAT3. When combined with paclitaxel, bazedoxifene may be a potential small molecule for the treatment of both estrogen receptor positive and triple-negative breast cancer.

1. Introduction

Breast cancer is the most common cancer among women, accounting for 30% all new cancer diagnoses in women and is the leading cause of cancer death from ages 20 to 59 [1]. At present, breast cancer treatments are based on the expression of biomarkers such as estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 [2]. Tumors expressing estrogen receptor are treated with anti-hormonal therapies and have been shown to exhibit good clinical outcomes; however, resistance to these therapies finally occur, and has become a major limitation for endocrine-based therapy [3]. A breast cancer subtype that does not express these three receptors is triple-negative breast cancer (TNBC). TNBC is the most aggressive breast cancer, with no approved targeted therapy, the highest mortality rates and the worst prognosis; the main treatment options include surgical resection, radiation therapy and chemotherapy, which has serious side effects, and treatment options are limited [4]. Therefore, it is of great importance to identify novel drugs that can effectively prevent and treat breast cancer, especially against TNBC, and that will have fewer side effects.

Bazedoxifene belongs to the third generation of selective estrogen receptor modulators [5]. Bazedoxifene (BZA) with conjugated equine estrogen (CEE) is approved by the FDA for the prevention and treatment for menopausal osteoporosis [6], which means that it has already undergone osteoporosis efficacy and safety trials in humans. Studies showed that bazedoxifene blocked the effects of estradiol on cell growth in the estrogen receptor positive breast cancer cell line MCF-7 [7]; these studies were mainly performed in MCF7 cells, and few were studied in other estrogen receptor positive breast cancer cells [7–9]. The effect of bazedoxifene on TNBC and its mechanism is still unknown.

We reported the discovery of bazedoxifene as novel inhibitor of interleukin-6/glycoprotein 130 (IL-6/GP130) protein-protein interactions [10]. We found that bazedoxifene significantly inhibited constitutive signal transducer and activator of transcription 3 (STAT3)

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activity induced by IL-6 and GP130 in SUM159 TNBC cells [10]. Because SUM159 does not express estrogen receptor, it is clear that the inhibition of constitutive p-STAT3 $^{(Y705)}$ level and cell proliferation by bazedoxifene is not through modulation of the estrogen receptor target. Previous studies indicate that IL-6/GP130/STAT3 is one of the major cancer proliferation pathways in TNBC [11]. We also observed that bazedoxifene induced apoptosis and suppressed tumor growth of pancreatic cancer cells; bazedoxifene combined with paclitaxel or gemcitabine synergistically inhibited cell viability and cell migration of pancreatic cancer, rhabdomyosarcoma and medulloblastoma [12–14].

Paclitaxel is considered one of the first line drugs in the treatment of breast cancer [15]. However, paclitaxel can cause side effects [15]. The main purpose of this study was to investigate the effect of improving drug efficacy and reducing the amount of paclitaxel dosage by combined administration with bazedoxifene, which may help reduce the side effects of paclitaxel.

2. Materials and methods

2.1. Cell lines and reagents

Human estrogen receptor positive breast cancer cell lines MCF7, T47D, and BT474, human TNBC cell lines MDA-MB-231 and MDA-MB-468 and mouse TNBC 4T1 cells were purchased from ATCC (American Type Culture Collection, Manassas, VA). All cell lines were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose L-glutamine and sodium pyruvate (DMEM, Mediatech Inc. A Corning Subsidiary, Manassas, VA), except for T47D, which was cultured in RPMI-1640 medium (Mediatech Inc. A Corning Subsidiary, Manassas, VA), and all media were supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt) and maintained in a humidified 37 °C incubator with 5% CO_2.

Reagents used in the present study were as follows: BZA/CEE was from Acesys Pharmatech (Fairfield, NJ, USA) and paclitaxel was from LC Laboratories (Woburn, MA). According to the instructions from the manufacturers, these drugs were dissolved in sterile dimethyl sulfoxide (DMSO, Sigma-Aldrich; Merck KGaA) to make a 20 mM stock solution and then stored at −20 °C. Additional reagents were 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich; Merck KGaA), crystal violet (Sigma-Aldrich; Merck KGaA), *N*, *N*-dimethylformamide (Fisher Scientific; Thermo Fisher Scientific, Inc., Waltham, MA), and lysis buffer (Cell Signaling Technology, Inc. Danvers, MA).

2.2. MTT cell viability assay

MCF7, T47D, BT474, MDA-MB-231, MDA-MB-468, and 4T1 breast cancer cells were seeded in 96-well microtiter plates in triplicate at a density of 3000 cells per well and grown overnight at 37 °C. The following day, the cells were treated with bazedoxifene as indicated and incubated for 48 h. MCF7, MDA-MB-231 and 4T1 cells were also treated with bazedoxifene or paclitaxel or their combination as indicated and incubated for 48 h. Then, 25 μL MTT was added to each well and incubated for 4 h, followed by the addition of 150 μL *N*, *N*-dimethylformamide solubilization solution. The plates were placed on a shaker at room temperature overnight to allow complete lysis of the cells and read at 595 nm the following day. Experiments and data processing were performed as described previously [16]. Half-maximal inhibitory concentrations (IC50) were determined using Sigma Plot 9.0 Software (Systat Software, Inc., San Jose, CA). Combination index (CI) was performed using data obtained from MTT assay with CompuSyn software [17]. The CI values indicate a synergistic effect when < 1, an antagonistic effect when > 1, and an additive effect when equal to 1.

2.3. Caspase-3/7 activity assay

MCF7, BT474, MDA-MB-231, MDA-MB-468, and 4T1 breast cancer cells were seeded in 96-well plates in triplicate at a density of 5000 cells per well and incubated overnight at 37 °C. The next day, cells were treated with bazedoxifene, paclitaxel or their combination as indicated and incubated for 5 h. The caspase-3/7 activity was detected using Caspase-3/7 Fluorescence Assay Kit (Cayman, Ann Arbor, MI) according to the manufacturer's instructions.

2.4. Colony formation assay

MCF7, BT474, MDA-MB-231, MDA-MB-468, and 4T1 cells were seeded (500 cells/well) in 6-well plates and incubated at 37 °C overnight. The following day, cells were incubated with bazedoxifene, paclitaxel, or their combination as indicated. The culture medium was changed in 3 days with fresh medium without drugs, and the medium was changed every week for another 2 weeks. Then, cells were washed twice with PBS, fixed with cold methanol for 30 min at 4 °C and stained with crystal violet dye (0.1% w/v) at room temperature for 1 h. The plates were washed with water, dried and scanned [16].

2.5. Wound-healing assay

MCF7, MDA-MB-231, and 4T1 cells were seeded in six-well plates and incubated at 37 °C overnight. The following day when cells reached 100% confluence, a straight line with the same width was scratched across the monolayer using a 100-μL pipette tip. After washing with PBS to remove non-adherent cells, cells were then treated with bazedoxifene, paclitaxel or their combination as indicated and incubated for 20–26 h. When the wound in the DMSO control was healed, the image from the original scratch line was captured by Nikon Eclipse TS100. Experiments and data processing were performed as described previously [16,18]. The MTT assay was used to determine if the effects of bazedoxifene and paclitaxel on cell migration were due to the inhibition of cell viability, and was performed as described previously [16].

2.6. Western blot analysis

Experiments were performed as described previously [16]. MCF-7 and 4T1 cells were seeded at 50% confluence in 10 cm dishes and incubated at 37 °C overnight; the following day, cells were treated with bazedoxifene as indicated for 24 h. Then, cells were harvested and lysed in ice-cold cell lysis buffer containing protease and phosphatase inhibitors. The protein lysates were separated by 10% SDS-PAGE, and the resolved proteins were transferred to a polyvinylidene fluoride membrane (GE Healthcare Life Sciences, Shanghai). Membranes were incubated at 4 °C overnight with primary antibodies (1:1000; all from Cell Signaling Technology, Inc. Danvers, MA) against phosphorylated signal transducer and activator of transcription 3 (Tyr705, p-STAT3^{Y705}, #9145, rabbit mAb), STAT3 (#9404, rabbit mAb), phosphorylated protein kinase B (Ser473) (p-AKT $^{(Ser473)}$, #4058, rabbit mAb), phospho-specific extracellular signal-regulated kinase (p-ERK) 1/2 (threonine 202/tyrosine 204, #4370, rabbit mAb), estrogen receptor (#13258, rabbit mAb), cyclin D1 (#2978, rabbit mAb), phospho-P70S6 kinase (p-P70S6K, #9234, rabbit mAb), survivin (#2808, rabbit mAb), c-Myc (#13987, rabbit mAb), B-cell lymphoma 2 (Bcl-2, #2876, rabbit mAb) and GAPDH (#2118, rabbit mAb), followed by incubation with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, HRP-linked antibody, #7074; Cell Signaling Technology, Inc. Danvers, MA) (1:10,000) for 1.5 h at room temperature. Proteins were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc. Waltham, MA).

2.7. Tumor suppressive experiments

The experiment was approved and conducted in accordance with the principles and standard procedures approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland, Baltimore (UMB) and the Care and Use of Laboratory Animals of UMB. Six-week-old female athymic nude (Foxn1nu) mice (strain: Hsd) were obtained from ENVIGO. After 2 days of adaption for the mice, 4T1 cells (1×10^6) cells suspended in 50μL Matrigel (BD Science, Franklin Lakes, NJ)/PBS (1:1, v/v) were injected bilaterally into the 4th mammary fat pads of mice as described previously [19]. The tumors developed 4 days after implantation, and the tumor size reached approximately 50 mm^3. Then, mice were randomly divided into two groups consisting of 4 mice per group: vehicle control group and bazedoxifene group administered by gavage once daily (8 mg/kg).

For MDA-MB-231 cells, 3×10^6 cells suspended in 50 μL Matrigel/PBS (1:1, v/v) were injected bilaterally into the 4th mammary fat pads of mice. The tumor developed 8 days after implantation, and tumor size reached approximately 50 mm^3. Then, mice were randomly divided into vehicle control group, bazedoxifene group, paclitaxel group and combination group (bazedoxifene combined with paclitaxel). Bazedoxifene (8 mg/kg) was administered by gavage once daily, and paclitaxel (5 mg/kg) was administered by intraperitoneal injection twice weekly.

Caliper measurements of the tumor size and mouse weight were calculated every other day; the behavior and activity were also observed. The tumor volume was calculated by two-dimensional measurements of length (L) and width (W) of the tumor using the following formula [13]: Tumor volume = $0.5236 \times L \times W^2$. Mice implanted with 4T1 or MDA-MB-231 were sacrificed by CO2 inhalation according to the IACUC approved protocol after final bazedoxifene treatment on the 18th and 30th day of drug administration, respectively. Tumors tissue were harvested, frozen on dry ice, and lysed for Western blotting analysis to detect the expression of p-STAT3$^{(Y705)}$, STAT3, p-ERK$^{(T\ 202/T204)}$, P-AKT $^{(Ser473)}$, cleaved caspase-3 and GAPDH.

2.8. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 software. Differences were analyzed with one-way analysis of variance followed by Tukey's post hoc test for multiple comparisons and Student's *t*-test for two comparisons. Data were presented as the means ± standard error of the mean. Significance was set at $P < .05$. *, ** and *** indicates $P < .05$, $P < .01$ and $P < .001$, respectively.

3. Results

3.1. Bazedoxifene inhibited cell viability of breast cancer cells and exhibited synergistic effects with paclitaxel treatment

To explore the inhibitory effect of bazedoxifene on breast cancer cells, we first performed MTT assay. Breast cancer cell viability were assessed upon exposure to different concentrations of bazedoxifene for 48 h. As shown in Fig. 1A, cell viability of MCF7, T47D, BT474, MDA-MB-231, MDA-MB-468, and 4T1 cells was remarkably reduced by bazedoxifene in a dose-dependent manner, with respective IC50 values of 7.14 μmol/L, 3.58 μmol/L, 8.29 μmol/L, 8.23 μmol/L, 7.45 μmol/L and 3.14 μmol/L.

To explore the synergistic inhibitory effects of bazedoxifene and paclitaxel on breast cancer cells, MCF-7, MDA-MB-231 and 4T1 cells were treated with bazedoxifene, paclitaxel or their combination for 48 h. As shown in Fig. 1B, cell viability was more inhibited in the combined group than in the monotherapy group. The CI values of all the combined treatments were less than 1, suggesting there was synergism in the combined treatment of bazedoxifene with paclitaxel, and bazedoxifene sensitized breast cancer cells to paclitaxel.

3.2. Bazedoxifene combined with paclitaxel induced apoptosis of breast cancer cells more significantly than monotherapy

Apoptosis is an important process in tumor growth. We found that bazedoxifene could induce apoptosis of cancer cells in a dose-dependent manner [12,13]; therefore, we next evaluated the effects of bazedoxifene, paclitaxel or their combination on apoptosis of breast cancer. As shown in Fig. 2, after 5 h of drug treatment, combination treatment induced cell apoptosis more significantly than single agents.

3.3. The combination of bazedoxifene and paclitaxel inhibited the colony formation of breast cancer cells better than either drug alone

In previous studies, we found that bazedoxifene could inhibit the colony formation of cancer cells [12]; therefore, we next investigated whether combined treatments also inhibited the colony forming capability of breast cancer cells, which is an important process in tumor growth. Fig. 3A shows the representative images in three repeated experiments. Fig. 3B shows the quantitative analysis of colony numbers, and the data are expressed as percentage relative to control cells. The results showed that the combined treatment can inhibit the formation of cell colonies better than either drug alone, which was consistent with the results from the viability and apoptosis assays.

3.4. Bazedoxifene and paclitaxel synergistically inhibited breast cancer cell migration

To explore whether bazedoxifene plus paclitaxel could synergistically inhibit breast cancer cell migration, we performed cell migration assays. Representative images and quantitative analysis are shown in Fig. 4. Cells migration was inhibited more significantly in the combined treatment groups than in those treated by single drugs (Fig. 4). The CI values of all the combination treatments were less than 1, suggesting there was synergism in the combination treatments of bazedoxifene with paclitaxel to inhibit cell migration. We performed MTT cell viability experiments and found that the drugs used in the migration experiments did not significantly affect cell viability (Fig. 4). Therefore, bazedoxifene and paclitaxel inhibited wound healing mainly due to the inhibition of cell migration rather than the indirectly results of inhibition of cell viability.

3.5. Bazedoxifene inhibited the expression of estrogen receptor, p-STAT3 $^{(Y705)}$, p-ERK $^{(T\ 202/T204)}$, P-AKT $^{(Ser473)}$ and other signaling molecules in breast cancer cells

To investigate the mechanism by which bazedoxifene inhibited breast cancer, we studied the expression of proteins after bazedoxifene was applied to treat breast cancer cells. Fig. 5A shows that T47D, BT474 and MCF7 cells had strong basal expression of estrogen receptor, while 4T1, MDA-MB-468 and MDA-MB-231 cells had no basal estrogen receptor expression. Fig. 5A also shows that 4T1, MDA-MB-468 and MDA-MB-231 cells had basal expression of p-STAT3 $^{(Y705)}$, while BT474, T47D and MCF7 cells did not express p-STAT3 $^{(Y705)}$. Actually, the expression of p-STAT3 $^{(Y705)}$ and STAT3 in T47D, BT474 and MCF7 cells was much less than in 4T1, MDA-MB-468 and MDA-MB-231 cells [20].

Previous reports have indicated that bazedoxifene inhibited MCF7 cell growth and functioned as a pure estrogen receptor antagonist [21]. In addition to MCF7, we also studied the mechanism by which bazedoxifene inhibited estrogen receptor-positive breast cancer cells in BT474 and T47D. Western blot (Fig. 5B, C, and D) showed that bazedoxifene downregulated the expression of estrogen receptor in all three estrogen receptor positive breast cancer cells, consistent with other findings [21,22]. Fig. 5B also showed that bazedoxifene dose-dependently inhibited the expression of p-P70S6K, survivin, c-Myc and Bcl-2 in MCF7 cells.

At present, there are few published studies about the inhibitory

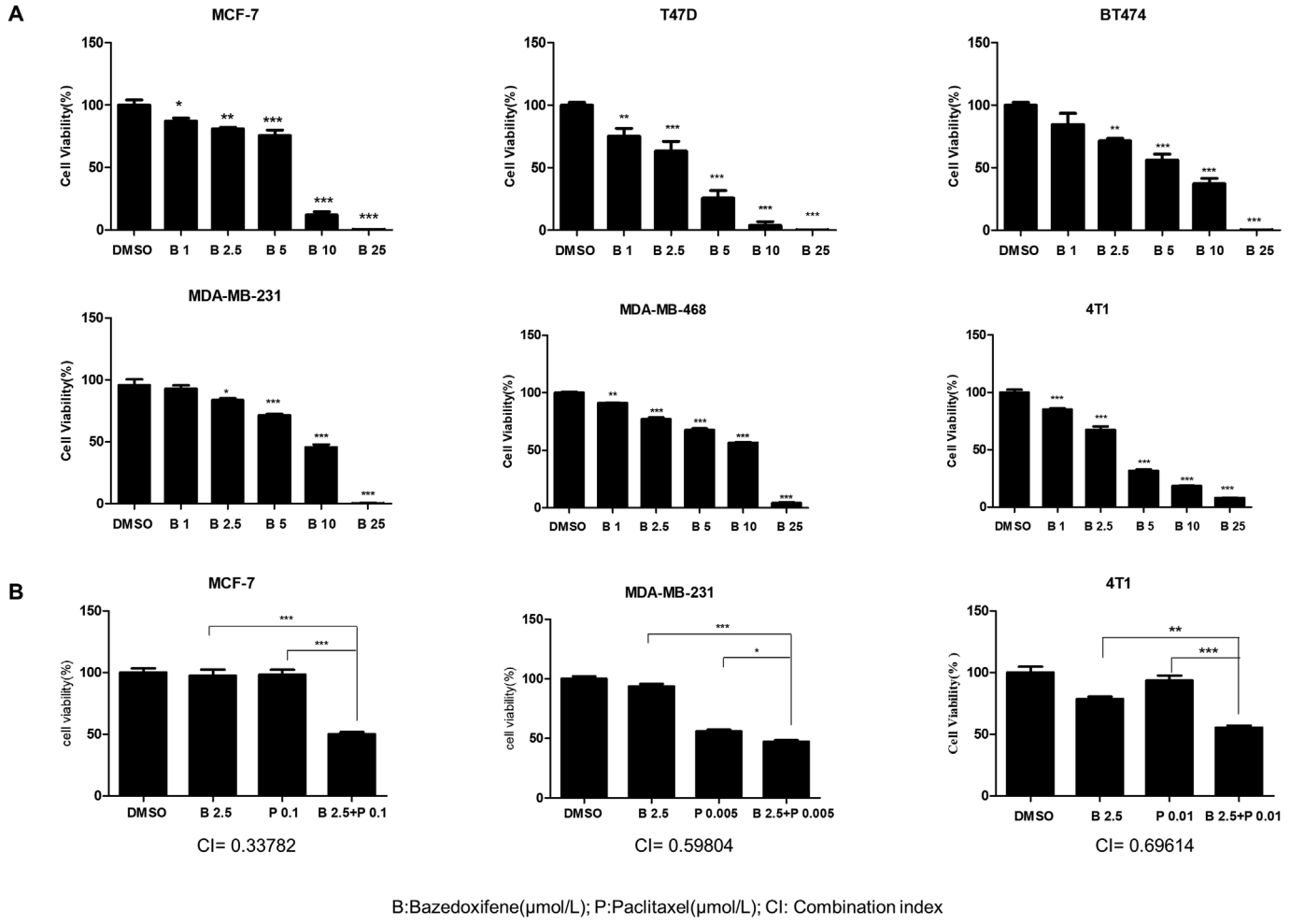

Fig. 1. Bazedoxifene inhibited cell viability of breast cancer cells and exhibited synergistic effects with paclitaxel treatment. (A) Breast cancer cells were treated with bazedoxifene at the indicated concentrations in triplicate for 48 h and processed for MTT assay to analyze cell viability (*, P < .05; **, P < .01; ***, P < .001). (B) MCF7, MDA-MB-231 and 4T1 cells were treated with bazedoxifene or/and paclitaxel at the indicated concentration in triplicate for 48 h and processed for MTT assay to analyze cell viability (*, P < .05; **, P < .01; ***, P < .001).

effect of bazedoxifene on TNBC and its mechanism. As shown in Fig. 5E, F, G, bazedoxifene dose-dependently inhibited the expression of p-STAT3 $^{(Y705)}$ without suppressing total STAT3 in MDA-MB-231, MDA-MB-468 and 4T1 cells. Bazedoxifene also inhibited the expression of p-AKT $^{(Ser473)}$ and p-ERK1/2$^{(T202/Y204)}$ (Fig. 5E, F, and G). Furthermore, bazedoxifene inhibited the expression of cyclin D1, p-P70S6K and c-Myc in 4T1 cells (Fig. 5E).

3.6. Bazedoxifene alone or combined with paclitaxel suppressed tumor growth of breast cancer in vivo

Studies have found that bazedoxifene can inhibit MCF7 mouse xenografts. To explore the inhibitory effect of bazedoxifene on transplanted tumors of triple-negative breast cancer in mice, we implanted 4T1 and MDA-MB-231 cells to establish mouse xenograft tumor models. Fig. 6A, B and C show data from 4T1 transplantation in the mouse mammary fat pad model; Fig. 6D, E and F show data from MDA-MB-231 transplantation in the mouse mammary fat pad model. As shown in Fig. 6A and D, tumor volume treated by bazedoxifene was significantly decreased compared with the tumor size in vehicle control group, indicating that bazedoxifene could suppress TNBC growth *in vivo*. Consistent with cell viability, cell proliferation and apoptosis experiments, Fig. 6D also showed that the combination of bazedoxifene and paclitaxel significantly inhibited the growth of TNBC *in vivo* more than monotherapy. Fig. 6B and E showed that bazedoxifene inhibited the increase of tumor weight, and bazedoxifene combined with paclitaxel was more effective in inhibiting tumor growth than either drug alone. In addition, Fig. 6C and F shows that bazedoxifene inhibited the expression of p-STAT3 $^{(Y705)}$, p-ERK $^{(T\ 202/T204)}$ and P-AKT $^{(Ser473)}$ in 4T1 and MDA-MB-231 cells, but did not affect the expression of STAT3, and upregulated the expression of cleaved caspase-3, thereby inhibiting the growth of these breast cancer cells *in vivo*.

4. Discussion

In this study, we found that bazedoxifene dose-dependently inhibited the cell viability of both estrogen receptor positive breast cancer cells and TNBC cells, as well as TNBC tumor growth *in vivo*. Combinational treatment of bazedoxifene along with the conventional chemotherapeutic agent paclitaxel synergistically impeded cell viability, colony formation, and cell migration and induced apoptosis far more significantly than single drugs alone. Western blotting revealed that bazedoxifene could inhibit the expression of estrogen receptor and downstream signaling molecules such as cyclin D1, p-P70S6K, survivin, c-Myc and Bcl-2 in estrogen receptor positive breast cancer cells, and bazedoxifene could inhibit the expression of p-STAT3 $^{(Y705)}$, p-ERK $^{(T202/T204)}$, P-AKT $^{(Ser473)}$ as well as the downstream signal molecules of STAT3, such as cyclin D1, p-P70S6K and c-Myc, in TNBC.

This study showed that bazedoxifene inhibited the expression of cyclin D1, p-P70S6K and c-Myc in both estrogen receptor positive and

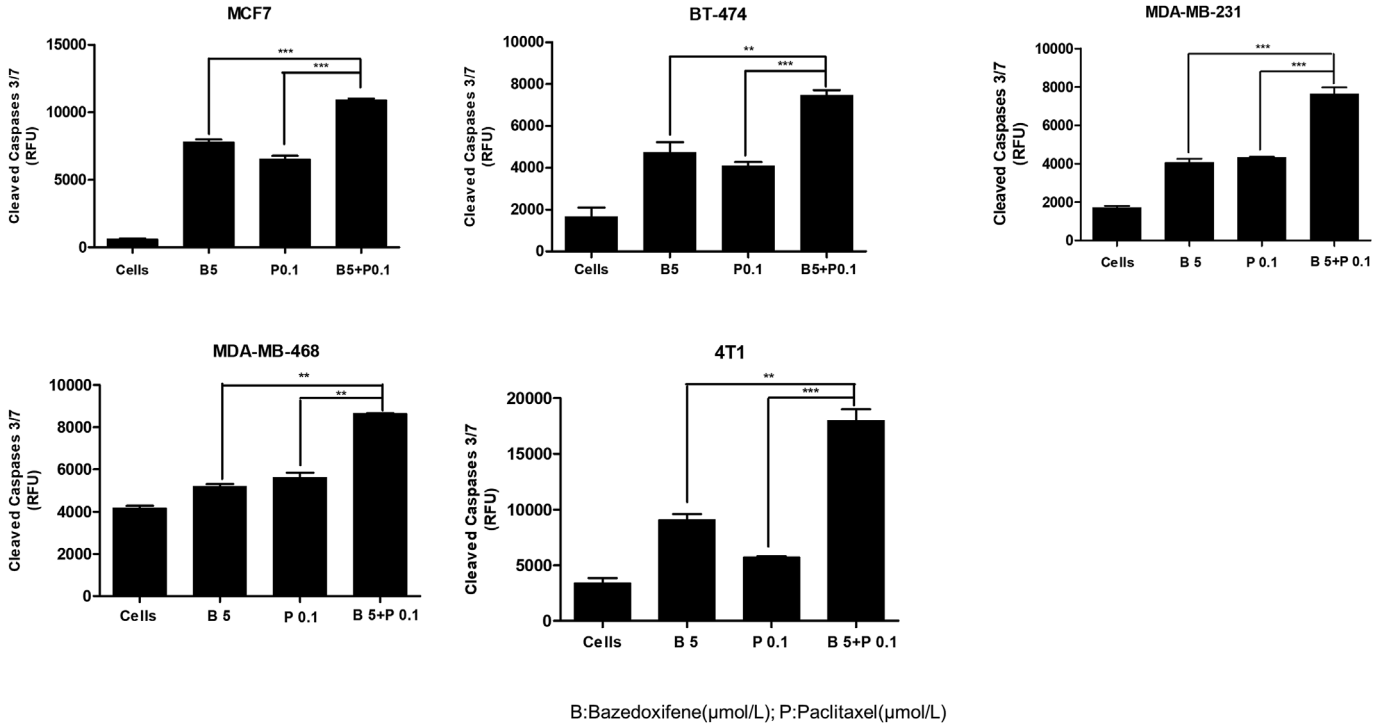

Fig. 2. Bazedoxifene combined with paclitaxel induced apoptosis of breast cancer cells more significantly than either drug alone. Breast cancer cells were treated with bazedoxifene (5 μmol/L) or/and paclitaxel (0.1 μmol/L) in triplicate for 5 h to detect cell apoptosis using Caspase-3/7 Fluorescence Assay Kit (**, P < .01; ***, P < .001).

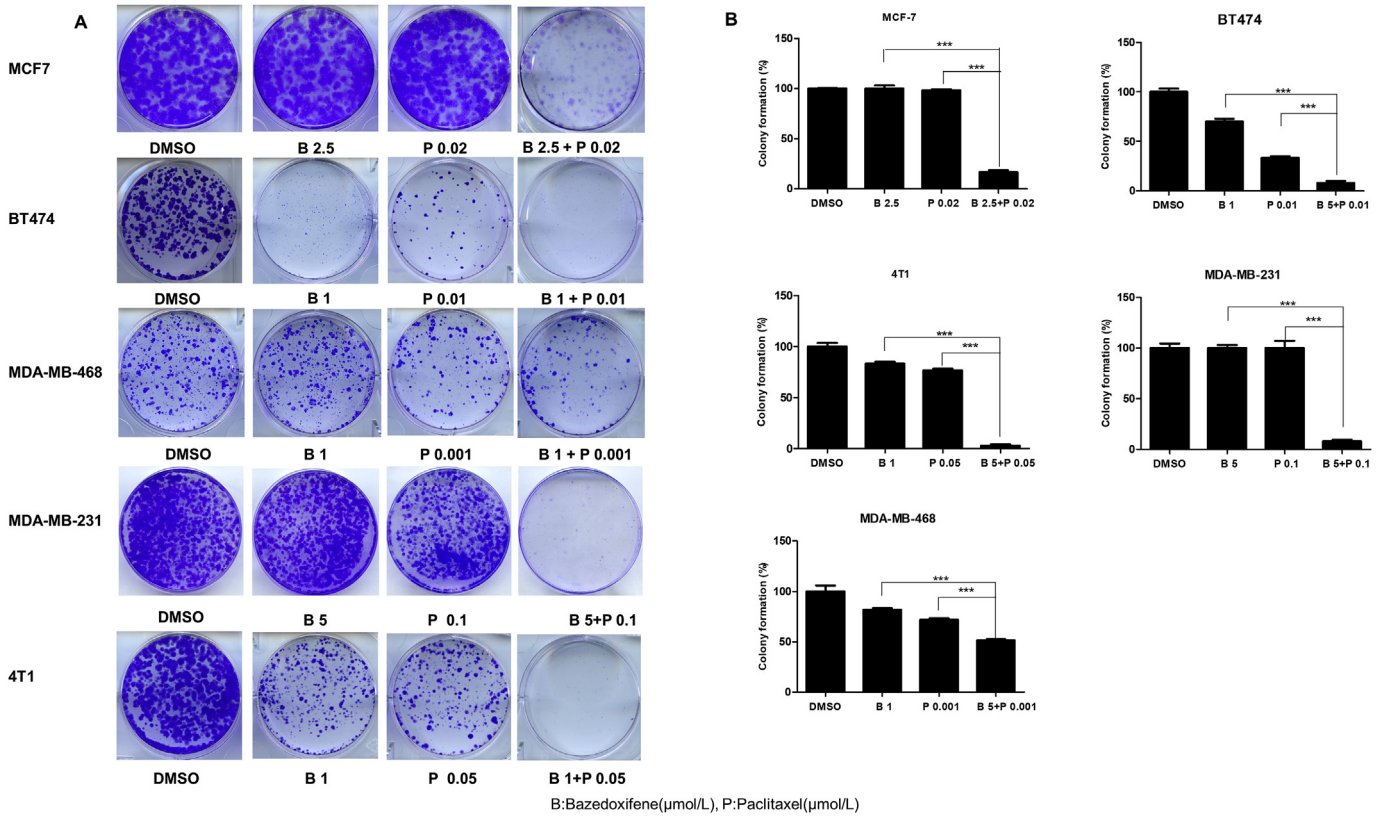

Fig. 3. The combination of bazedoxifene and paclitaxel inhibited the colony formation of breast cancer cells better than either drug alone. (A) The representative images of three repeated experiments, showing colony formation assay in breast cancer cells treated with bazedoxifene, paclitaxel or their combination. (B) The quantitative analysis of colony numbers; data are expressed in percentage relative to control cells.

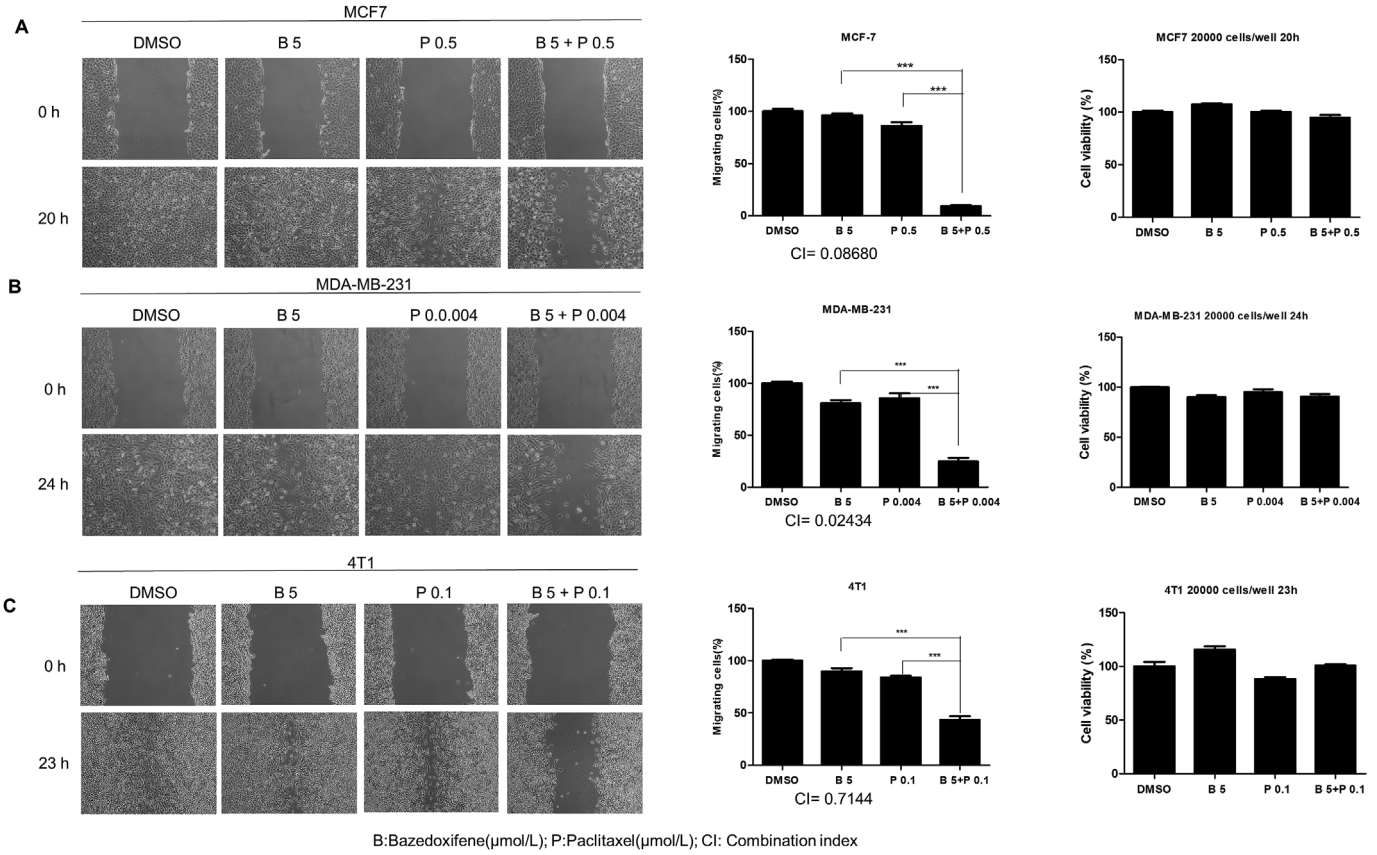

Fig. 4. Bazedoxifene and paclitaxel synergistically inhibited breast cancer cell migration. MFC7 (A), MDA-MB-231 (B) and 4T1(C) cells were treated with bazedoxifene, paclitaxel or their combination and allowed to migrate into the scratched area for 20–26 h. When the scratch in DMSO healed, pictures were taken with 100× magnification (***, P < .001).

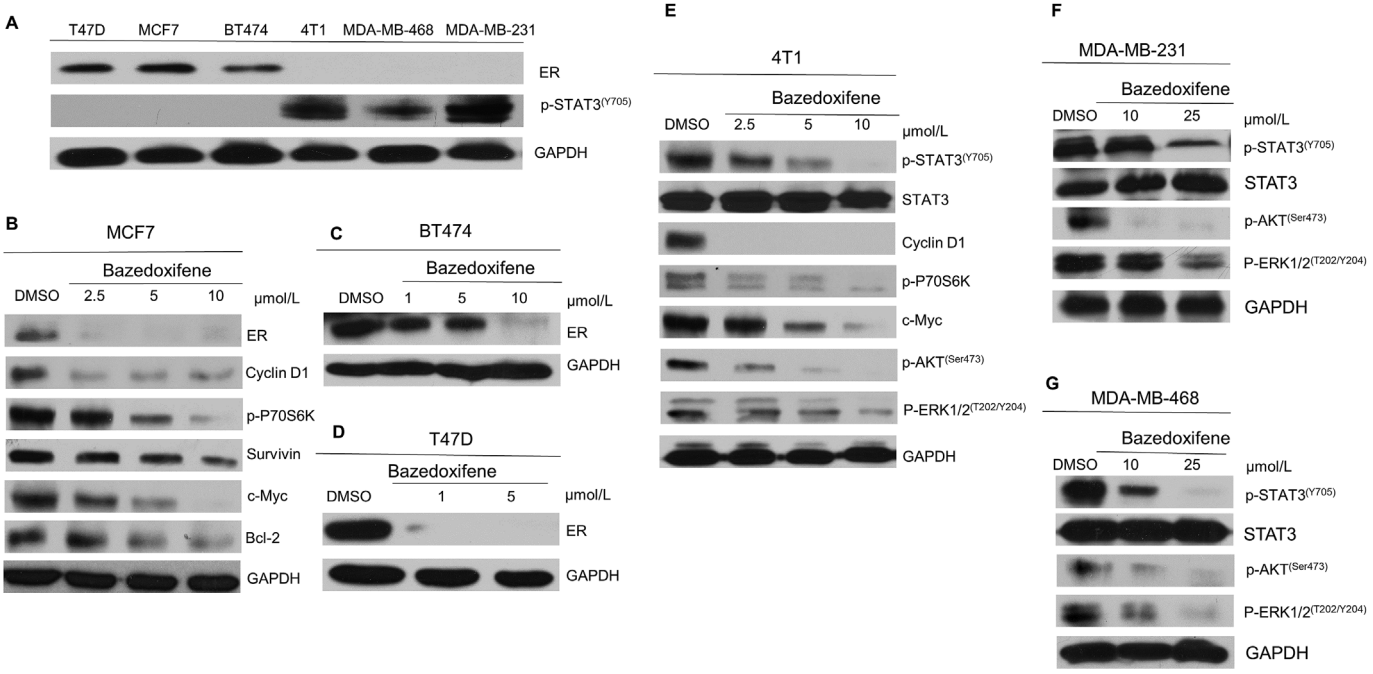

Fig. 5. Bazedoxifene inhibited the expression of estrogen receptor, p-STAT3 $^{(Y705)}$, p-ERK $^{(T202/T204)}$, p-AKT $^{(Ser473)}$ and other molecules in breast cancer cells. (A) Basal expression levels of estrogen receptor and p-STAT3 $^{(Y705)}$ in MCF7, BT474, T47D, MDA-MB-231, MDA-MB-468 and 4T1 cells. Bazedoxifene decreased the expression of estrogen receptor and several other proteins in MCF7 (B), BT474 (C) and T47D (D) cells after treatment with bazedoxifene for 24 h. Bazedoxifene decreased the expression of p-STAT3 $^{(Y705)}$, p-AKT $^{(Ser473)}$ and p-ERK $^{(T202/Y204)}$ and several other proteins in 4T1 (E), MDA-MB-231 (F) and MDA-MB-468 (G) cells after treatment with bazedoxifene for 24 h.

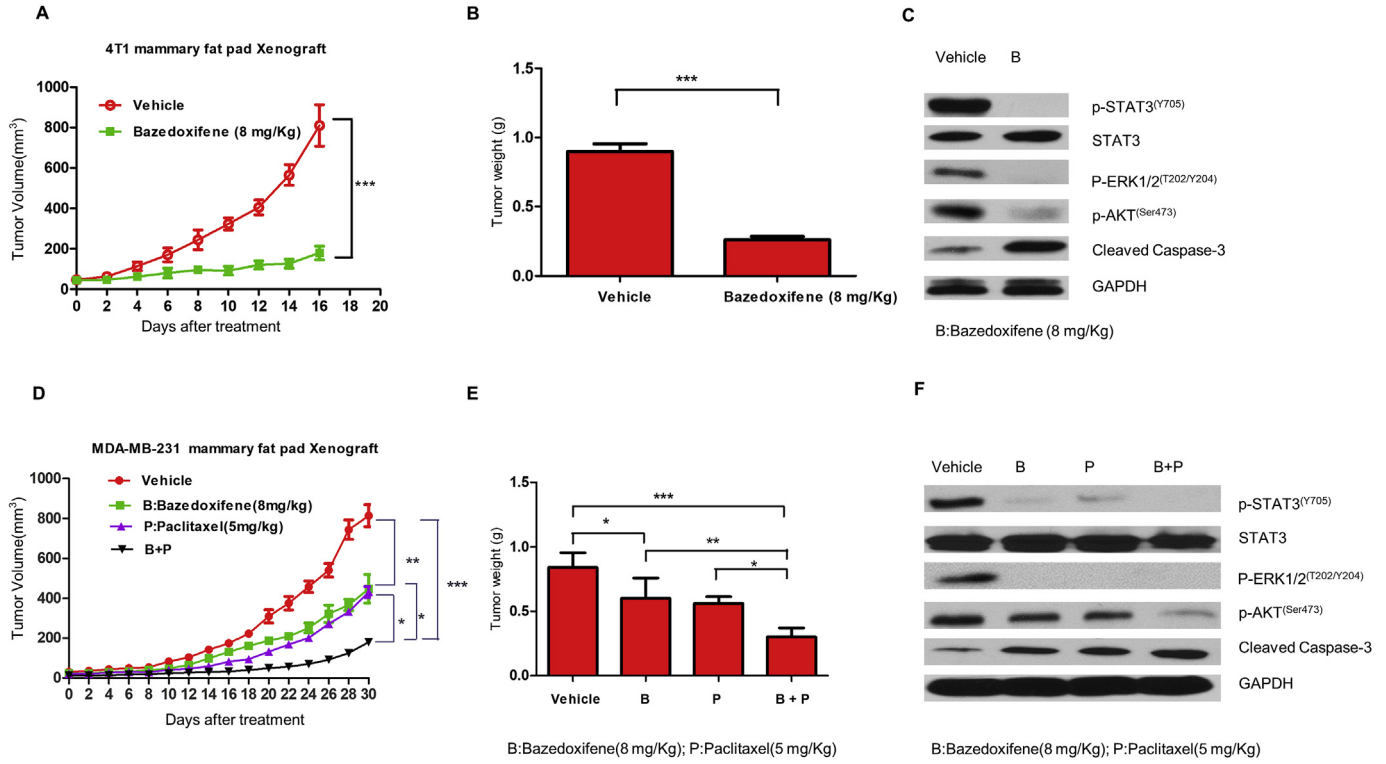

Fig. 6. Bazedoxifene alone or combined with paclitaxel suppressed tumor growth of breast cancer *in vivo*. Tumor volume (A), tumor weight (B) and proteins (C) of 4T1 tumors in mouse mammary fat pads treated with bazedoxifene (***, P < .001). Tumor volume (D), tumor weight (E) and proteins (F) of MDA-MB-231 tumor in mouse mammary fat pads treated with bazedoxifene, paclitaxel, or their combination (*, P < .05; **, P < .01; ***, P < .001).

TNBC cells, but not through the same signaling pathway. Study showed that E2 upregulated the expression of cyclin D1, Bcl-2, survivin, p-P70S6K and c-Myc in MCF7 cells with enhanced cell growth and reduced apoptosis, and bazedoxifene inhibited the proliferation of MCF7 cells as well as the expression of these molecules by suppressing estrogen receptor [23]. Our study also showed that bazedoxifene inhibited cell viability, proliferation, and migration and induced apoptosis of estrogen receptor positive breast cancer cells by inhibiting the expression of estrogen receptor and its downstream signaling molecules. Consistent with other studies, we found that MCF7, BT474 and T47D cells expressed no or a small amount of p-STAT3 (Y705) relative to triple-negative breast cancer cells depending on the exposure time in the western blot analysis [20]. Therefore, as a selective estrogen receptor modulator, bazedoxifene acts as an antagonist of the estrogen receptor in estrogen receptor positive breast cancer and inhibits the expression of the estrogen receptor and its downstream signaling molecules to inhibit cell viability, proliferation and migration and to induce apoptosis.

As 4T1, MDA-MB-231 and MDA-MB-468 cells do not express estrogen receptor, it is clear that the inhibition of cyclin D1, p-P70S6K, c-Myc and cell viability of TNBC by bazedoxifene is not through modulation of the estrogen receptor signaling cascade. Bazedoxifene inhibited the expression of these three proteins while inhibiting the phosphorylation of STAT3. Activated STAT3 is frequently found in various human cancer cell lines and tissues promoting cell proliferation, invasion and migration, angiogenesis, and increasing resistance to apoptosis [24]. The activation of STAT3 correlated with the elevated expression of survivin and cyclin D1 in breast cancer [25,26]. In fact, studies have shown that survivin and cyclin D1 are downstream signaling molecules of STAT3 in TNBC. STAT3 promotes tumor progression by deregulating cell growth proteins such as c-Myc and cyclin D1 [27]. Therefore, bazedoxifene inhibits STAT3 phosphorylation and its downstream signaling molecules such as c-Myc and cyclin D1, thereby inhibiting TNBC cells. Bazedoxifene inhibits the phosphorylation of STAT3 in TNBC cells, which is consistent with the mechanism by which we previously studied bazedoxifene inhibition of tumors. We previously found that bazedoxifene bound to GP130 and selectively inhibited IL-6 induced STAT3 phosphorylation in SUM159 TNBC cells [10].

STAT3 is activated in all breast cancer subtypes, but it is most often associated with TNBC [28]. Activation of STAT3 plays a critical role in cell proliferation, metastasis, apoptosis, and immune response in breast cancer [29]. IL-6 family cytokines, including IL-6 and others, are the main upstream regulators of the STAT3 signaling cascade [30]. Their promotion of breast cancer is related to hormone receptors expressed on the cell surface and the production of these cytokines through autocrine and/or paracrine signaling [31]. These cytokines activate STAT3 mainly through autocrine and paracrine signaling in TNBC [32,33] and through paracrine signaling in estrogen receptor positive breast cancer with low basal p-STAT3 $^{(Y705)}$ levels [34].

AKT and ERK signaling pathways are involved in tumorigenesis, and they regulate cellular functions such as cell survival, metabolism, growth and tumor development; the phosphorylated AKT and ERK are overexpressed in breast cancer, and they predict worse prognosis [35]. A study also showed that AKT and ERK were more highly phosphorylated in TNBC; this was similar to our study in which p-AKT $^{(Ser473)}$ and p-ERK $^{(T202/T204)}$ were expressed in 4T1, MDA-MB-231 and MDA-MB-468 cells, but not in MCF7 cells [36]. Researchers emphasized the importance of combined inhibition of these two signaling pathways, which may lead to better inhibition and clinical outcome [35]. In this study, bazedoxifene not only inhibited p-STAT3 $^{(Y705)}$ but also inhibited the expression of p-AKT $^{(Ser473)}$ and p-ERK $^{(T202/T204)}$. This is another mechanism by which bazedoxifene inhibited TNBC, similar to the mechanism by which bazedoxifene inhibited pancreatic cancer growth [13].

Bazedoxifene was originally used clinically to prevent and treat postmenopausal osteoporosis, and we newly found it as selectively inhibitor of IL-6 induced STAT3 phosphorylation in the GP130/JAK/STAT3 signaling pathway in cancer [10]; in this way, bazedoxifene

inhibited cell viability, proliferation and migration, and promoted apoptosis of pancreatic cancer and medulloblastoma cells [12–14]. We also found that it could inhibit the growth of transplanted tumors in immunosuppressive mice and enhance the sensitivity of traditional anti-cancer drugs and have a synergistic effect [12–14]. Thus, we investigated the inhibitory effect of bazedoxifene alone or combined with the traditional drug paclitaxel on estrogen receptor positive and TNBC in this study. We found that bazedoxifene alone inhibited cell viability as well as TNBC tumor growth *in vivo*; the combination of bazedoxifene and paclitaxel treatments generated more potent inhibition of cell viability, colony formation, cell migration and induced more apoptosis than single agents in estrogen receptor positive and TNBC cells. Thus, bazedoxifene could increase the sensitivity of breast cancer to paclitaxel.

Because bazedoxifene is an FDA-approved drug for clinical application with an overall favorable safety and tolerability profile [37], the discovery of anti-tumor effects of bazedoxifene on breast cancer may greatly facilitate breast cancer therapy and accelerate the clinical application of bazedoxifene. Studies have shown BZA/CEE reduced hot flush frequency and severity, and treated vulvar-vaginal atrophy and its symptoms; BZA/CEE showed a neutral effect on breast and endometrium without increasing breast tenderness, breast density, breast cancer, or endometrial stimulation, with good endometrial and breast safety [38,39]. Interestingly, bazedoxifene effectively antagonizes estrogen-induced uterine endometrial stimulation, and animal studies have found that BZA may be an effective new drug for the treatment of endometriosis [40]. The results support BZA/CEE for the relief of menopausal symptoms and prevention of postmenopausal osteoporosis, and it is safe for the breast and endometrium. The largest study with the longest duration reported that the incidence of venous thromboembolism (VTE) and cardiovascular events was similar in women treated with BZA/CEE or placebo [39]. Compared with placebo intervention for osteoporosis of postmenopausal women, bazedoxifene intervention resulted in no increased serious adverse events, myocardial infarction or stroke [41].

The production of estrogen in women after menopause is almost stopped, and estrogen supplementation in women who have lost estrogen for a long time induces apoptosis. *In vivo* studies have shown that BZA/CEE blocks the maturation of the mammary gland of ovariectomized immature mice [8]. Interestingly, xenograft studies have shown that BZA/CEE can prevent the growth of occult hormone-dependent tumors in nude mice [8]. BZA/CEE may also be used to prevent breast cancer.

Bazedoxifene has a good inhibitory effect on both estrogen receptor positive breast cancer and TNBC and can be used in combination with classic chemotherapeutic drugs paclitaxel to inhibit breast cancer. It may enhance the curative effects and reduce the toxicity of chemotherapeutic drugs. In view of the safety and these preclinical studies of bazedoxifene, clinical trials may consider the application of bazedoxifene alone or in combination with paclitaxel for the treatment of breast cancer, especially TNBC. Bazedoxifene may help prevent estrogen-dependent breast cancer, which needs further study.

Conflict of interest

The authors declare no potential conflicts of interest.

CRediT authorship contribution statement

Shengling Fu: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Xiang Chen:** Investigation, Validation. **Hui-Wen Lo:** Methodology, Writing – review & editing. **Jiayuh Lin:** Funding acquisition, Methodology, Resources, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2019.01.026.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, Ca - Cancer J. Clin. 68 (2018) 7–30 [PubMed: 29313949].
[2] G. Tinoco, S. Warsch, S. Gluck, K. Avancha, A.J. Montero, Treating breast cancer in the 21st century: emerging biological therapies, J. Canc. 4 (2013) 117–132 [PubMed: 23386910].
[3] C.Y. Liu, C.Y. Wu, K. Petrossian, T.T. Huang, L.M. Tseng, S. Chen, Treatment for the endocrine resistant breast cancer: current options and future perspectives, J. Steroid Biochem. Mol. Biol. 172 (2017) 166–175 [PubMed: 28684381].
[4] E.A. O'Reilly, L. Gubbins, S. Sharma, R. Tully, M.H. Guang, K. Weiner-Gorzel, J. McCaffrey, M. Harrison, F. Furlong, M. Kell, A. McCann, The fate of chemoresistance in triple negative breast cancer (TNBC), BBA Clin. 3 (2015) 257–275 [PubMed: 26676166].
[5] P.Y. Maximov, T.M. Lee, V.C. Jordan, The discovery and development of selective estrogen receptor modulators (SERMs) for clinical practice, Curr. Clin. Pharmacol. 8 (2013) 135–155 [PubMed: 23062036].
[6] E.M. Umland, L. Karel, N. Santoro, Bazedoxifene and conjugated equine estrogen: a combination product for the management of vasomotor symptoms and osteoporosis prevention associated with menopause, Pharmacotherapy 36 (2016) 548–561 [PubMed: 27027527].
[7] R.J. Santen, Y. Song, J.P. Wang, W. Yue, Preclinical breast effects of a tissue selective estrogen complex (TSEC) including conjugated estrogen with bazedoxifene, J. Steroid Biochem. Mol. Biol. 170 (2017) 61–64 [PubMed: 27174719].
[8] R.J. Santen, Y. Song, W. Yue, J.P. Wang, D.F. Heitjan, Effects of menopausal hormonal therapy on occult breast tumors, J. Steroid Biochem. Mol. Biol. 137 (2013) 150–156 [PubMed: 23748149].
[9] Y. Song, R.J. Santen, J.P. Wang, W. Yue, Effects of the conjugated equine estrogen/bazedoxifene tissue-selective estrogen complex (TSEC) on mammary gland and breast cancer in mice, Endocrinology 153 (2012) 5706–5715 [PubMed: 23070546].
[10] H. Li, H. Xiao, L. Lin, D. Jou, V. Kumari, J. Lin, C. Li, Drug design targeting protein-protein interactions (PPIs) using multiple ligand simultaneous docking (MLSD) and drug repositioning: discovery of raloxifene and bazedoxifene as novel inhibitors of IL-6/GP130 interface, J. Med. Chem. 57 (2014) 632–641 [PubMed: 24456369].
[11] T.H. Heo, J. Wahler, N. Suh, Potential therapeutic implications of IL-6/IL-6R/gp130-targeting agents in breast cancer, Oncotarget 7 (2016) 15460–15473 [PubMed: 26840088].
[12] H. Xiao, H.K. Bid, X. Chen, X. Wu, J. Wei, Y. Bian, C. Zhao, H. Li, C. Li, J. Lin, Repositioning Bazedoxifene as a novel IL-6/GP130 signaling antagonist for human rhabdomyosarcoma therapy, PLoS One 12 (2017) e180297.[PubMed: 28672024].
[13] X. Wu, Y. Cao, H. Xiao, C. Li, J. Lin, Bazedoxifene as a novel GP130 inhibitor for pancreatic cancer therapy, Mol. Canc. Therapeut. 15 (2016) 2609–2619 [PubMed: 27535971].
[14] X. Chen, J. Wei, C. Li, C.R. Pierson, J.L. Finlay, J. Lin, Blocking interleukin-6 signaling inhibits cell viability/proliferation, glycolysis, and colony forming activity of human medulloblastoma cells, Int. J. Oncol. 52 (2018) 571–578 [PubMed: 29207075].
[15] E. Saloustros, D. Mavroudis, V. Georgoulias, Paclitaxel and docetaxel in the treatment of breast cancer, Expert Opin. Pharmacother. 9 (2008) 2603–2616 [PubMed: 18803448].
[16] S. Fu, X. Chen, H.J. Lin, J. Lin, Inhibition of interleukin 8/CX-C chemokine receptor 1,/2 signaling reduces malignant features in human pancreatic cancer cells, Int. J. Oncol. 53 (2018) 349–357 [PubMed: 29749433].
[17] T.C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies, Pharmacol. Rev. 58 (2006) 621–681 [PubMed: 16968952].
[18] S. Fu, J. Lin, Blocking interleukin-6 and interleukin-8 signaling inhibits cell viability, colony-forming activity, and cell migration in human triple-negative breast

cancer and pancreatic cancer cells, Anticancer Res. 38 (2018) 6271–6279 [PubMed: 30396947].
[19] Y. Zhang, S. Miwa, N. Zhang, R.M. Hoffman, M. Zhao, Tumor-targeting Salmonella typhimurium A1-R arrests growth of breast-cancer brain metastasis, Oncotarget 6 (2015) 2615–2622 [PubMed: 25575815].
[20] A.K. Sasser, N.J. Sullivan, A.W. Studebaker, L.F. Hendey, A.E. Axel, B.M. Hall, Interleukin-6 is a potent growth factor for ER-alpha-positive human breast cancer, FASEB J. 21 (2007) 3763–3770 [PubMed: 17586727].
[21] S.E. Wardell, E.R. Nelson, C.A. Chao, D.P. McDonnell, Bazedoxifene exhibits antiestrogenic activity in animal models of tamoxifen-resistant breast cancer: implications for treatment of advanced disease, Clin. Canc. Res. 19 (2013) 2420–2431 [PubMed: 23536434].
[22] U. Akar, B. Ozpolat, K. Mehta, G. Lopez-Berestein, D. Zhang, N.T. Ueno, G.N. Hortobagyi, B. Arun, Targeting p70S6K prevented lung metastasis in a breast cancer xenograft model, Mol. Canc. Therapeut. 9 (2010) 1180–1187 [PubMed: 20423989].
[23] Y. Song, R.J. Santen, J.P. Wang, W. Yue, Inhibitory effects of a bazedoxifene/conjugated equine estrogen combination on human breast cancer cells in vitro, Endocrinology 154 (2013) 656–665 [PubMed: 23254198].
[24] H. Yu, R. Jove, The STATs of cancer–new molecular targets come of age, Nat. Rev. Canc. 4 (2004) 97–105 [PubMed: 14964307].
[25] T. Gritsko, A. Williams, J. Turkson, S. Kaneko, T. Bowman, M. Huang, S. Nam, I. Eweis, N. Diaz, D. Sullivan, S. Yoder, S. Enkemann, S. Eschrich, J.H. Lee, C.A. Beam, J. Cheng, S. Minton, C.A. Muro-Cacho, R. Jove, Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells, Clin. Canc. Res. 12 (2006) 11–19 [PubMed: 16397018].
[26] K. Leslie, C. Lang, G. Devgan, J. Azare, M. Berishaj, W. Gerald, Y.B. Kim, K. Paz, J.E. Darnell, C. Albanese, T. Sakamaki, R. Pestell, J. Bromberg, Cyclin D1 is transcriptionally regulated by and required for transformation by activated signal transducer and activator of transcription 3, Cancer Res. 66 (2006) 2544–2552 [PubMed: 16510571].
[27] J. Turkson, STAT proteins as novel targets for cancer drug discovery, Expert Opin. Ther. Targets 8 (2004) 409–422 [PubMed: 15469392].
[28] S.R. Walker, M. Xiang, D.A. Frank, Distinct roles of STAT3 and STAT5 in the pathogenesis and targeted therapy of breast cancer, Mol. Cell. Endocrinol. 382 (2014) 616–621 [PubMed: 23531638].
[29] P. Yue, J. Turkson, Targeting STAT3 in cancer: how successful are we? Expert Opin. Investig. Drugs 18 (2009) 45–56 [PubMed: 19053881].
[30] J.L. Bishop, D. Thaper, A. Zoubeidi, The multifaceted roles of STAT3 signaling in the progression of prostate cancer, Cancers (Basel) 6 (2014) 829–859 [PubMed: 24722453].
[31] Y. Guo, F. Xu, T. Lu, Z. Duan, Z. Zhang, Interleukin-6 signaling pathway in targeted therapy for cancer, Cancer Treat Rev. 38 (2012) 904–910 [PubMed: 22651903].
[32] M. Berishaj, S.P. Gao, S. Ahmed, K. Leslie, H. Al-Ahmadie, W.L. Gerald, W. Bornmann, J.F. Bromberg, Stat3 is tyrosine-phosphorylated through the interleukin-6/glycoprotein 130/Janus kinase pathway in breast cancer, Breast Cancer Res. 9 (2007) R32 [PubMed: 17531096].
[33] J.C. Lieblein, S. Ball, B. Hutzen, A.K. Sasser, H.J. Lin, T.H. Huang, B.M. Hall, J. Lin, STAT3 can be activated through paracrine signaling in breast epithelial cells, BMC Canc. 8 (2008) 302 [PubMed: 18939993].
[34] A.K. Sasser, N.J. Sullivan, A.W. Studebaker, L.F. Hendey, A.E. Axel, B.M. Hall, Interleukin-6 is a potent growth factor for ER-alpha-positive human breast cancer, FASEB J. 21 (2007) 3763–3770 [PubMed: 17586727].
[35] K.S. Saini, S. Loi, E. de Azambuja, O. Metzger-Filho, M.L. Saini, M. Ignatiadis, J.E. Dancey, M.J. Piccart-Gebhart, Targeting the PI3K/AKT/mTOR and Raf/MEK/ERK pathways in the treatment of breast cancer, Cancer Treat Rev. 39 (2013) 935–946 [PubMed: 23643661].
[36] S. Umemura, S. Yoshida, Y. Ohta, K. Naito, R.Y. Osamura, Y. Tokuda, Increased phosphorylation of Akt in triple-negative breast cancers, Cancer Sci. 98 (2007) 1889–1892 [PubMed: 17892507].
[37] T.J. de Villiers, A.A. Chines, S. Palacios, P. Lips, A.Z. Sawicki, A.B. Levine, C. Codreanu, N. Kelepouris, J.P. Brown, Safety and tolerability of bazedoxifene in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled phase 3 trial, Osteoporos. Int. 22 (2011) 567–576 [PubMed: 20535606].
[38] C.L. Smith, R.J. Santen, B. Komm, S. Mirkin, Breast-related effects of selective estrogen receptor modulators and tissue-selective estrogen complexes, Breast Cancer Res. 16 (2014) 212 [PubMed: 25928299].
[39] H.S. Taylor, K. Ohleth, Using bazedoxifene plus conjugated estrogens for treating postmenopausal women: a comprehensive review, Menopause 19 (2012) 479–485 [PubMed: 22278343].
[40] J.J. Kulak, C. Fischer, B. Komm, H.S. Taylor, Treatment with bazedoxifene, a selective estrogen receptor modulator, causes regression of endometriosis in a mouse model, Endocrinology 152 (2011) 3226–3232 [PubMed: 21586552].
[41] L. Peng, Q. Luo, H. Lu, Efficacy and safety of bazedoxifene in postmenopausal women with osteoporosis: a systematic review and meta-analysis, Medicine (Baltim.) 96 (2017) e8659. [PubMed: 29245225].