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Original Article

Lidocaine enhances the efficacy of palbociclib in triple-negative breast cancer

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Abstract: The use of anesthetics in the surgical resection of tumors may influence the prognosis of cancer patients. Lidocaine, a local anesthetic, is known to act as a chemosensitizer and relieve pain in some cancers. In addition, palbociclib, a potent cyclin-dependent kinase (CDK) 4/6 inhibitor, has been approved for chemotherapy of advanced breast cancer. However, recent studies have revealed the acquired resistance of breast cancer cells to palbociclib. Therefore, the development of combination therapies that can extend the efficacy of palbociclib or delay resistance is crucial. This study investigated whether lidocaine would enhance the efficacy of palbociclib in breast cancer. Lidocaine synergistically suppressed the growth and proliferation of breast cancer cells by palbociclib. The combination treatment showed an increased cell cycle arrest in the G0/G1 phase by decreasing retinoblastoma protein (Rb) and E2F1 expression. In addition, it increased apoptosis by loss of mitochondrial membrane potential as observed by increases in cytochrome *c* release and inhibition of mitochondria-mediated protein expression. Additionally, it significantly reduced epithelial-mesenchymal transition and PI3K/AKT/GSK3β signaling. In orthotopic breast cancer models, this combination treatment significantly inhibited tumor growth and increased tumor cell apoptosis compared to those treated with a single drug. Taken together, this study demonstrates that the combination of palbociclib and lidocaine has a synergistic anti-cancer effect on breast cancer cells by the inhibition of the PI3K/AKT/GSK3β pathway, suggesting that this combination could potentially be an effective therapy for breast cancer.

Keywords: Lidocaine, palbociclib, breast cancer, cell cycle arrest, AKT/GSK3β pathway

Introduction

Breast cancer is curable in 70-80% of patients with early-stage, non-metastatic disease; however, advanced breast cancer with distant organ metastasis is regarded as incurable with recently available therapies [1-3]. Triple-negative breast cancer (TNBC) is an aggressive type of breast cancer that does not express the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) [4]. TNBC accounts for approximately 15-20% of all breast cancer patient and the survival period of TNBC patients is shorter, and the mortality rate is 40% within the first 5 years after diagnosis compared with other subtypes of breast cancer [1, 3]. Also, TNBC is highly invasive, and approximately 46% of TNBC patients have distant metastasis and the average survival time after metastasis is only 13.3 months, and the recurrence rate after surgery is high at 25% [1, 2, 4]. It contributes significantly to tumorigenesis and resistance to chemotherapy and increases the risk of disease recurrence or death within 5 years after treatment. As there are no validated efficient targeted therapies against TNBC, current treatments rely only on chemotherapy. Therefore, the discovery of novel combination treatments to maximize TNBC therapy is urgently needed.

A hallmark of cancer is the dysregulation of the cell cycle, resulting in abnormal proliferation that ultimately promotes tumorigenesis and disease progression. Cell cycle control is also frequently dysregulated in breast cancer [5]. CDK4/CDK6, cyclin-dependent kinases that function in the form of cyclin D1-bound com-

pounds at a cell cycle checkpoint, can promote G1-S phase transition in the cell cycle by phosphorylation of the tumor suppressor retinoblastoma (Rb) protein [6]. Therefore, the inhibition of CDK4/6 may prove to be an effective therapeutic intervention against breast cancer. Palbociclib is a highly selective CDK4/6 inhibitor that blocks the phosphorylation of Rb and subsequently arrests the cell cycle at the G1-phase. It was approved by the Food and Drug Administration (FDA) to treat ER-positive and HER2-negative advanced breast cancer [7, 8]. A previous study reported that palbociclib in combination with tamoxifen or trastuzumab effectively inhibited ER-positive and HER2-amplified breast cancers [9]. However, combination therapy is required to achieve effective and durable disease control because TNBC is characterized by a group of highly proliferative tumors that are enriched in cell cycle genes and resistance to CDK4/6 inhibitor monotherapy has been observed [10-12].

Lidocaine is an aminoamide-type anesthetic that is widely used in regional anesthesia, peripheral nerve blocks, and epidural anesthesia with excellent pain relief due to its rapid onset of action and intermediate efficacy [13]. In addition, lidocaine has shown other pharmacological effects, such as anti-inflammatory action [14], protection of acute lung injury [15], neuroprotection [16], alleviation of postoperative cognitive dysfunction [17, 18]. In addition, recent studies have reported the anti-cancer properties of lidocaine in various cancers including lung [19], colon [20], gastric [21], and breast cancers [22]. Moreover, some studies have revealed that lidocaine sensitized the anticancer drugs in melanoma, bladder, and breast cancers [23-25]. However, to date, no studies have investigated the chemosensitization activity of lidocaine with palbociclib in breast cancer, especially TNBC.

Therefore, the present study aimed to investigate whether lidocaine, in combination with palbociclib, inhibits the proliferation of TNBC and attenuates tumor growth in TNBC-implanted mice through any mechanism. The findings of our study may provide a new therapeutic strategy for breast cancer treatment.

Materials and methods

Cell culture

Human breast cancer cells (MDA-MB 231 and MDA-MB 453) and mouse mammary cancer cells (4T1) were purchased from the American Type Culture Collection (ATCC, Manassas, VO, USA) and Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Welgene, Gyeongsan, Korea) and Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco, Waltham, MA, USA) medium supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA) and 1% antibiotic-antimycotic (Gibco, Waltham, MA, USA). The cultures were maintained at 37°C in a 5% CO_2/95% air incubator. The WST assay kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Palbociclib (PD332991) and lidocaine (2% lidocaine HCl injection) were supplied by LC Laboratories (Woburn, MA, USA) and Huons Co., Ltd. Pharmaceutical Company (Seongnam, Korea). respectively. Palbociclib was kept at -20°C after being dissolved in dimethyl sulfoxide (DMSO).

Cell viability assay

Cell viability was assessed using a WST assay. Briefly, the TNBC cells were seeded at a density of 7×10^2 or 3×10^3 cells/well in a 96-well plate for 96 h. The medium was removed and treated with palbociclib for 48 h, followed by lidocaine for an additional 48 h. The final dose of DMSO in the medium was ≤0.1% (v/v). After the cells were incubated for 48 h, 10 µl of WST solution (2 mg/mL) was added to each well and incubated for another 1 h at 37°C. Subsequently, the plate was read on a microplate reader at a wavelength of 450 nm. Three replicate wells were used for each analysis. The dose-response curves were used to calculate the median inhibitory concentration (IC50, defined as the drug concentration that inhibited cell growth by 50%).

Cell proliferation assay

The proliferation of cells was demonstrated by growth curves using the JULI™ Stage real-time image recording system which automatically captured cell morphologies at every configured time point using a camera equipped with a CO_2 incubator.

Three-dimensional (3D) spheroid formation

TNBC cells (MDA-MB-231 and MDA-MB-453) were cultured in flasks at 37°C and 5% CO_2. The attached cells were trypsinized and seeded into a 96-well ultralow attachment microplate (Corning, Hartford, NC, USA) at 1000 cells/well

in a complement medium supplemented with basic fibroblast growth factor, human epidermal growth factor, N-2, and B-27. The cells were incubated for 24 h at 37°C and then trea ted with palbociclib (0.1 or 0.5 μM) and/or lidocaine (2 or 3 mM) for 14 days, respectively. The diameter of the spheroids formed was measured using computer software (Image J software).

Bromodeoxyuridine (BrdU) cell proliferation assay

TNBC cells (MDA-MB-231 and MDA-MB-453) were seeded at 2×10^3 and 3×10^3 cells/well in 96-well plates, respectively. The subsequent day, the media was removed, and the cells were treated with palbociclib (0.1 or 0.5 μM) and/or lidocaine (2 or 3 mM) for 48 h. Following incubation, the cells were treated with BrdU solution for 4 h at 37°C and a proliferation assay was performed using a BrdU cell proliferation assay kit (Cell Signaling Technologies). Each well was incubated with a fixing/denaturing solution for 30 min at room temperature, followed by treatment with BrdU detection antibody solution for 1 h. The cells were washed thrice and incubated with horseradish peroxidase (HRP)-conjugated antibody solution for 30 min, followed by washing with a washing buffer. Tetramethylbenzidine substrate was added to each well for 20 min, and then it was terminated by the addition of 2 N sulfuric acid. To measure the absorbance a wavelength of 450 nm, we used a microplate reader.

Clonogenic assay

TNBC cells (MDA-MB-231 and MDA-MB-453) were seeded at a density of 7×10^4 or 7×10^5 cells in 100-mm dishes and incubated for 24 h at 37°C. The media was removed, and the cells were treated with palbociclib (0.1 or 0.5 μM) and/or lidocaine (2 or 3 mM) for 96 h. The cells were re-plated in triplicates at 1×10^2 and 1×10^3 cells/well into six-well plates and incubated for 14 days. Colonies in each well were washed twice with Dulbecco's phosphate-buffered saline (DPBS), fixed with 4% paraformaldehyde, stained with crystal violet for approximately 20 min, quantified, and photographed.

Western blotting

TNBC cells (MDA-MB-231 and MDA-MB-453) were washed three times with ice-cold DPBS before lysis. The cells were lysed in a solution containing 1% Triton X-100, 1% Nonidet P-40, and protease and phosphatase inhibitor cocktails (GenDEPOT, Barker, TX, USA). Equal amounts of protein were separated by 8% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Immunostaining was performed by incubating the blots with primary antibodies, followed by HRP-conjugated secondary antibody, and detected with enhanced chemiluminescence (ECL) plus system (Amersham Biosciences, Piscataway, NJ, USA). The secondary antibodies were purchased from Cell Signaling Technologies.

Survivin (71G4B7) Rabbit mAb (cat. #2808), p-Rb (Ser780) Rabbit mAb (cat. #9307) were purchased from Cell Signaling Technology (Beverly, MA, USA). XIAP (H-202) Rabbit mAb (cat. #sc-11426), Mcl-1 (S-19) Rabbit mAb (cat. #sc-819), Bcl-2 (C-2) Mouse mAb (cat. #sc-7382), vimentin Mouse mAb (cat. #V2258) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Twist (10E4E6) Mouse mAb (cat. #NBP2-37364) was purchased from Novus (St. Louis, MI, USA), and β-actin Mouse mAb (cat. #ab8227) was purchased form Abcam (Cambridge, MA, USA). Primary rabbit polyclonal antibodies against the following proteins were also used: cleaved caspase-3 (Asp175) Rabbit mAb (cat. #9661), p-AKT (Ser473) Rabbit mAb (cat. #9771), AKT Rabbit mAb (cat. #9272), GSK3β Rabbit mAb (cat. #9315), E-cadherin (24E10) Rabbit mAb (cat. #4065), GAPDH Rabbit mAb (cat. #5174) and histone H3(D1H2) Rabbit mAb (cat. #4499). ZEB1 Rabbit mAb (cat. #NBP1-05987) was purchased from Novus (St. Louis, MI, USA) and p-GSK3β (Ser9) Rabbit mAb (cat. #MA5-14873) was purchased from Invitrogen (St. Louis. MO, USA). The secondary antibodies were purchased from Cell Signaling Technologies.

Cytochrome c oxidase assay

MDA-MB 231 cells were seeded on 18-mm glass coverslips and grown to approximately 70% confluence. Palbociclib was pre-incubated for 24 h, and lidocaine was added immediately after palbociclib withdrawal. The cells were incubated with 500 nM MitoTracker Red probe (Molecular Probes Inc., Eugene, OR, USA) for 30 min at 37°C. The cells were washed twice with DPBS, fixed in acetic acid:ethanol (1:2 v/v) solu-

tion for 10 min at -20°C, and then incubated overnight at 4°C with an anti-cytochrome c antibody (1:50 dilution) (Santa Cruz Biotechnologies, Dallas, TX, USA). The following day, the cells were washed twice with DPBS and incubated with fluorescently labeled anti-mouse secondary antibody (1:100 dilution) (Dianova, Hamburg, Germany) for 1 h at room temperature.

The cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Subsequently, the slides were washed twice with DPBS, mounted with 1,4-diazabicyclo[2.2.2]octane (DABCO) (Sigma-Aldrich, St. Louis, MO, USA), and viewed under a confocal laser-scanning microscope (Fluo View 1000; Olympus).

Measurement of the mitochondrial membrane potential by JC-1 staining

TNBC cells (MDA-MB-231 and MDA-MB-453) were seeded on 18-mm glass coverslips and grown to approximately 70% confluence. The cells were treated with palbociclib for 24 h; and then were exposed to lidocaine for a further 24 h, followed by incubation with JC-1 solution (Cayman Chemical, Ann Arbor, MI, USA) for 20 min at 37°C. After staining with DAPI to visualize the nuclei, the slides were washed twice with DPBS, mounted with DABCO (Sigma-Aldrich), and viewed by confocal laser-scanning microscopy (Fluo View 1000; Olympus).

Cell cycle analysis

TNBC cells (MDA-MB-231 and MDA-MB-453) were seeded at a density of 2×10^5 or 7×10^5 cells in 100-mm dishes and incubated for 24 h at 37°C. The next day, MDA-MB 231 cells were treated with palbociclib for 36 h; and subsequently were exposed to lidocaine for a further 12 h. MDA-MB 453 cells were pre-incubated with palbociclib for 12 h, and lidocaine was added immediately. The cells were fixed in 70% ethanol, resuspended in DPBS along with RNase A (10 μg/mL), and stained with propidium iodide (PI, 50 μg/mL) for 30 min at 37°C. The cell cycle portions were analyzed using a fluorescence-activated cell sorting (FACS) analyzer (FACS verse, Beckman Coulter, Indianapolis, IN, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Apoptotic cells were detected using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Burlington, MA, USA). Briefly, the cells were seeded onto 18-mm cover glasses and cultured up to approximately ~70% confluency for 24 h at 37°C. The cells were subsequently treated with palbociclib (0.1 or 0.5 μM) and lidocaine (2 or 3 mM) for 96 h, fixed in an ice-cold mixture of acetic acid and ethanol solution, washed with DPBS, and stained with the TUNEL assay solution. The slides were mounted and examined for nuclear fragmentation under a light microscope.

Annexin V assay

TNBC cells (MDA-MB-231 and MDA-MB-453) were seeded in 100-mm culture dishes at ~approximately 70% confluence and incubated at 37°C for 24 h. The next day, the cells were treated with palbociclib for 48 h. The medium from the attached cells was removed, and the cells were briefly washed twice with cold DPBS, and double-stained with Annexin V (APC) and PI (BD Biosciences, San Jose, CA, USA) in Annexin V binding buffer and analyzed on a FACS Calibur flow cytometer (Beckman Coulter, Indianapolis, IN, USA) equipped with a 488 nm argon laser. Live cells were gated using forward and side scatter to avoid non-specific fluorescence from dead cells.

Phospho-kinase array

A human phospho-kinase array kit was used to evaluate the relative phosphorylation levels of 43 different kinases (R&D Systems, Minneapolis, MN, USA). MDA-MB 231 cells were plated in 100-mm culture dishes at ~approximately 70% confluence and incubated at 37°C for 24 h. The next day, the cells were treated with palbociclib for 36 h, exposed to lidocaine for a further 36 h, and subsequently lysed as mentioned earlier. After blocking for 1 h using an array buffer 1, the membranes were incubated with 500 μg of protein lysates overnight at 4°C, washed, and incubated with a streptavidin-HRP detection antibody. Membranes were developed using ECL western blotting detection reagents (Chemi reagent A and Chemi reagent

B) supplied by the manufacturer, and the expression levels were quantified using ImageJ software.

Mouse orthotopic models

Animal care and experimental procedures were performed in accordance with the approval and guidelines of the Institutional Animal Care and Use Committee of Inha University (INHA 200831-714). Mouse breast orthotopic models were prepared by inoculating 1×10^4 4T1 cells suspended in DPBS (50 µl/mouse) into the mammary fat pad of 4-week-old female BALB/c mice (OrientBio, Gyeonggi-do, Korea). After the tumors reached approximately 50 mm^3 in size, the mice were randomly divided into four groups: group 1, control; group 2, palbociclib only (100 mg/kg); group 3, lidocaine only (50 mg/kg); group 4, co-treatment with palbociclib and lidocaine. The control group was treated with 200 µl of 0.5% carboxymethyl cellulose by oral gavage, and the treatment groups were administered palbociclib (100 mg/kg, P.O.) and/or lidocaine (50 mg/kg, I.P.) daily for 23 days. In particular, the lidocaine and co-treatment groups were administered from 4 days after treatment to the control group. The tumor size was measured with fine calipers three times a week, and the tumor volume was measured using the formula 0.5× (length) × $(width)^2$. At the end of each experiment, animals were sacrificed, and the primary tumors were harvested, weighed, photographed, and stored at -80°C.

Immunohistochemistry

Paraffin-embedded sections of tumors were blocked with normal goat or horse serum (Vector Laboratories, Burlingame, CA, USA) for 1 h, and incubated at 4°C overnight with 1:30 dilutions of primary antibodies against cleaved caspase-3 (Cell Signaling Technologies), forkhead box protein M1 (FOXM-1), p-Rb, and cyclin B1 (Santa Cruz Biotechnologies, Dallas, TX, USA). The sections were then incubated with biotinylated secondary antibodies (1:60 dilution) for 1 h. The immunoreactive proteins were visualized by first applying an avidin-biotin peroxidase complex solution (ABC kit; Vector Laboratories, Burlingame, CA, USA), washed with PBS, and developed with a diaminobenzidine tetrahydrochloride substrate for 30 min with subsequent hematoxylin counterstaining. At least three random fields in each section were examined at a magnification of 200×.

Statistical analysis

Statistical significance was determined using analysis of variance (ANOVA) or the unpaired Student's t-test, as appropriate. Results are presented as means ± standard deviations (SD), and are considered statistically significant at $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

Results

The combination of palbociclib and lidocaine inhibits the growth of TNBC cells

To evaluate the anti-cancer effect of palbociclib and lidocaine co-treatment in breast cancer cells, TNBC cells (MDA-MB-231 and MDA-MB-453) were treated with palbociclib for 48 h and were subsequently treated with lidocaine for a further 48 h. To identify the synergistic effects of the two drugs, combination index (CI) values were calculated using CompuSyn V1.0 (Biosoft). The co-treatment showed significant synergistic effects, with CI <1 for the combination of 0.5 µM palbociclib and 3 mM lidocaine in MDA-MB 231 cells (CI = 0.706) and 0.1 µM palbociclib and 2 mM lidocaine in MDA-MB 453 cells (CI = 0.594), respectively (**Figure 1A**, **1B**). These combinations significantly inhibited cell proliferation in both TNBC cell lines, measured using BrdU cell proliferation assay (**Figure 1C**), which was confirmed by 3D spheroid cultures (**Figure 1D**). In addition, the survival of TNBC cells treated with by this combination was effectively inhibited compared with either single agent (**Figure 1E**). Therefore, these findings indicate that lidocaine enhances the anti-cancer efficacy of palbociclib and synergistically inhibits the proliferation of TNBC cells.

The combination of palbociclib and lidocaine highly induces cell cycle arrest

To understand the anticancer mechanism responsible for the synergistic anti-proliferative activity of palbociclib and lidocaine in TNBC cells, the cell cycle distribution was evaluated using flow cytometric analysis. As shown in **Figure 2A**, the combination treatment synergistically induced G0/G1 cell cycle arrest (80-93%) in TNBC cells. Furthermore, the combined treatment decreased the expression of Rb,

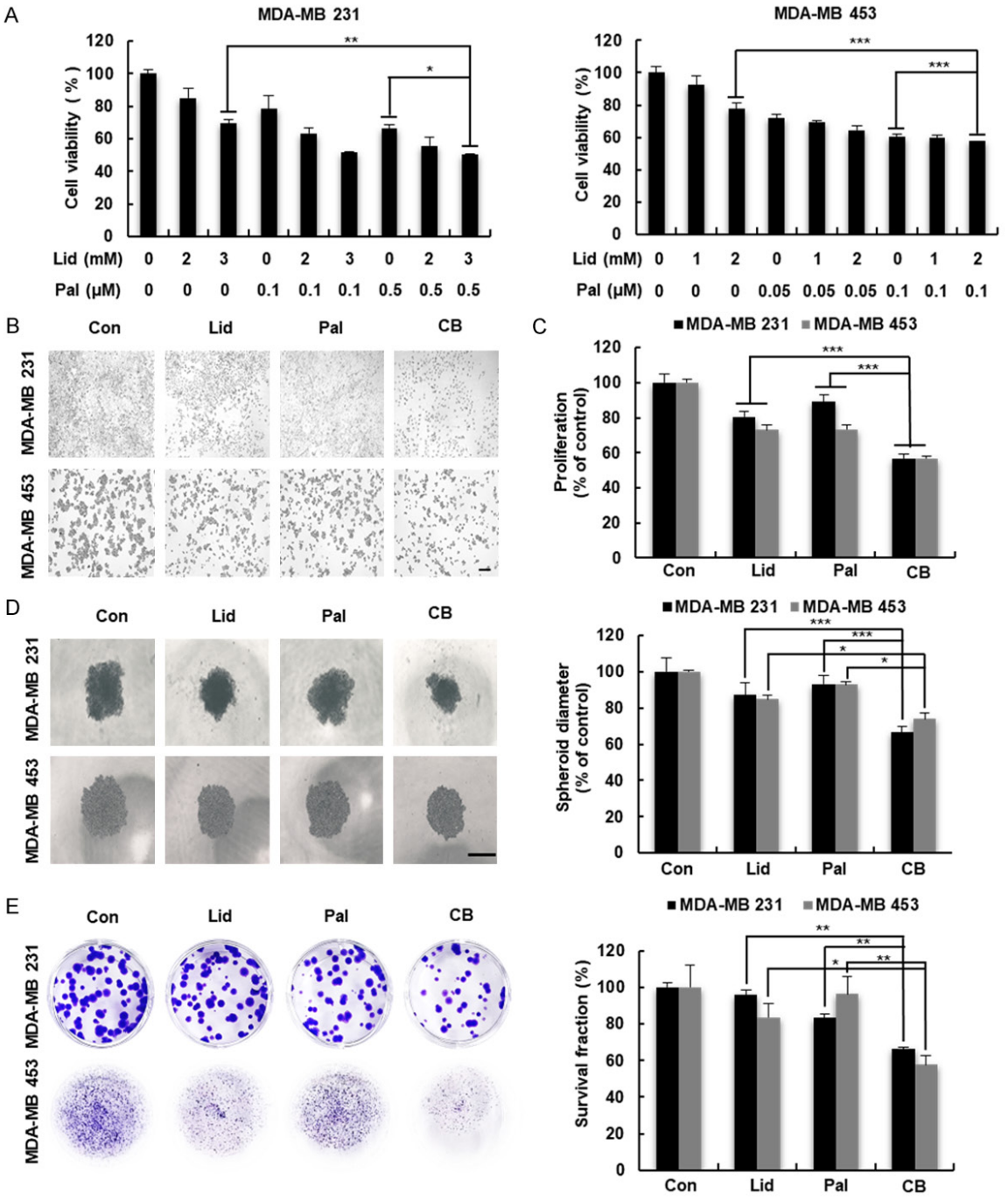


Figure 1. Co-treatment of palbociclib and lidocaine inhibits the growth of triple-negative breast cancer (TNBC) cells. A. MDA-MB 231 and MDA-MB 453 cells were treated with palbociclib for 48 h and were subsequently exposed to lidocaine for a further 48 h. Additionally, the viability in TNBC cells was measured using the WST assay. B. Effect of co-treatment on TNBC proliferation was estimated using JULI™ stage real-time cell recorder. The distinct cells in each group were imaged (Magnification 20×; scale bar 200 μm). C. Bromodeoxyuridine assay was performed to determine the anti-proliferative effects of palbociclib and lidocaine. TNBC cells were treated with palbociclib for 48 h and then lidocaine for additional 48 h. D. The cells were treated with palbociclib and/or lidocaine for 96 h, and were then re-seeded into ultraround detachment plates for 14 days. The cell size (diameter) was measured by optical microscopy (Magnification 40×; scale bar 100 μm). E. The cells were seeded in 100-mm culture dishes and treated with palbociclib and lidocaine for 4 days were then re-seeded into six-well plates and cultured for 14 days. The number of colonies (~50 cells/colony) was counted and analyzed. Data are presented as means ± SD (*P<0.05, **P<0.01, ***P<0.001).

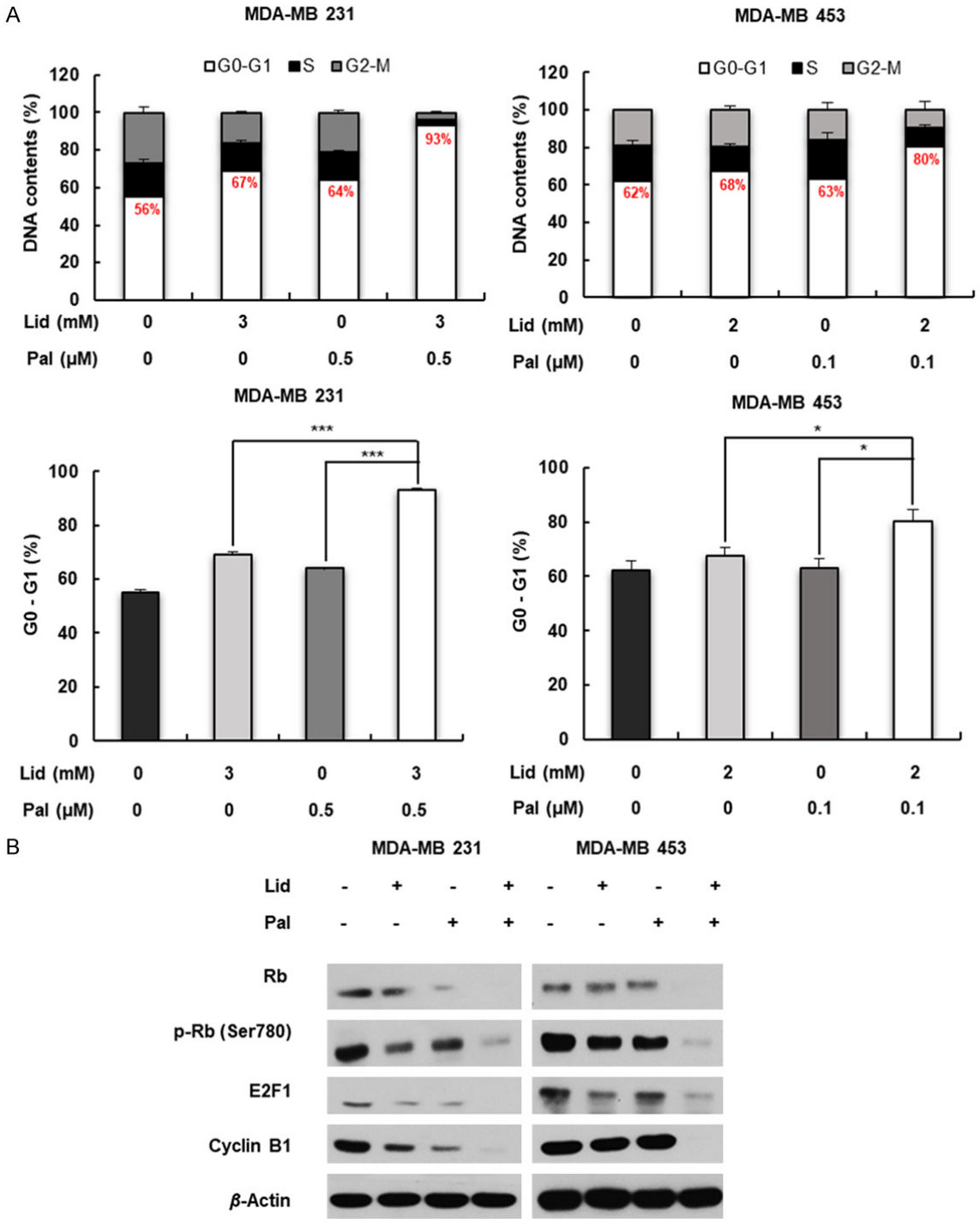


Figure 2. Co-treatment of palbociclib and lidocaine synergistically induces cell cycle arrest. A. MDA-MB 231 cells were treated with palbociclib for 36 h, and/or lidocaine for 12 h. MDA-MB 453 cells were treated with palbociclib for 12 h, and/or lidocaine for 12 h. The cell cycle distribution was determined by flow cytometry, and each phase was analyzed. The data are presented as means ± standard deviation (*P<0.05, **P<0.01, ***P<0.001). B. The cell lysates were prepared and the expression levels of Rb, p-Rb (Ser780), E2F1, cyclin B1 were determined by western blotting.

E2F1, and cyclin B1, which typically caused cell cycle arrest in the G0/G1 phase (**Figure 2B**). These results indicate that the synergistic effect of palbociclib and lidocaine on cell proliferation was induced through the G0/G1 phase arrest.

The combination of palbociclib and lidocaine synergistically induces mitochondria-mediated apoptosis

As co-treatment significantly reduced cell proliferation, we investigated whether it could induce apoptosis. TUNEL staining showed that the combination treatment induced apoptosis by causing DNA strand breaks (**Figure 3A**). Immunofluorescent staining revealed that co-treatment resulted in significant activation of cleaved-caspase3 (**Figure 3B**). In addition, it induced a higher percentage of Annexin V-positive cells compared to treatment alone in TNBC cells (**Figure 3C**). In **Figure 3D**, JC-1 was confirmed by fluorescence in living cells in the form of a dimer. In the combined treatment, a significant change in the mitochondrial membrane potential was induced in most cells by decreasing red fluorescence or increasing green fluorescence. In addition, the combined treatment significantly increased the release of cytochrome c in MDA-MB 231 cells, while simultaneously reducing the co-localization of cytochrome c and mitochondria. Additionally, as shown in **Figure 3E**, the combined treatment significantly inhibited the expression of anti-apoptotic factors such as survivin, XIAP, Mcl-1, and Bcl-2, compared to single agents. Collectively, our findings suggest that the synergistic effects of the combined treatment are mediated by the mitochondrial apoptotic pathway in TNBC cells.

The combination of palbociclib and lidocaine downregulated the PI3K/AKT/GSK3β signaling pathway

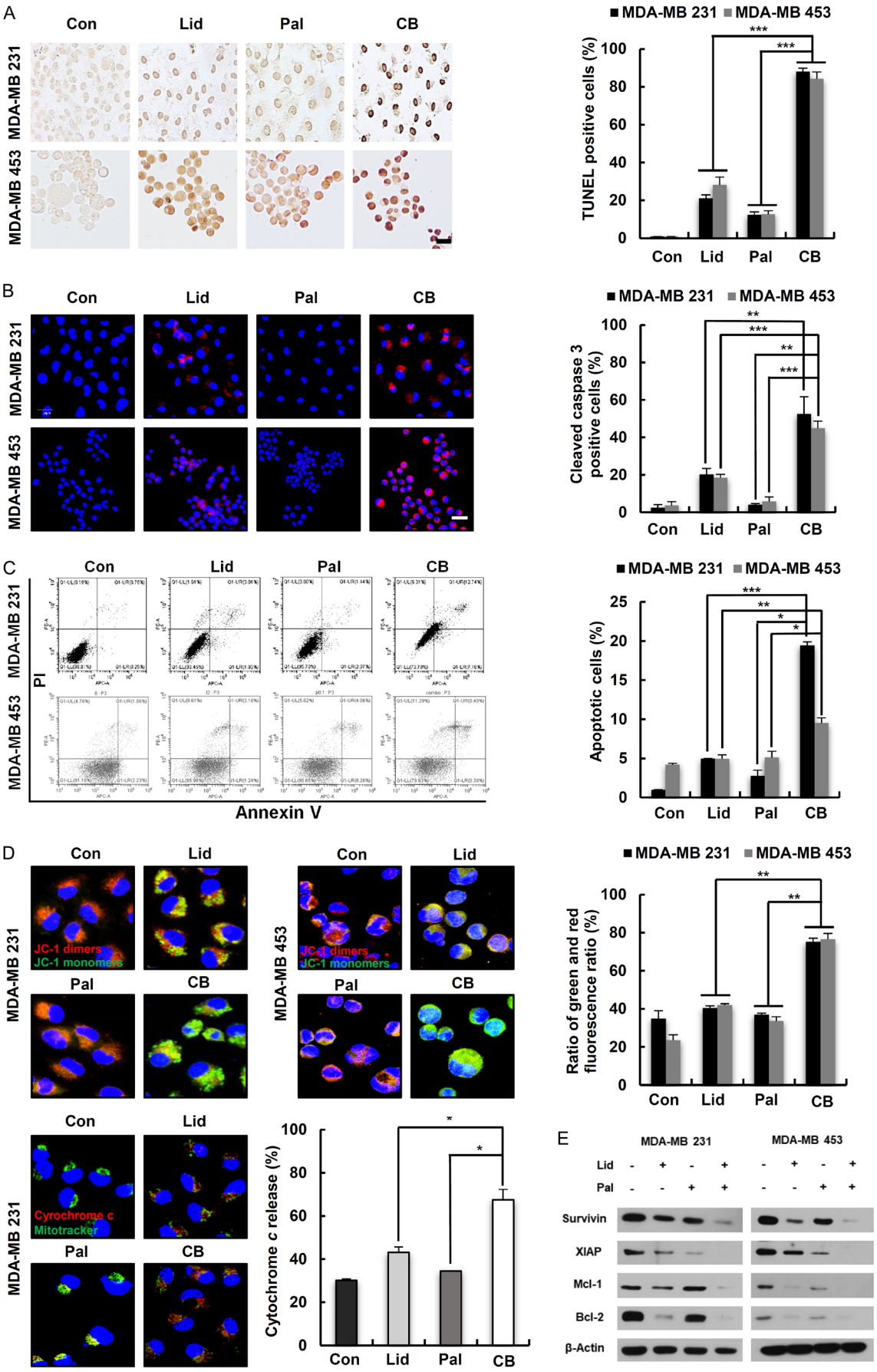
To investigate the mechanisms by which the combination of palbociclib and lidocaine caused anticancer activity in TNBC, we performed a phospho-kinase analysis. Notably, among the 43 different kinases, co-treatment significantly inhibited the expression of p-AKT (Ser473) and p-GSK3β (Ser9) by 20-80% in TNBC cells (**Figure 4A**). These results were confirmed by showing that co-treatment reduced the levels of p-AKT (Ser473) and p-GSK3β (Ser9) by western blotting analysis in TNBC cells (**Figure 4B**). These results suggest that the anti-proliferative and pro-apoptotic effects of the combination might be mediated by inhibition of the PI3K/AKT/GSK3β pathway in TNBC cells.

The combination of palbociclib and lidocaine inhibited metastasis by blocking epithelial mesenchymal transition (EMT) signaling

Since previous studies have reported that lidocaine suppressed cancer recurrence or metastasis when used during surgery [26], we assessed whether the combination of palbociclib and lidocaine may increase the anti-metastatic activity by inhibiting cell migration and invasion. When the transwell migration and invasion assays were performed on TNBC cells, cell migration and invasion were inhibited by combined treatment rather than every single treatment (**Figure 5A**). Subsequently, we investigated whether the combined treatment inhibited the viability of cells in response to shear stress and anchorage-independent growth (cell cluster), which circulating tumor cells undergo *in vivo*. As shown in **Figure 5B**, we found that cell spheroid growth and cell attachment on collagen-coated plates were significantly decreased in the combined treatment, as compared to the single treatment alone. In cancer cells, reduced E-cadherin and increased vimentin expression are known as the main EMT characteristics, which are responsible for the progression of EMT, such as cell migration and invasion [27]. To explore whether the combined treatment with palbociclib and lidocaine affected the expression of E-cadherin and vimentin, we used western blotting and immunofluorescence. We found that the combined treatment significantly increased expression of cytokeratin 18 and E-cadherin but decreased that of vimentin, ZEB1, and twist in MDA-MB 231 cells (**Figure 5C**, **5D**).

The combination of palbociclib and lidocaine suppressed tumor growth in breast cancer orthotopic models

To investigate whether lidocaine enhances the anti-cancer effect of palbociclib, 4T1 mouse breast cancer cells were injected into the mammary fat pad of BALB/C mice. As shown in **Figure 6A**, the tumor growth of mice treated with palbociclib or lidocaine alone was slightly delayed compared with the control group for approximately 3 weeks of treatment; however, the combination of palbociclib and lidocaine significantly reduced the tumor volume. In particular, the combination treatment group showed a decrease of over 50% in the tumor


A
Con
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Annexin V
Apoptotic cells (%)
D
JC-1 dimers
JC-1 monomers
Ratio of green and red fluorescence ratio (%)
Cyrochrome c
Mitotracker
Cytochrome c release (%)
E
Survivin
XIAP
Mcl-1
Bcl-2
β-Actin

Figure 3. Co-treatment of palbociclib and lidocaine synergistically induces mitochondria-mediated-apoptosis. (A) Triple-negative breast cancer (TNBC) cells were treated with palbociclib for 48 h and subsequently with lidocaine for an additional 48 h. Apoptosis by the co-treatment was determined by TUNEL staining (magnification 100×; scale bar 100 μm). (B) The cells were treated as in (A). Fluorescence images of cleaved caspase-3 (red) were assessed in TNBC cells (magnification 100× scale bar 30 μm). (C) Annexin V positive cell population was assessed by flow cytometry. (D) TNBC cells were stained with a JC-1 and analyzed with an Olympus confocal laser scanning microscope. Increased apoptosis resulted in an increase in the ratio of green to red fluorescence. Changes in the ratio of green to red fluorescence (%) after JC-staining were quantitatively measured. MDA-MB 231 cells were stained with anti-cytochrome c antibody, Mitotracker and DAPI (magnification 100×; scale bar 30 μm). Data are presented as means ± SD (*P<0.05, **P<0.01, ***P<0.001). (E) The cell lysates were prepared, and levels of anti-apoptotic molecules such as survivin, XIAP, Mcl-1, and Bcl-2, were analyzed by western blotting.

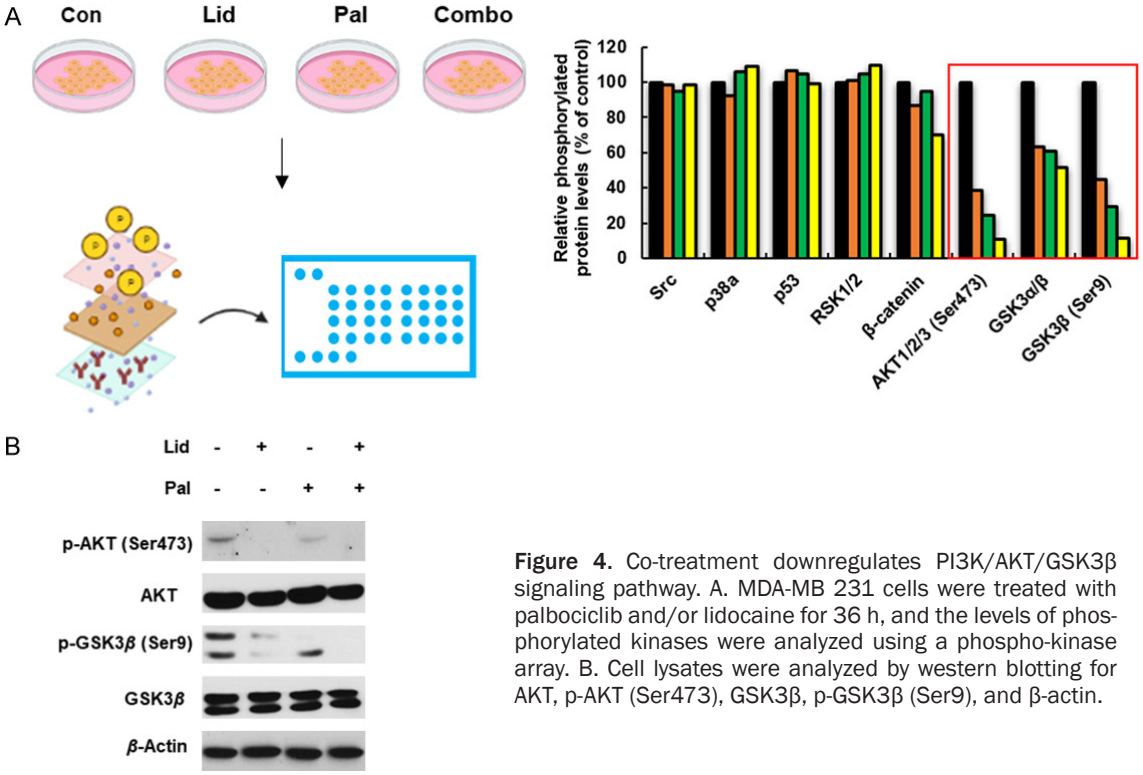

Figure 4. Co-treatment downregulates PI3K/AKT/GSK3β signaling pathway. A. MDA-MB 231 cells were treated with palbociclib and/or lidocaine for 36 h, and the levels of phosphorylated kinases were analyzed using a phospho-kinase array. B. Cell lysates were analyzed by western blotting for AKT, p-AKT (Ser473), GSK3β, p-GSK3β (Ser9), and β-actin.

growth compared with the control group, which was line with the changes in tumor weight. Throughout the study, all the treatment groups were well tolerated, and there was no significant difference in the body weight compared with the control group. In histopathological analysis, the combination treatment showed a decrease in the proliferating cell nuclear antigen (PCNA), a cell proliferation marker, and increased the number of apoptotic cells in tumor tissues, identified as cleaved caspase-3. Given that palbociclib inhibits cell cycle progression through G0/G1 arrest, we identified the expression levels of well-known G0/G1 arrest cell cycle molecules such as p-Rb, cyclin B1, and FOXM1. We found that palbociclib decreased the expression of cell cycle regulatory molecules, whereas the levels of these molecules were completely suppressed following co-treatment with both agents, suggesting their synergistic activity. Moreover, the combination treatment significantly attenuated the expression of p-AKT and p-GSK3β (**Figure 6B**).

Discussion

TNBC remains a challenging breast cancer subtype because of its higher risk of distant recurrence, and poorer outcome after recurrence or metastasis compared with other breast cancer subtypes. Due to the negative expression of ER, PR, and HER2, TNBC is not sensitive to endocrine and targeted treatments and faces problems of resistance or recurrence [4]. In

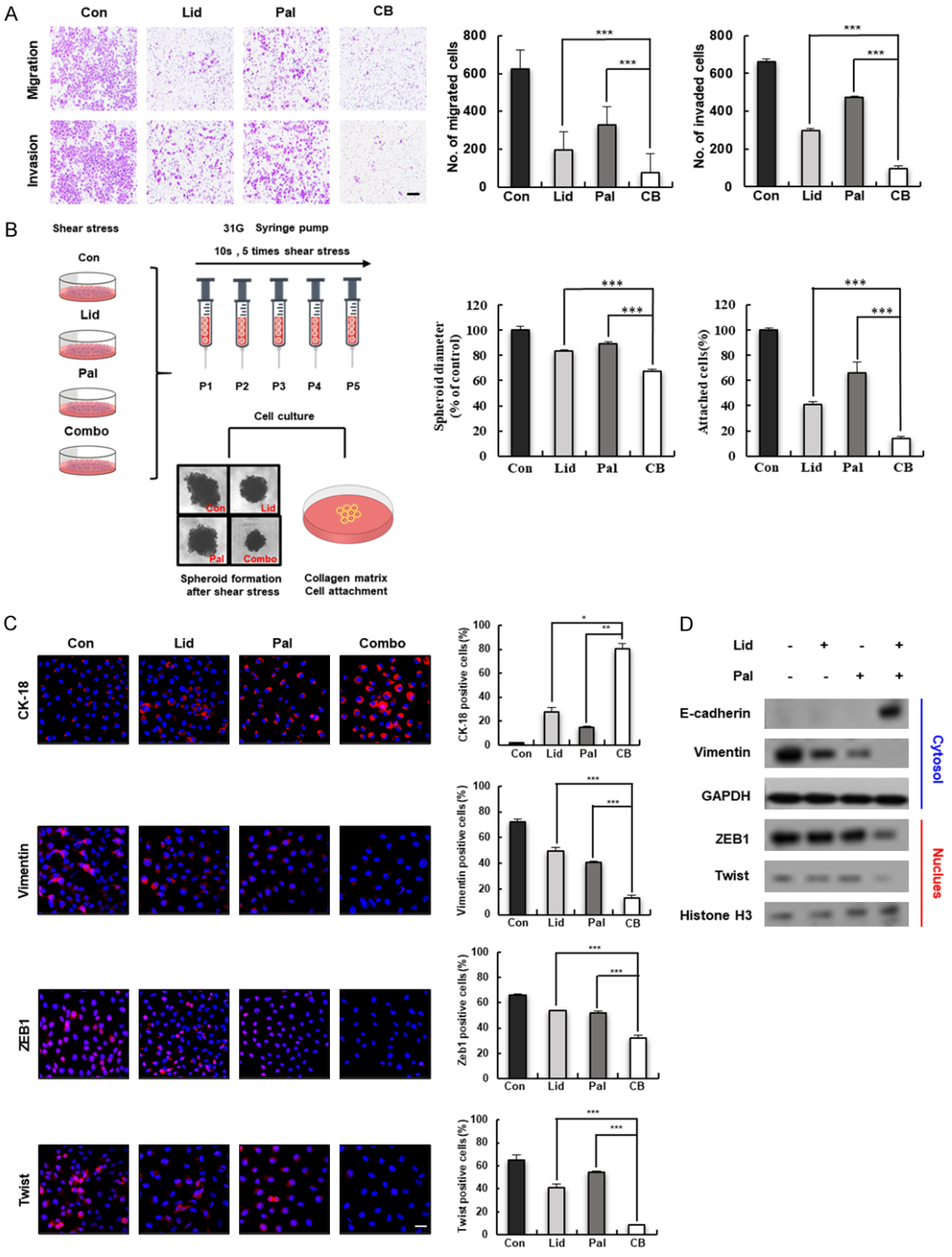

Figure 5. Lidocaine treatment synergistically enhances the inhibition of the epithelial mesenchymal transition (EMT) by palbociclib treatment. A. Migration and invasion assay of MDA-MB 231 cells using a transwell system (magnification 200×; scale bar 100 μm). The number of migrated or invaded cells was presented as the mean ± SD (*P<0.05, **P<0.01, ***P<0.001). B. Quantification of cell attachment and spheroid formation after shear stress of MDA-MB231 cells with the co-treatment. P0, cells not subjected to shear stress; Pn, cells subjected to n-time consecutive exposures to shear stress. DAPI was stained for cell attachment on collagen-coated plates, and spheroid formation after shear stress of MDA-MB 231 cells with co-treatment. C. The expression of EMT markers was evaluated in

MDA-MB 231 cells by immunofluorescence staining after the co-treatment of palbociclib and lidocaine for 96 h. D. Cell lysates were analyzed by western blotting. The expression level of E-cadherin, vimentin, and EMT transcriptional factors including ZEB1 and twist was detected by western blot analysis in MDA-MB 231 cells.

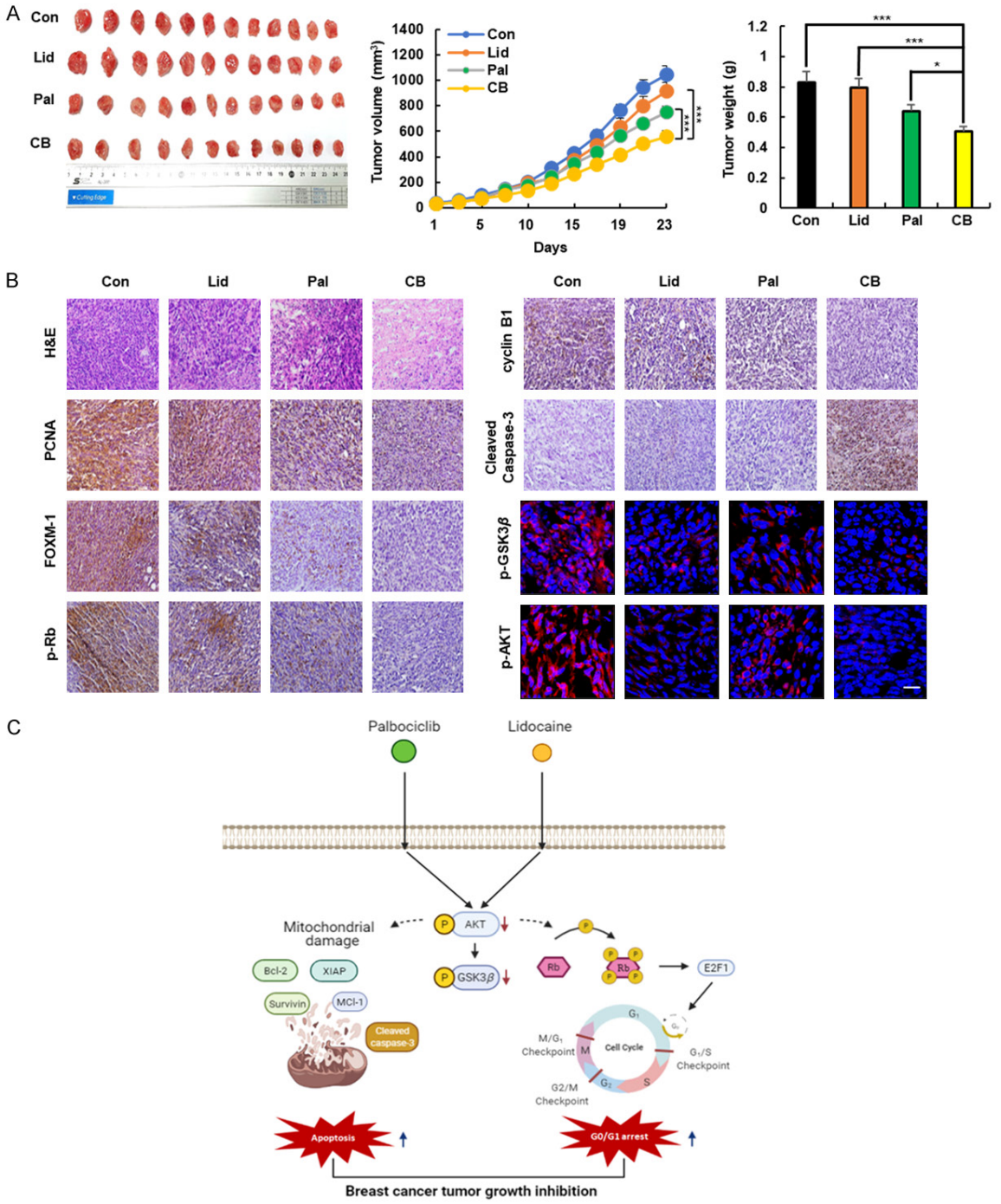

Figure 6. Co-treatment suppresses tumor growth in breast cancer orthotopic models. A. Effects of palbociclib (100 mg/kg), lidocaine (50 mg/kg), and co-treatment for 23 days on 4T1 orthotopic model tumor growth curve and body weight. Data are presented as means ± standard deviation ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). Representative isolated tumors and tumor weight. B. Histological analysis of orthotopic breast tumor tissue by hematoxylin and eosin staining, immunohistochemical detection of PCNA, FOXM-1, p-Rb, cyclin B1, cleaved caspase-3, and p-AKT (Ser473), p-GSK3β (Ser9) (magnification 200×; scale bar 50 μm). C. Scheme for how palbociclib combined with lidocaine induces apoptosis and inhibits the growth of triple-negative breast cancer cells.

particular, although palbociclib is FDA-approved for the treatment of patients with ER-positive and HER2-negative advanced breast cancer, it is not well documented in TNBC [7, 8]. Recently, lidocaine as an anesthetic has been shown to be associated with improved outcomes after surgery for different cancers [28]. Perioperative lidocaine infusion reduces postoperative pain, decreases the need for opioids, and reduces nausea/vomiting [29]. Additionally, there is increasing evidence that the antiproliferative effects of lidocaine can mitigate perioperative tumor growth by effectively inhibiting cell proliferation and metastasis [30]. Given the previous studies that lidocaine could reduce the risk of cancer proliferation, metastasis, and recurrence [31], we hypothesized that the combination of lidocaine and palbociclib might achieve synergistic effects in TNBC, and further investigated its mechanism of action. Herein, we found that the combination synergistically inhibited TNBC cell proliferation by inducing G1 arrest and apoptosis and enhanced the anti-cancer and anti-metastatic effects through the inhibition of the PI3K/AKT/GSK3β pathway along with inhibition of EMT signaling. Furthermore, it synergistically inhibited tumor growth in an animal model of TNBC.

CDK4/6 is an important regulator of the cell cycle and plays an important role in the transition from the G1 to the S phase. It binds to cyclin D and forms the CDK4/6-cyclin D complex. Previous studies have shown that palbociclib inhibits CDK-related pathways, including the formation of the CKD4/6-cyclin D complex [32, 33]. Additionally, since alterations of the components of the cell cycle machinery such as Rb, CDK4/6, and CDK2 have been frequently reported in TNBC, these genetic features might be suitable targets for the TNBC treatment with CDK4/6 inhibitors [34, 35]. However, the simultaneous treatment of palbociclib and other chemotherapeutic agents has shown mainly an antagonistic effect due to the reduced sensitivity of non-cycling cells to chemotherapeutic drugs [36, 37]. Therefore, combinatorial strategies with CDK4/6 inhibitors and other chemotherapeutics must be designed carefully, due to the potential interference between the action of CDK4/6 inhibitors and the activity of chemotherapeutics acting on cycling cells.

Recent studies have reported that lidocaine inhibits cell proliferation by inducing the cell cycle in various cancers, such as melanoma, colon, and lung cancer [38-40]. Accordingly, we attempted to identify that the combination of lidocaine and palbociclib effectively decreased cell proliferation by inducing cell cycle arrest. In this study, we found that the combination of these two agents synergistically induced G1 phase arrest through the Rb/E2F1 signaling pathway, probably through the influence of each agent on cell cycle arrest [39-41]. Previous studies have shown that lidocaine inhibited cell proliferation by inducing cell cycle arrest in various cancer cells. It seems that lidocaine could sufficiently improve the antiproliferative effect of palbociclib. Collectively, given that cell proliferation is tightly regulated by the cell cycle machinery, our results show that this combination effect on cell cycle arrest could eventually affect cell proliferation and viability, thereby exerting anticancer effects against TNBC cells.

Despite strong evidence for CDK4/6 activation in various cancer cells, CDK4/6 inhibition was not sufficient to induce apoptosis. However, in the current study, the combined treatment resulted in a more significant increase in cell death in TNBC than alone treatment, consistent with observations of nuclear fragmentation for DNA damage in TUNEL staining and Annexin V. Additionally, apoptosis is highly regulated by different pro-apoptotic and anti-apoptotic proteins such as members of the inhibitor-of-apoptosis protein family, including XIAP, survivin, Mcl-1, and Bcl-2 through changes in the mitochondrial membrane potential [42]. Our study showed that the combination of two agents induced marked changes in the mitochondrial membrane potential (ψm) and synergistically increased cytochrome c release from mitochondria along with the decreased expression of anti-apoptotic molecules such as XIAP, survivin, Mcl-1, and Bcl-2, suggesting that the combined treatment synergistically induced mitochondria-mediated cell death in TNBC. More importantly, the in vitro anticancer efficacy of the combination was also observed in vivo in 4T1 breast cancer orthotopic animal models. The synergistic effects of lidocaine on apoptosis were in line with previous studies by Xing et al. and Wang et al., who found that a combination of lidocaine and chemotherapeutic agents, such as cisplatin and fluorouracil could enhance apoptosis by changing the expression levels of apoptosis-related molecules in hepatocellular carcinoma and melanoma [23, 30].

Although the anti-cancer effect of lidocaine is known to be mediated by various signaling pathways, including PI3/AKT, MAPK, and SRC [30, 40, 43, 44], there are few studies on the exact mechanism of lidocaine in the combined treatment with other chemotherapeutics. To address the molecular mechanism underlying the effect of this combination regimen, we analyzed a phospho-kinase array. We found that the combination of two agents effectively triggered PI3K/AKT/GSK3β signaling by decreasing the expression of p-AKT and p-GSK3β, which was confirmed by *in vivo* findings that it inhibited the phosphorylation of AKT and GSK3β in tumor tissues isolated from 4T1 orthotopic mice. Given that the PI3K/AKT/p-GSK3β pathway plays an important role in tumor malignancy and chemo-resistance in breast cancer [45, 46], its inhibition by the combination is expected to inhibit the growth of tumor cells and overcome palbociclib-resistance in combination therapy with lidocaine in TNBC.

Meanwhile, there is increasing evidence that lidocaine inhibits metastasis and cell proliferation in several cancers [41, 44, 47]. The molecular mechanism involved in metastasis has been shown to dysregulate EMT-related signaling pathways [48]. Activation of EMT endows with migratory and invasive properties upon cancer cells, inducing resistance to conventional chemotherapy [49, 50]. In breast cancers, EMT markers, such as vimentin, E-cadherin, and transcription factors including ZEB1, Snail, and Twist, are highly expressed [51, 52]. Therefore, the inhibition of EMT is considered important in cancer treatment in terms of improving the drug sensitivity and blocking metastasis. Recently, considering that lidocaine inhibited metastasis of ovarian cancer by inhibiting EMT signaling [53], we investigated whether this combination could inhibit metastasis processes, including migration and invasion in TNBC. As expected, our study demonstrated that the combination of lidocaine and palbociclib significantly inhibited the mobility and invasiveness of TNBC cells by increasing E-cadherin expression, reducing that of vimentin, and inhibiting the expression of Twist and ZEB1, finally leading to the blockade of metastasis. These results were supported by a study by Freeman et al., in which lidocaine enhanced the metastasis-inhibition action of cisplatin in breast cancer [25]. Accordingly, our study demonstrates that lidocaine enhances the metastatic effect of palbociclib by regulating EMT signaling.

In conclusion, to our knowledge, we show for the first time that combined treatment with palbociclib and lidocaine significantly inhibited the growth of TNBC cells, and showed synergistic anticancer activities by inhibiting cell proliferation and inducing apoptosis *in vitro and in vivo*. In addition, this combination may augment the therapeutic effect by inhibiting the PI3K/AKT/GSK3β and EMT pathways. Furthermore, these results suggest that the use of lidocaine during surgery or perioperative conditions may be beneficial to the treatment efficiency of cancer patients receiving palbociclib in TNBC (**Figure 6C**).

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Disclosure of conflict of interest

None.

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