



Bazedoxifene is a novel IL-6/GP130 inhibitor for treating triple-negative breast cancer

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Abstract

Purpose Triple-negative breast cancer (TNBC) has been ranked as one of the devastating malignancy worldwide. Its disease progression and treatment obstacle is associated with the negligible expression of estrogen receptors (ER⁻), progesterone receptors (PR⁻), and HER2 (HER2⁻). Due to a lack of growth hormone receptors, TNBC is desperately demanding effective therapeutic regimens. A growing body of evidence indicated that glycoprotein 130 kDa (GP130), the pivotal mediator involved in interleukin 6 (IL-6) and signal transducer and activator of transcription 3 (STAT3) signaling pathways, is strongly correlated with tumor progression. Therefore, GP130 could become a novel target for treating TNBC. In our earlier studies, we demonstrated bazedoxifene as being a novel GP130 inhibitor.

Methods In the current report, anti-tumor effect of bazedoxifene on TNBC was further evaluated in TNBC cell lines SUM159, MDA-MB-231, and MDA-MB-468. We assessed anti-TNBC potency of bazedoxifene by carrying out various analysis encompassing western blot, cell proliferation, cell migration, colony formation, and growth of tumors in the xenograft mice.

Results Our findings demonstrated that bazedoxifene not only decreased the expression of P-STAT3, IL-6/GP130-mediated downstream target genes P-AKT and P-ERK, but also blocked mitogen effects stimulated by IL-6, including cell viability, and overall cell survive, proliferation as well as cell migration. Likewise in laboratory animal model, tumor growth in mice was remarkably suppressed by bazedoxifene via an oral administration route. Combinational treatment of bazedoxifene plus the conventional chemotherapeutic agent, paclitaxel, synergistically impeded cell viability, colony formation, and cell migration far more significantly than the one from single-drug alone.

Conclusions Taken together, our data suggest that bazedoxifene may be developed as a promising small molecular therapeutic agent for eradicating TNBC intrinsically associated with constitutively active IL-6/GP130/STAT3 signaling cascade.

Keywords Bazedoxifene · GP130 · Triple-negative breast cancer · P-STAT3 · Paclitaxel

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Introduction

Breast cancer is the second-leading cause of death in women in the United States. It represents 29% of all new female cancer cases and accounts for 14% of all female cancer death [1]. Breast cancer shows high morbidity, despite its survival factors are correlated with the stage, grade, histology, as well as expression of estrogen/progesterone receptor status (ER/PR) and human epidermal growth factor receptor 2 (HER2) [2]. Moreover, treatment of breast cancer with the conventional chemotherapy agents often lead to off-target side-effects, such as impaired fertility and premature menopause, osteoporosis, neuropathy, cardiomyopathy and congestive heart failure, myalgia and arthralgia [2]. Being one of the most abundant and fatal malignancy, the innovative treatment regimen for eradicating breast cancer is attracting a great research attention.

Based on the presence or absence of the expression of growth hormone receptors, breast cancer is largely categorized into estrogen receptor positive (ER⁺), HER2-positive (HER2⁺), or triple-negative breast cancer (TNBC), all of which demand IL-6 signaling at varying degrees [3]. IL-6, a 23- to 30-kDa pleiotropic cytokine, is overexpressed on or secreted by various cancer cells, cancer-associated stromal cells, drug-refractory cancer cells as well as cancer stem-like cells. Through recruiting glycoprotein 130 kDa (GP130), binding of IL-6 with IL-6 receptor (IL-6R) forms a hexameric IL-6/IL-6R/GP130 complex which further activates downstream effector cascade [4, 5], including phosphorylation of specific tyrosine residues on STAT3 (P-STAT3) to exert its tumor-promoting effects. Constitutive activation of STAT3, by phosphorylation of Tyr 705, has been frequently detected in human breast cancer cell lines as well as clinical breast cancer specimens [6]. The IL6/GP130/STAT3 signaling pathway not only advances many human cancers, but also promotes tumor survival, proliferation, differentiation, metastasis, angiogenesis, apoptosis, immunosuppression, and drug resistance [7–12]. GP130, also known as CD130, is a transmembrane protein, which is ubiquitously expressed in various tumors and is a common receptor subunit shared by 8 well-known cytokines (IL-6, IL-11, IL-27, LIF, CNTF, OSM, CT-1, and CLC) [13]. Recently, increasing attention has focused on targeting the IL-6/GP130 hexameric complex, with the aim of developing a new attractive option for anti-cancer therapy.

Based on the computational program of fragment-based drug design and drug repositioning using the multiple ligand simultaneous docking (MLSD) software, we previously discovered that two selective estrogen receptor (ER) modulator drugs, known for treating osteoporosis,

raloxifene (brand name: Evista) and bazedoxifene, could effectively disrupt the IL-6R/GP130 interactions [14]. The finding was recently validated in pancreatic cancer as well as in the head and neck cancer cell lines [14–16]. Among them, bazedoxifene binds GP130 D1 domain through the spots Ile83, Phe36, Tyr94, and Asn92. Further in vitro and in vivo findings implicated bazedoxifene as being a novel inhibitor specifically for blocking GP130 signaling, and thus it could become a promising anti-cancer agent. Bazedoxifene is primarily an estrogen-receptor antagonist in breast and endometrial tissue, and approved in 2013 by FDA, as a third generation of selective estrogen receptor modulator (SERM) against the postmenopausal osteoporosis.

As TNBC remains the lethal malignancy and that it is lacking effective treatment agents, and ER-negative breast cancer cells mainly express the transmembrane GP130/IL-6 receptor [7], GP130 could be developed into a novel target for the therapy. Although the GP130-specific small molecule inhibitors encompassed B-R3 and B-P4, madindoline A, SC144, raloxifene and bazedoxifene, as well as LMT-28 [3], their action modes were classified into monoclonal antibodies, natural product, and synthetic compounds. Earlier finding reported that co-administration of SC144 plus paclitaxel delayed breast tumor growth in an SC144 dose-dependent manner [17]. In the current study, SC144, a small-molecule inhibitor selectively targeting GP130 [18], was used to set the baseline outcome, for comparing the efficacy of bazedoxifene as either a single agent or in combination with paclitaxel for treating TNBC breast cancer. Because bazedoxifene impedes the binding of IL-6 to GP130 or to IL-6 R α , the downstream signaling cascade effectors including JAK/STAT3, PI3-K/AKT/mTOR, and MEK/ERK were assessed. Likewise, the neoplastic effects on cell viability, proliferation, migration were evaluated. This study demonstrated the significant therapeutic effects of bazedoxifene on treating TNBC both in vitro as well as in vivo.

Materials and methods

Cell lines and reagents

Human TNBC cell lines, SUM159, MDA-MB-231, and MDA-MB-468, were subjected to this study. MDA-MB-231 and MDA-MB-468 cells were purchased from ATCC (the American Type Culture Collection, Manassas, VA, USA) while SUM159 cells were obtained from Dr. Max S. Wicha (University of Michigan, Ann Arbor, MI). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as well as 1% Penicillin/Streptomycin and maintained in a humidified 37 °C incubator with 5% CO₂.

Bazedoxifene acetate was purchased from Acesys Pharmatech (Fairfield, NJ, USA). SC144 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Paclitaxel was from LC Laboratories (Woburn, MA, USA). According to the instructions provided from the manufacturers, these drugs were dissolved in sterile dimethyl sulfoxide (DMSO) to constitute a 20 mM stock agents and the aliquots were stored at -20°C .

Western blotting assay

Human TNBC cell lines (SUM159, MDA-MB-231, and MDA-MB-468) were propagated in the routine growth medium to reach 70–80% confluence and then were treated with bazedoxifene (10 and 20 μM) or with DMSO control. After an overnight incubation (about 12 h), cells were harvested by mechanical scraping and then lysed in a cold RIPA lysis buffer (Cell Signaling Technology, #9803) supplemented with inhibitors to block proteases and phosphatases activities intrinsic to cell lysate.

For IL-6 treatments, MDA-MB-231 and MDA-MB-468 first underwent serum starvation for 24 h and then were added with bazedoxifene at 0, 5, and 10 μM for 5 h. Cells were next incubated in 50 ng/mL of IL-6 for 30 min and then lysed. 50 ng/mL of EGF was also incubated with in parallel. The lysates were subjected to 10% SDS-PAGE to resolve various proteins and then the proteins migrated and adhered to gels were further transferred to a PVDF membrane (GE Healthcare, #10600023). Membranes were probed with a 1:1000 dilution of specific primary antibody followed by with 1:10,000 HRP conjugated secondary antibody. The primary antibodies, specifically recognize phosphorylated STAT3 (Tyr705), total STAT3, phosphorylated STAT5, total STAT5, phosphorylated-AKT (Ser473), phospho-specific extracellular signal-regulated kinase (ERK) 1/2 (Threonine 202/Tyrosine 204), and cleaved caspase-3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as well as the secondary antibody, were all from Cell Signaling Technology (Beverly, MA, USA). The resultant Immunoreactive complex was revealed by using enhanced chemiluminescence plus reagents (SuperSignal™ West Femto Maximum Sensitivity Substrate, Thermo, #34096) and the discrete bands, corresponding to immune-complex, were quantified by using the Storm Scanner (Amersham Pharmacia Biotech Inc, Piscataway, NJ) system. P-STAT3, P-AKT, and P-ERK were quantitated and normalized by total STAT3 using Image J.

Growth inhibition assay

Cells proliferations were assessed by BrdU (Bromodeoxyuridine) Cell Proliferation Assay Kit (Cell Signaling Technology, # 6813S). Briefly, cells were seeded in 96-well plates in triplicate at a density of 8000 cells per well and

then continuously grew for an overnight in the same complete medium. The next morning, cells were incubated in an incomplete medium devoid of FBS for another 24 h, followed by a treatment with either bazedoxifene or SC144, at the doses indicated elsewhere. 5 h after the drug treatment, cells were supplemented with IL-6 (10 ng/mL) for additional 24 h and fixed in solution (100 μL per well). Fixed cells were further treated with anti-BrdU antibody followed by secondary antibody, and then incubated with TMB substrate at room temperature for 30 min. After adding 100 μL /well of stop solution, the absorbance at 450 nm, associated with each well, was measured and the readout served as an indicative of cell proliferation.

The growth inhibition effect of GP130 knockdown on MDA-MB-231, SUM159, and MDA-MB-468 cells, effect of bazedoxifene on GP130 knocked down cells, were investigated using MTT assay. Briefly, cancer cells were seeded in each well of 96-well plates in triplicates at a density of 8000 cells per well and grew for an overnight. The cells were then treated with control siRNA (Invitrogen) and GP130 siRNA (Santa Cruz Biotechnology) at 37°C for 24 h, respectively, and then incubated in 10% FBS DMEM for additional 72 h or treated with different doses of drugs for 48 h. Each well was further incubated with 25 μL of thiazolyl blue tetrazolium dye (MTT, Sigma) for 4 h, followed by the addition of 150 μL of *N,N*-Dimethylformamide (Sigma). The resultant mixture was placed on a shaker for overnight at room temperature. The colorimetric readouts associated with each well and measured from the absorbance at 595 nm served as an indicative of cell viability in given well.

Furthermore, effects of bazedoxifene and paclitaxel single-drug alone or in combination, on the cell viability were also assayed by MTT.

Caspase-3/7 assay

Cellular apoptosis was determined using Caspase-3/7 Fluorescence Assay Kit (Cayman, Ann Arbor, MI) according to the manufacturer's instruction. MDA-MB-231 and SUM159 cells were seeded in 96-well plates (8000 cells/well), respectively. And bazedoxifene at 0, 10, and 15 μM was treated. After 5 h of treatment, caspase 3/7 activity was measured at an excitation wavelength of 485 nm and an emission of 535 nm.

STAT3-dependent transcriptional luciferase activity assays

Plasmid pLucTKS3, comprising seven copies of the STAT3 binding site driven from the thymidine kinase minimal promoter, was used in this study for assessing the STAT3-dependent transcriptional activities [6, 19]. pLucTKS3 plus the control pLucSV40 luciferase reporter plasmids were

concurrently transfected into MDA-MB-231 cell line, by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The stable clones, which showed high luciferase activity, were selected for evaluating the effect how bazedoxifene impacts STAT3 transcription. After exposing to various doses (5, 10, and 20 μM) of bazedoxifene for 24 h, the STAT3 transcriptional activities were inferred from the luciferase activity measured by using a Promega luciferase kit.

Colony formation assay

Cells were grown to 60–80% confluent in six-well cell culture plates and then pre-treated with SC144, bazedoxifene, or the combination of bazedoxifene and paclitaxel for 4–8 h. Drug-treated cells were harvested by trypsinization and 1000 of the resultant viable cells were re-plated in each of 60 mm plate followed by a continuous cultivation to reach the cell confluence. Two to three weeks later, cells were washed with PBS twice and fixed with cold methanol for 30 min followed by a staining with 1% crystal violet dye (dissolved in 25% methanol) at room temperature for 1 h. The plates were then rinsed with distilled water, dried, followed by a quantification using colorimetric scanning.

Cell migration and invasion assays

MDA-MB-231 and SUM159 were seeded in each of six-well plates. When cells reached 100% confluent, a straight line with approximately the same width was scratched across the monolayer using a pipette tip. After rinsing, cells were then treated with the control DMSO or with different doses of bazedoxifene alone or in combination with paclitaxel. 24 h later when the wound in the control group (treated with DMSO) was closed, the image of the scratch line in the drug-treated cells was captured by Leica Microsystems and the width of the scratched line was quantified by three independent observers. By comparing the width of scratch lines obtained from the drug-treated cells to the one from DMSO-treated cells (set as 100%), the relative migratory potential was derived.

Cell invasion assays were performed using CytoSelect™ 24-well cell invasion assay (basement membrane) kit (Cell Biolabs Inc., Germany) according to the manufacturer's instructions. Briefly, cell suspension containing 1.5×10^5 (MDA-MB-231) and 0.3×10^5 (SUM159) in 300 μL serum-free medium with bazedoxifene were seeded in the inserts which were placed over the lower well containing 500 μL of culture medium. Cells were cultured for 24 h and stained with the Cell Stain Solution at room temperature for 10 min. Invasive cells were counted under a light microscope under high magnification objective from multiple randomly selected visual fields. MTT assay was

also conducted. 0.3×10^5 /well cells were seeded into 96-well plate with bazedoxifene and MTT assay was performed after 24 h of culture.

In vivo tumor xenograft studies in tumor-bearing mice

All animal studies were conducted in accordance with the principles and standard procedures set off by IACUC of the University of Maryland, Baltimore, Baltimore, MD, USA. Briefly, SUM159 or MDA-MB-231 cells (3×10^6) contained in Matrigel (BD Science Franklin Lakes, NJ) were injected subcutaneously into mammary fat pads and both sides of flank area, respectively, of 6-week-old female athymic nude mice, respectively, which were all without ovariectomized, and were purchased from Harlan (Indianapolis, IN, USA). One week after the initial implantation, eight mice were equally divided into two treatment groups, respectively, consisting of saline vehicle control and bazedoxifene treatment. For SUM159 tumor-bearing mice, the dosage of bazedoxifene (pure bazedoxifene, not bazedoxifene acetate) was 8.8 mg/kg/d and for MDA-MB-231 group, it was 4.4 mg/kg/d. All were treated orally each day. Tumor growth was quantified by measuring the length (L) and width (W) of the tumor every other day with a caliper, and the tumor volume was calculated based on the formula as being $0.52 \times LW^2$. After 22 days of treatment, tumors were harvested, and weight. Further, tumors tissue homogenates were lysed and separated by SDS-PAGE to examine the expression of STAT3 phosphorylation.

Statistical analysis

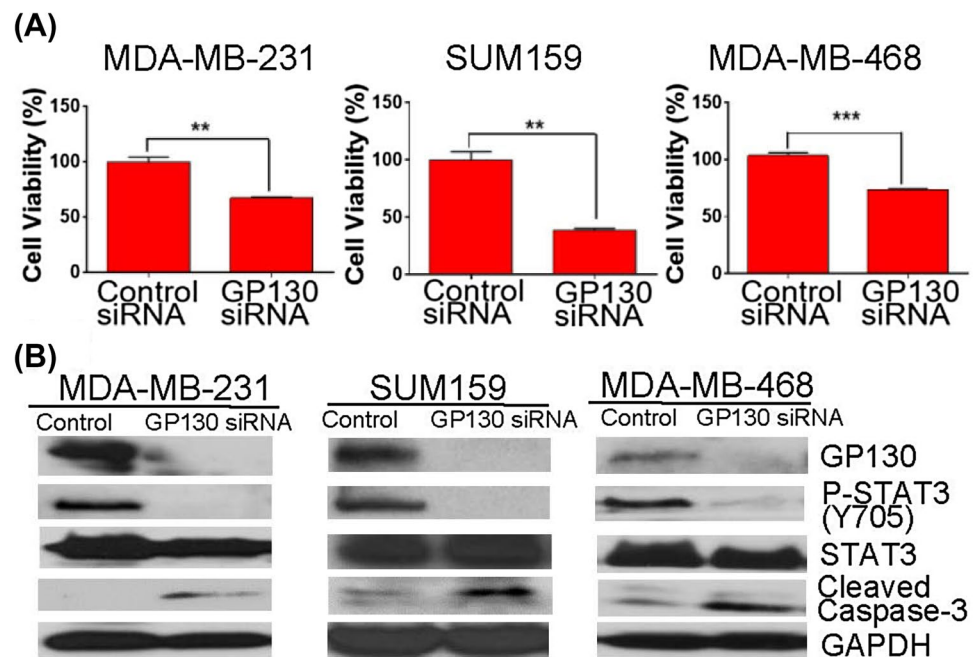
The Student's *t* test was used for conducting statistical analysis and *p* value was determined via Prism 7.0 (GraphPad Software, Inc.). Differences were considered to be statistically significant, if *p* values were < 0.05 .

Results

GP130 is essential for the TNBC cell viability

We used GP130 siRNA to knock down the expression of GP130 to verify the effect of GP130 on cell viability. Results are shown in Fig. 1. Figure 1a displayed that the cell viabilities in all experimental cell lines were reduced when GP130 siRNA treated. Furthermore, Fig. 1b confirmed the expressions of GP130 were exactly reduced after GP130 siRNA treatment, which suggest the important role of GP130 in cell growth. Targeted GP130 inhibitor will affect the cell viability, reduce expression of P-STAT3, and increase caspase-3.

Fig. 1 Knockdown of GP130 inhibited TNBC cell viability. **a** Cell viability of MDA-MB-231, SUM159, and MDA-MB-468 were significantly inhibited by GP130 siRNA. **b** The expression levels of GP130 were reduced when with GP130 siRNA incubated, while p-STAT3 were increased in MDA-MB-231, SUM159, and MDA-MB-468 compared with control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, mean \pm SD



Bazedoxifene hampered activation of STAT3, AKT, and ERK, a phenomenon inferred by their reduced phosphorylation

IL-6 enhances tumor progression through the activation of JAK/STAT3, RAS/MAPK, and PI3K/AKT oncogenic pathways resulting in a gain of malignant features including proliferation, survival, migration, invasion, angiogenesis, and cancer promoting inflammation [20]. Herein, we examined the impact of bazedoxifene on downstream effector molecules of IL-6/GP130 including P-STAT3 (Y705), P-AKT (S473), and P-ERK. As shown in Fig. 2a, b, levels of P-STAT3 (Y705), P-AKT (S473) and P-ERK were all suppressed when bazedoxifene treated at 20 μ M. The expression of total STAT3 in each cell line group was, however, largely abundant and unaffected by the drug treatment, indicating bazedoxifene only inhibited STAT3 activation via phosphorylation rather than suppressing its overall expression. IL-6 was able to elevate P-STAT3 and this occurrence was, however, reverted when bazedoxifene was introduced (Fig. 2c). At a concentration of 10 μ M, bazedoxifene inhibited P-STAT3 that were initially stimulated by IL-6 (Fig. 2c). Figure 1d shows that the bazedoxifene had no effects on inhibition of P-STAT3, P-STAT5, and P-AKT, which were initially stimulated by EGF. The results suggest bazedoxifene may play a potent effect via targeting to inhibit IL-6/GP130.

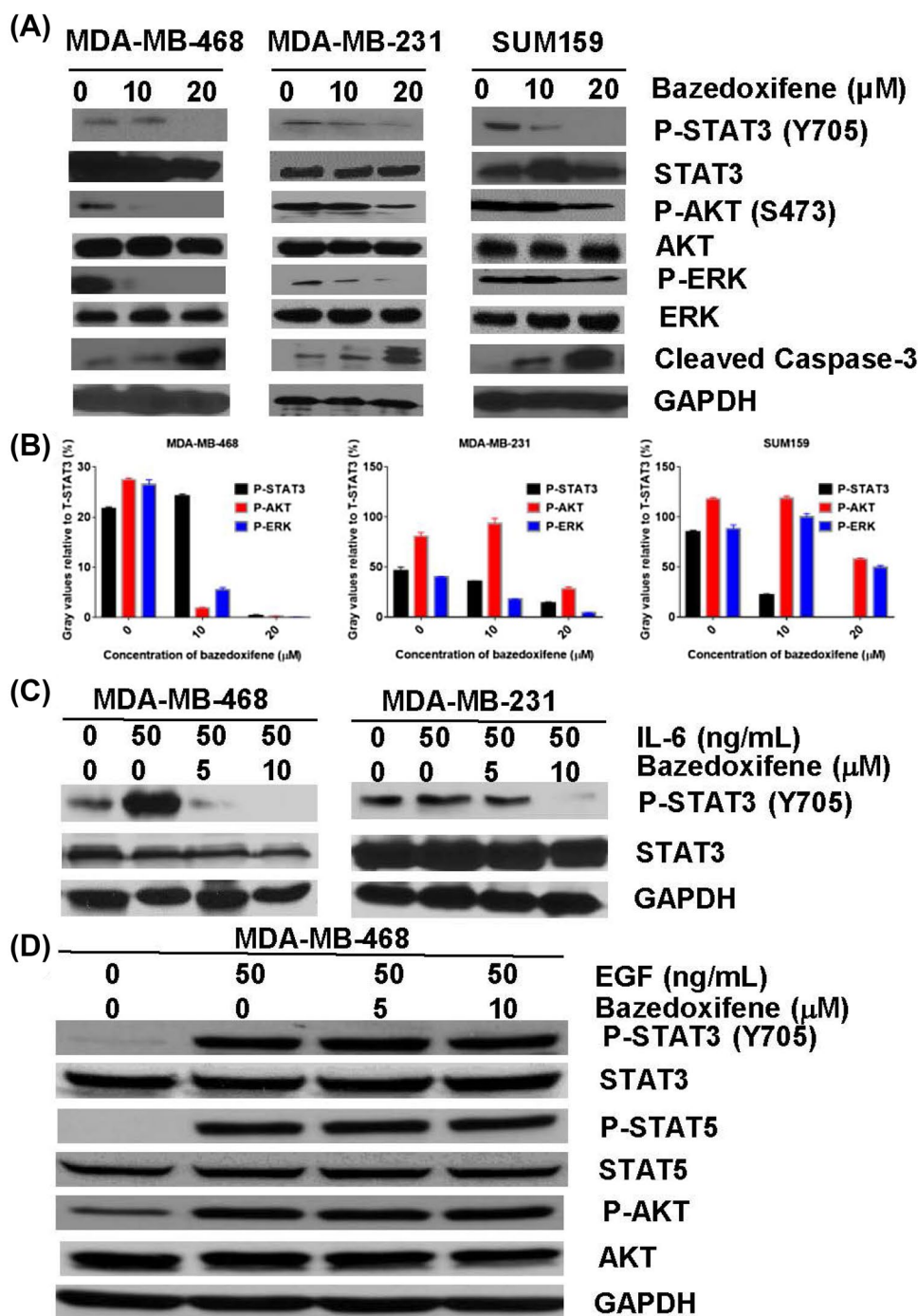
Cell apoptosis occur in a concentration-dependent manner in response to bazedoxifene

Figure 3 displayed the treatment of MDA-MB-231 and SUM159 with bazedoxifene resulted in a significant concentration-dependent increase in caspase-3/7 which demonstrated bazedoxifene can affect cell viability through induction of apoptosis.

GP130 expression is essential for bazedoxifene-mediated inhibition of cell viability

To further verify the selectivity of bazedoxifene on GP130, the growth inhibition effects of bazedoxifene on GP130 knockdown cells were investigated. Figure 4 shows that the cell viability of SUM159 cells were reduced when bazedoxifene was added compared with control siRNA. However, bazedoxifene had no inhibited effects in GP130 siRNA groups. Western blot results showed that the P-STAT3 and cyclin D1 were reduced in GP130 siRNA treatment group. Results indicate that the GP130 is essential for bazedoxifene-mediated inhibition effects.

Fig. 2 Bazedoxifene hindered STAT3, AKT, and ERK signaling pathways. Western blot results (a) and quantification (b) described inhibition of P-STAT3, P-AKT, and P-ERK by bazedoxifene at 20 μ M in TNBC and this effect was also responsive to IL-6-treatment in MDA-MB-231 breast cancer cells (c). However, bazedoxifene cannot inhibit P-STAT3, P-STAT5 and P-AKT induced by EGF (d)



Cell proliferation induced by IL-6 was inhibited by bazedoxifene

Furthermore, how bazedoxifene affects cell proliferation in TNBC cells was assessed by BrdU incorporation method in MDA-MB-468, SUM159, and MDA-MB-231 cell lines. After 5-h incubation with bazedoxifene or with SC144 in the growth medium devoid of FBS, cells were further treated with IL-6. At 24 h later, the cells that underwent proliferation and thus incorporated BrdU were quantified. As shown

in Fig. 5, proliferated cells derived from the prior incubation with IL-6 (2nd bar in Fig. 5a–c) were more evident than the ones in the untreated controls (1st bar and set as 100%). Our data indicate that IL-6 could promote TNBC proliferation and this stimulatory effect can be blocked by introducing bazedoxifene or SC144 in a concentration-dependent manner in cell level (** $p < 0.001$, IL-6 plus bazedoxifene or plus SC144, as compared to IL-6 mono-treatment alone). Thus, results demonstrate the mechanism of bazedoxifene action again related with IL-6 [16].

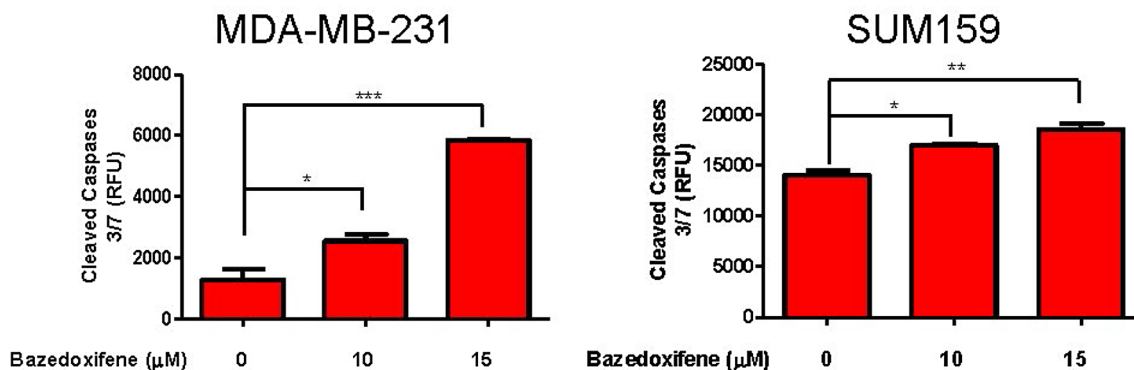


Fig. 3 Cell apoptosis occurs in a concentration-dependent manner in response to bazedoxifene. Cellular apoptosis was determined using the Apo-ONE homogeneous caspase 3/7 assay. Bazedoxifene were

treated with 10 and 15 μM in MDA-MB-231 and SUM159 groups. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, mean ± SD

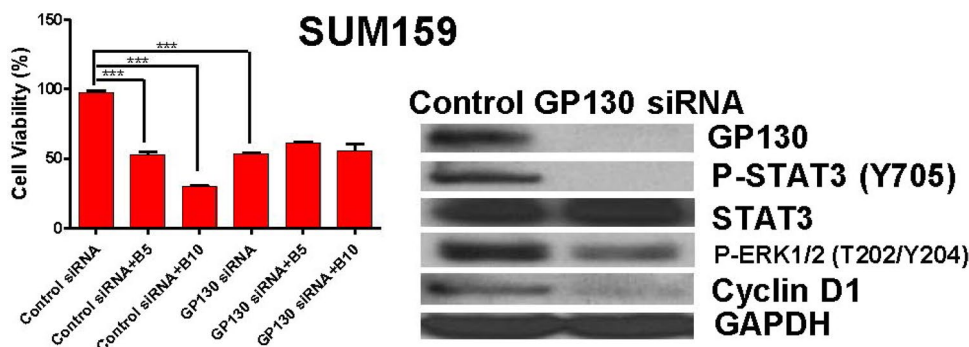


Fig. 4 GP130 expression is essential for bazedoxifene-mediated inhibition of cell viability. The left section is the cell viability of SUM159 under the treatment of bazedoxifene, GP130 siRNA, and

their combination. B5 and B10 means the treated bazedoxifene at 5 or 10 μM. The right section is the western blotting assay after GP130 siRNA treated. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, mean ± SD

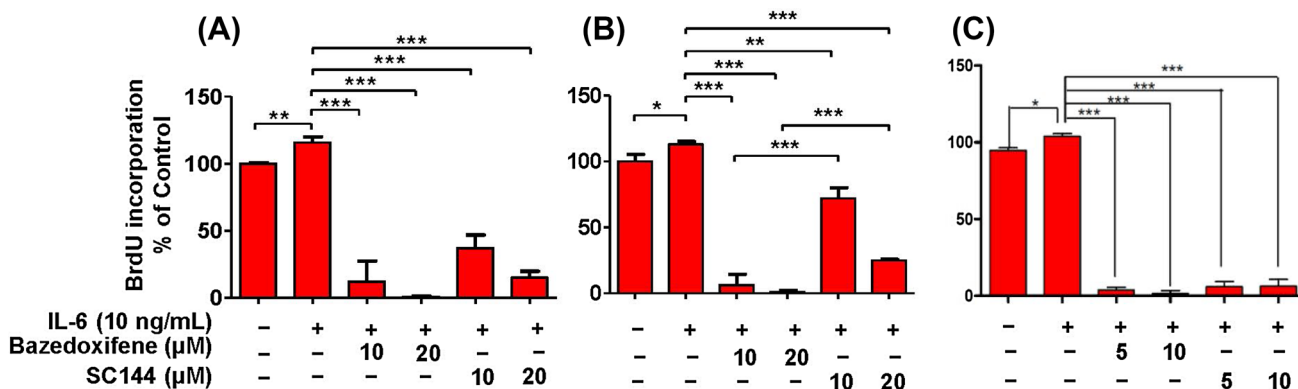


Fig. 5 Bazedoxifene suppressed TNBC proliferation induced by IL-6 and this inhibitory effect was more potent than the one exerted from SC144. MDA-MB-468 (a), SUM159 (b), and MDA-MB-231 (c) were seeded in routine culture medium for an overnight. Next morning, cells were switched to the medium devoid of FBS. 24 h later, cells were further incubated with bazedoxifene or with SC144 for 5 h followed by with IL-6 for 24 h. To quantify proliferation, BrdU incor-

poration assay was conducted. Cells in each well were subjected to spectrophotometric analysis to obtain absorbance at 450 nm that not only reflected BrdU incorporation but also served as an indicative of cell proliferation. Relative cell proliferation was obtained by comparing the absorbance derived from IL-6 or drug-treated cells to the one from untreated control cells (set as 100%). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, mean ± SD

Bazedoxifene is a potent inhibitor repressing STAT3-mediated gene transcription in a concentration-dependent manner

After binding of ligand IL-6 with its receptor GP130, the downstream effector like STAT3 can be constitutively activated [12]. The effect of bazedoxifene on the STAT3-targeted transcriptional activation was conducted by using STAT3-dependent luciferase assays in MDA-MB-231, one of TNBC cell lines that expresses constitutively activated STAT3 [19]. Towards this aim, our laboratory established cloned cells by stably transfecting a STAT3-dependent luciferase reporter construct, pLucTKS3, in MDA-MB-231.

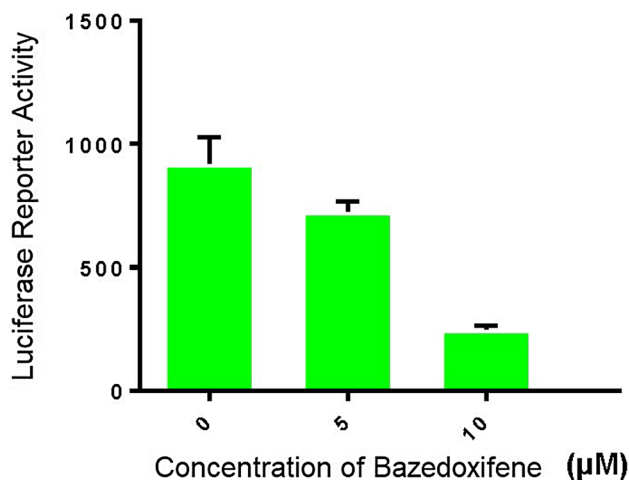


Fig. 6 Bazedoxifene inhibited STAT3-mediated gene transcription in MDA-MB-231 cell engineered to constitutively express STAT3-dependent transcription reporter construct. The engineered MDA-MB-231 construct was transfected with pLucTKS3 and pLucSV40 luciferase reporter plasmids by using Lipofectamine 2000 reagent and the resultant cells were incubated with various concentrations of bazedoxifene for 24 h followed by luciferase assays by using a Promega luciferase kit. Renilla luciferase, which was normalized by firefly luciferase, was used as experimental signal

As shown in Fig. 6, in a concentration-dependent fashion, the inhibitory effects of bazedoxifene on the downstream STAT3-mediated gene transcription were prominent. Together, we suggest that suppression of STAT3 signaling pathway by bazedoxifene therapy can develop a potent anti-cancer regiment, particularly for eradicating TNBC [21].

Bazedoxifene inhibits the cell growth and forming colonies

Furthermore, we examined how bazedoxifene influences the ability of cancer cells to grow into colonies. As a comparison, the control GP130 inhibitor, SC144, was carried out concurrently. SUM159, MDA-MB-231, and MDA-MB-468 were treated with respective agents at various doses and the same numbers of viable cells were cultivated for additional 2–3 weeks. The resultant colonies were fixed by ice-cold methanol and stained by crystal violet solution. Representative images of results are shown in Fig. 7. Interestingly, in a dosage-dependent fashion, we observed negligible colonies in SUM159 and MDA-MB-468 cells upon a treatment with 20 µM bazedoxifene as well as in MDA-MB-231 with 15 µM bazedoxifene. This inhibitory effect from bazedoxifene was far more prominent than the ones from SC144, suggesting the former is a far more potent inhibitor for cell growth and for forming colonies, than the latter.

Bazedoxifene inhibited cell migration and invasion

A growing body of evidence suggested that IL-6/GP130/STAT3 is involved in cell migration, a characteristic required for tumor invasion and metastasis [22, 23]. We then examined whether bazedoxifene can impede cell migration and invasion. As shown in Fig. 8a in MDA-MB-231 and in SUM 159, there were significant loss of migration in cells treated with bazedoxifene 5 µM (** $p < 0.01$), and that this inhibitory effect became more

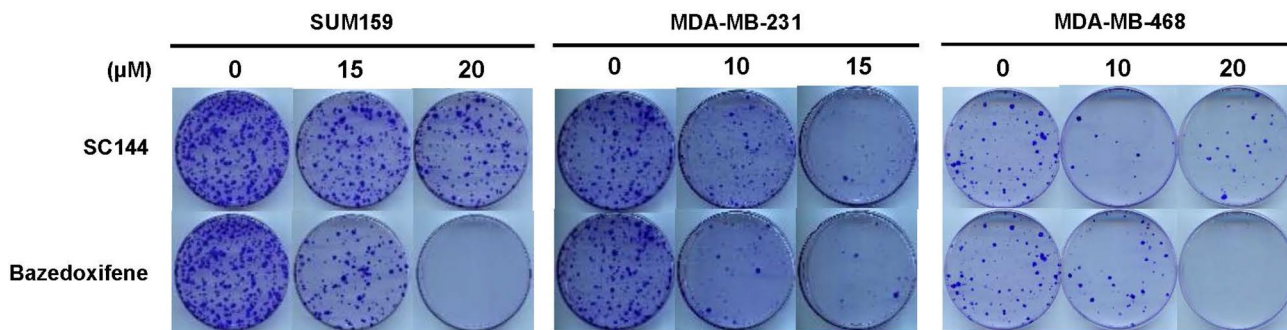


Fig. 7 Colony forming ability associated with TNBC was significantly inhibited by bazedoxifene, an agent more potent than SC144. SUM159, MDA-MB-231, and MDA-MB-468 cells were treated with SC144 and bazedoxifene at the indicated concentration, respectively.

After treatment, viable cells were counted and the same cell numbers were seeded then cultured under normoxic conditions for about 2–3 weeks. Colonies were fixed by ice-cold methanol and were stained by crystal violet solution

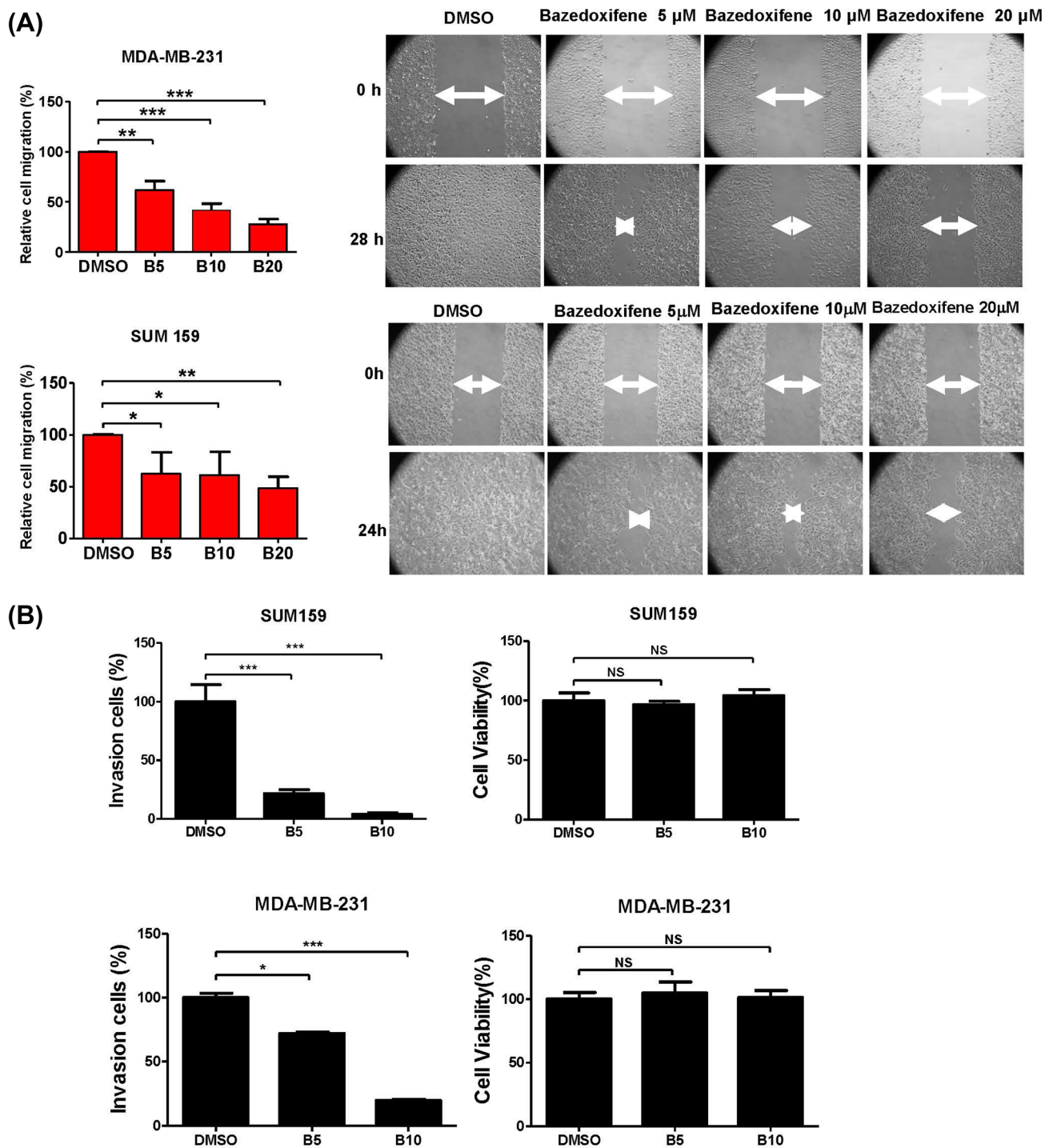


Fig. 8 Bazedoxifene suppressed migratory and invasion ability associated with MDA-MB-231 and SUM159. **a** Bazedoxifene inhibited cell migration by wound healing assay. **b** Bazedoxifene inhibited the

cell invasion ability. Cell growth were not affected under the treated concentration of 5 and 10 μM of bazedoxifene. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, mean \pm SD. NS not significant

prominent as the drug dose increased (10 and 20 μM ; *** $p < 0.001$). Our data indicated that 20 μM bazedoxifene resulted in the least motility in both cell lines tested. From Fig. 8b, we can see bazedoxifene (5 and 10 μM) could inhibit cell invasion significantly compared with control,

but without inducing any cell death. Our data suggest that inhibition of GP130 by the inhibitor bazedoxifene can block the migratory ability and invasion associated with TNBC.

Synergistic therapeutic effects exerted from bazedoxifene plus paclitaxel

Paclitaxel was evidenced as a microtubule-targeted drug [24], and it was shown to carry the clinical potential in improving survival and better life quality [25]. Herein, the synergistic effects of bazedoxifene plus paclitaxel were investigated in the aspect of cell proliferation, migration, and colony formation. As shown in Fig. 9, cell proliferation was synergistically hindered in the dual agent-treated cells than in the ones treated with single drug (Fig. 9a). Likewise, inhibitory effects on migratory potential (Fig. 9b) as well as on colony formation (Fig. 9c) concluded the same synergistic outcomes. Our findings thus provided a proof-of-principle that combinational treatments, with bazedoxifene plus paclitaxel, may improve the therapeutic outcomes for eradicating TNBC.

Bazedoxifene inhibited SUM159 and MDA-MB-231 tumor growth in mouse models in vivo

Next, we examined whether bazedoxifene can suppress the tumor growth in vivo. SUM159 and MDA-MB-231 cells were injected, respectively, into both sides of flanks of laboratory mice. Approximately one week after the injection

and when the tumors reached a size of 0.05–0.1 cm³, mice were then administered with bazedoxifene (8.8 mg pure bazedoxifene/kg for SUM159 mouse and 4.4 mg pure bazedoxifene/kg for MDA-MB-231 mouse) in the treated group or vehicles in the control group daily. As shown in Fig. 10, growth of tumor was impeded in drug-treated mice, as compared to the ones treated with control agent. From Fig. 7a, we can see the differences of tumor sizes started after treatment and this difference reached peak on 22th day ($***p < 0.001$). And tumor weight in bazedoxifene-treated group was significantly lower than that of control (Fig. 10b). P-STAT3(Y705) of tumor tissue sample in bazedoxifene-treated group was reduced, and caspase-3 was induced (Fig. 10c), indicating the anti-cancer effects of bazedoxifene. Figure 10d, e, displays the similar results in SUM159 tumor model, which showed a significant difference in tumor volume on 22th day ($***p < 0.001$, Fig. 10d) and tumor tissue western blotting results indicated P-STAT3(Y705) was induced in bazedoxifene-treated group (Fig. 10e), suggesting that the anticancer effects of bazedoxifene correlated with the expression levels of p-STAT3. Our findings demonstrate that bazedoxifene could significantly suppress SUM159 and MDA-MB-231 tumor growth not only in vitro, but also in vivo.

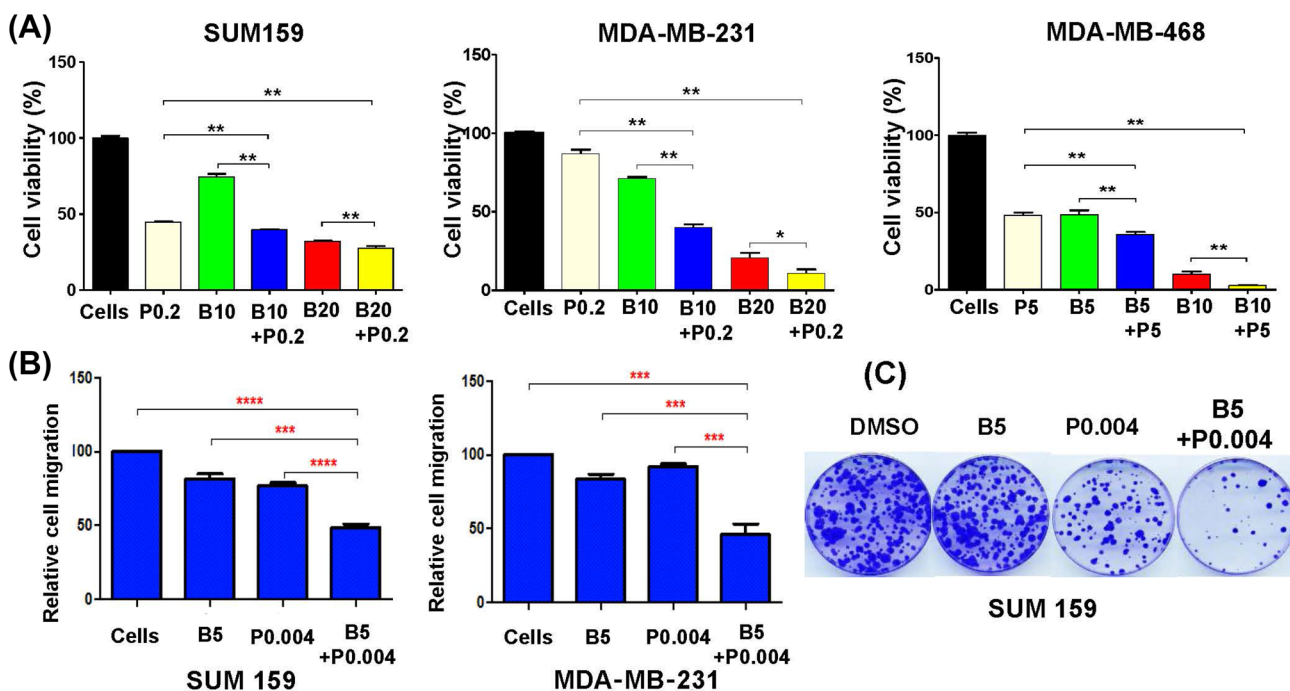


Fig. 9 Bazedoxifene plus paclitaxel exerted synergistic anti-cancer effects on cell viability, migration, and colony forming ability of TNBC, in a way more potent than single-agent alone. Combinational treatment with bazedoxifene (B) plus paclitaxel (P) with respective concentration (denoted with number in a unit of μM) exerted

enhanced suppressive effects on proliferative ability of SUM159, MDA-MB-231, and MDA-MB-468 cells (a) cell migration ability associated with SUM159 and MDA-MB-231 (b), as well as colony formation ability (c). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, mean \pm SD

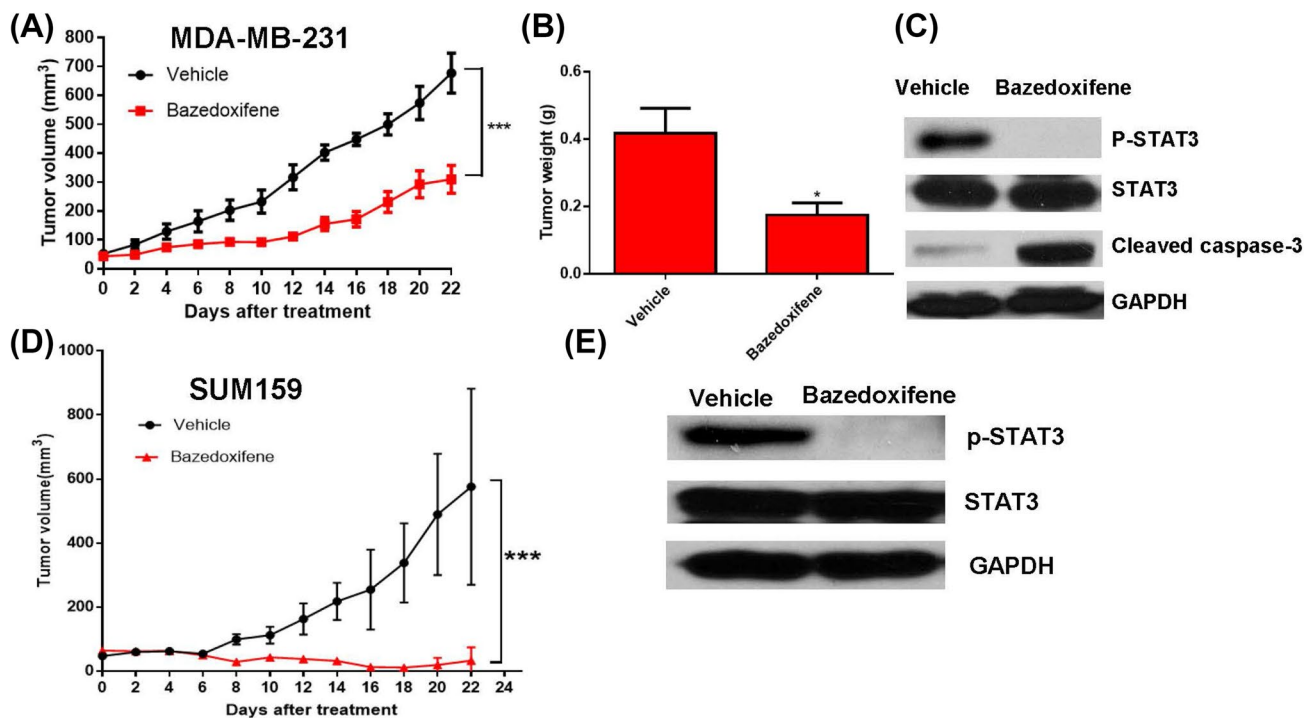


Fig. 10 Bazedoxifene inhibited tumor growth in TNBC-implanted mice. 6-week-old mice were inoculated with 3×10^6 /mL of SUM159 and MDA-MB-231 cells, respectively. One week after initial implantation, mice of each model were divided into two treatment groups, each was, respectively, treated with normal saline vehicle (as control) or with bazedoxifene. Tumor growth was assessed by measuring the length (L) and width (W) of the tumor every other day with a caliper,

and the tumor volume was calculated based on the formula as being: tumor size = $0.52 \times LW^2$. **a** MDA-MB-231 tumor volumes varied after treatment. **b** MDA-MB-231 tumor weight after 22 days of treatment. **c** The expression of P-STAT3 in MDA-MB-231 tumor tissue. **d** Tumor volumes of SUM159 varied after treatment. **e** The expression of P-STAT3 in separated SUM159 tumor. $n=8$, $*p<0.05$, and $***p<0.001$, mean \pm SD

Discussion

The fact that a lack of hormone receptors, ER, PR, and HER2, rendered disappointing outcomes evolved from molecular targeted chemotherapies for eradicating breast cancer. Patients with TNBC typically have poorer prognosis compared to those with different breast cancer subtypes. Identifying the novel molecular targets for TNBC therapy has become a desperate clinical demand. Over the past years, potential targetable pathways in TNBC, such as DNA repair, PI3K/mTOR, RAS/RAF/MEK, and JAK/STAT signaling, have undergone extensive studies [26]. As IL-6 is expressed in approximately 50% of breast cancers, inhibitors blocking its receptor GP130 signaling could evolve a promising treatment regimen [3]. One of GP130 inhibitors, bazedoxifene, is a FDA-approved drug and was initially administered as SERMs [27]. Yet, it was further identified as a potent molecular candidate to abrogate GP130 in our previous work [14]. In the current report, we expanded studies to assess its therapeutic effects on TNBC devoid of ER expression.

The growth stimulatory effect of TNBC by IL-6 was significantly inhibited by bazedoxifene as well as by SC144, despite the former exerting more potent effects than the

latter. Bazedoxifene-dependent growth suppression not only associated with IL-6 signaling cascade, but was also involved with STAT3-regulated gene transcription. Perhaps, concurrent blockage of IL-6 plus STAT3 signaling pathways could exert the improved therapeutic outcomes due to the synergistic effect.

In a close agreement of this notion, bazedoxifene not only impeded STAT3 activation by phosphorylation, but also suppressed activation of downstream factors of IL6/GP130 indicated by lowered P-AKT and P-ERK. Despite bazedoxifene being initially discovered by computer modeling for targeting IL-6 [16], current report further substantiates its anti-cancer effects, in the aspect of suppressing cell proliferation, cell motility (wound healing, migration), colony formation in vitro, as well as tumor growth in the mouse model in vivo. Although the mice used in this study were not oophorectomized, it was reported the pairing of bazedoxifene with estrogens will reduce the chance of getting cancer in the uterus induced by estrogens will increase the benefits of estrogens with prevention of bone loss [28–31].

Although it was reported that 5–10% of the ER α -negative breast cancers have also shown sensitivity to tamoxifen treatment, ER β expression status determines the effects of

tamoxifen in treatment of ER-negative breast cancer patient population [32], moreover, tamoxifen does not bind to GP130 in our computational modeling (Data not shown). Additionally, tamoxifen is with a major adverse effect that it increased incidence of uterine cancer [33–35]. In contrary to tamoxifen, in phase III clinical studies, bazedoxifene showed a favorable endometrial, ovarian, and breast safety profile in postmenopausal women over 3 [36] and 7 years [37], respectively. Bazedoxifene is with improved selectivity and safety, over tamoxifen [38, 39].

Bazedoxifene was demonstrated to harbor versatile anti-tumor effects in part by inhibiting the growth of both tamoxifen-sensitive and tamoxifen-resistant breast tumors in the xenograft model [40]. Yet, the prior studies centered on the ER-positive cohort [40, 41]. Hence, investigating the impact of bazedoxifene on ER-negative TNBC cells could reveal its anti-tumor effects by abrogating GP130-dependent signaling pathway. Clinical applications of this study include the development of a promising treatment regimen for TNBC by eradicating the IL-6/GP130 signaling cascade.

Of note, although bazedoxifene was used as 10–20 μM in vitro to inhibit GP130 downstream targets, which is higher than its ER antagonistic activity, it was used as 4.4 and 8.8 mg/kg by our in vivo experiments and showed anti-tumor growth and reduced P-STAT3 activity, which are approximately equivalent to the doses of 20 and 40 mg that are being used in human based on dose conversion between mice and human [42]. Therefore, the bazedoxifene doses used in vivo may still be achievable for suppressing TNBC tumor growth in human with acceptable toxicity. In addition, our combination results indicated the synergistic anti-cancer effect of reduced doses of bazedoxifene plus paclitaxel on the overall neoplastic features such as cell proliferation, migratory potential, and colony formation ability. Lower doses of each drug achieved the similar tumor suppressive activity as the higher doses of bazedoxifene or paclitaxel monotherapy were used. Indeed, chemo-toxicity is a fact but it is also true that it significantly reduces the risk of death in the adjuvant setting more or less in any breast cancer subtype and in a relevant proportion of patients, alone or added to endocrine [43]. On the other hand, as earlier finding reported that both exogenous and endogenous IL-6 conferred paclitaxel resistance [44], administering combination therapy with IL-6R inhibitor (like bazedoxifene) plus paclitaxel [45], could develop a novel treatment regimen for eradicating TNBC with persistently activated IL-6/GP130/STAT3 signaling.

Since the doses for bazedoxifene in our in vivo experiment are approximately equivalent to the doses that are being used in human, it may be sufficient for TNBC therapy or prevention with acceptable safety profile [36–39, 46]. Bazedoxifene may also provide an additional benefit of preventing bone loss in postmenopausal patients when use

to target the IL-6/GP130 signaling in TNBC. However, the potential toxicity of bazedoxifene to other tissues in the body that express ER, should still be examined and addressed before the TNBC clinical trials. Furthermore, precise delivery and targeted release to TNBC but not to other normal tissues of body may represent an alternative strategy for the use of bazedoxifene as an inhibitor of IL-6/GP130 in future. Collectively, (a) the safety profile of bazedoxifene in breast cancer patients may be acceptable but will need further evaluation, in healthy women the only dangerous one is venous thromboembolism [47]; (b) No clinical data are available on the concomitant use of bazedoxifene with agents in the treatment of early or advanced breast cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal studies were conducted in accordance with the principles and standard procedures set off by IACUC of the University of Maryland, Baltimore, Baltimore, MD, USA.

Informed consent For this type of study, formal consent is not required.

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