

Tunicamycin suppresses breast cancer cell growth and metastasis via regulation of the protein kinase B/nuclear factor- κ B signaling pathway

XIAOLI WANG^{1*}, WEI XIONG^{1*} and YIYIN TANG²

¹Radiotherapy Department; ²The First Department of Mammary Surgery, The Third Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650118, P.R. China

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Abstract. Breast cancer is one of the most common metastatic tumor types. Reports have suggested that Tunicamycin may inhibit the aggressiveness of cancer cells by promoting their apoptosis. In the present study, the inhibitory effects of Tunicamycin were investigated and the potential molecular mechanism underlying the Tunicamycin-inhibited growth and aggressiveness of breast cancer cells was explored. *In vitro* assays demonstrated that Tunicamycin significantly inhibited growth and arrested the cell cycle of breast cancer cells in a dose-dependent manner, compared with control cells. Results revealed that Tunicamycin treatment suppressed the migration and invasion of breast cancer cells. Significantly increased apoptosis of breast cancer cells was observed subsequent to Tunicamycin treatment, as compared with control cells. Mechanism analysis demonstrated that Tunicamycin inhibited the protein kinase B (Akt) and nuclear factor- κ B (NF- κ B) signaling pathways, whilst Akt overexpression significantly cancelled out the Tunicamycin-inhibited growth and aggressiveness of breast cancer cells, as compared with control cells. *In vivo* assays revealed that Tunicamycin treatment significantly inhibited tumor growth and significantly prolonged the survival of tumor-bearing mice, compared with the PBS-treated group. In conclusion, these results indicate that Tunicamycin may inhibit the growth and aggressiveness of breast cancer cells via regulation of the Akt/NF- κ B signaling pathway.

Introduction

Metastatic breast cancer is one of the most common metastatic tumor types, and is a threat to female health globally (1). Human breast carcinoma remains a highly lethal disease due to local invasion and distant metastasis at diagnosis, and the fact that >30% of patients with breast carcinoma will develop metastasis during the development of their disease (2,3). Although the potential function of circulating tumor cell detection and monitoring in breast cancer has been explored in clinical studies, the mortality and recurrence rates remain high (4,5). A systematic review has summarized the treatments currently available for breast cancer-associated lymphedema, which provides potential anti-cancer strategies for patients with breast cancer (6). However, efficient clinical treatments for breast cancer are required in order to improve the survival rate for patients.

Tunicamycin is a nucleotide antibiotic produced by *Streptomyces lysosuperficus*. Numerous published reports have indicated that Tunicamycin may be widely used to inhibit tumor cell growth and aggressiveness (7,8). Evidence has revealed that Tunicamycin may inhibit the growth of breast cancer cells through promoting apoptosis (9,10). Hasegawa *et al* (11) suggested that Tunicamycin enhances the apoptosis induced by TNF-related apoptosis-inducing ligand in endometriotic stromal cells. The unfolded protein response is required in the nu/nu mouse microvasculature when treating a breast tumor with Tunicamycin, which supports the potential of Tunicamycin to be a powerful glycotherapeutic treatment for breast cancer (12). However, the underlying anti-tumor mechanism mediated by Tunicamycin in breast cancer cells has been poorly understood thus far.

In the present study, it was revealed that Tunicamycin may be efficient for the treatment of breast cancer. Research has demonstrated that Tunicamycin exerts anti-tumor efficacy by inhibiting tumor cell growth and enhancing the apoptosis of tumor cells (13,14). The present study investigated the potential signaling mechanisms mediated by Tunicamycin in breast cancer cells. Findings revealed that Tunicamycin may be an efficient agent for the treatment of breast cancer via regulation of the protein kinase B (Akt)/nuclear factor- κ B (NF- κ B) signaling pathway.

Correspondence to: Professor Yiyin Tang, The First Department of Mammary Surgery, The Third Affiliated Hospital of Kunming Medical University, 519 Kunzhou Road, Kunming, Yunnan 650118, P.R. China
E-mail: tangyiyinprof@sina.com

*Contributed equally

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Materials and methods

Ethical statement. The present study was performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of China (15). All animal experiments were performed in accordance with the National Institute of Health, and approved by the Committee on the Ethics of The Third Affiliated Hospital of Kunming Medical University (Kunming, China).

Cells and reagents. MCF-7 and SKBR-3 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). All tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were cultured in a 37°C humidified atmosphere of 5% CO₂.

Flow cytometry. MCF-7 (1x10⁶) and SKBR-3 (1x10⁶) cells were cultured in 6-well plates and treated with Tunicamycin (2 mg/ml) or phosphate-buffered saline (PBS) for 12 h at 37°C. Apoptosis of MCF-7 and SKBR-3 cells was evaluated using an Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Jose, CA, USA). MCF-7 and SKBR-3 cells were isolated from Tunicamycin- or PBS-treated mice and treated with an Annexin V-FITC and PI kit, according to the manufacturer protocol. Fluorescence was measured with a FACScan flow cytometer (BD Biosciences) and analyzed using FCS Express™ IVD software (version 4; De Novo Software, Los Angeles, CA, USA).

Endogenous expression of Akt. MCF-7 and SKBR-3 cells were cultured to 90% confluency, following which the media was removed. MCF-7 and SKBR-3 cells were transfected with lentivirus-AKT (p-AKT) or lentivirus-vector (Control) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). MCF-7 and SKBR-3 cells with stable overexpression of Akt (OPAKT) were treated using Tunicamycin (5 mg/ml) for 24 h at 37°C to allow analysis of the protein expression via western blotting, as subsequently detailed.

MTT assays. MCF-7, SKBR-3 or Akt-overexpressing MCF-7 or SKBR-3 cells (1x10⁶ of each) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) were cultured in 96-well plates for 48 h at 37°C. Growing MCF-7 and SKBR-3 cells were treated using 3, 5 or 8 mg/ml Tunicamycin (Sigma-Aldrich; Merck KGaA) for 48 or 72 h at 37°C. Following 48 h of incubation, 20 μ l MTT (5 mg/ml) in PBS solution was added to each well, and the plate was further incubated for 4 h at 37°C. The majority of the medium was removed and 100 μ l DMSO was added into the wells to solubilize the crystals. The optical density was measured using a Bio-Rad (ELISA) reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 450 nm.

Cell migration and invasion assays. Stable Akt-overexpressing MCF-7 and SKBR-3 cells were cultured with Tunicamycin (5 mg/ml) or PBS for 48 h at 37°C. For migration assays, cells (1x10⁶) suspended in FBS-free medium were plated in the

upper wells of 24-well polycarbonate Transwell inserts (EMD Millipore, Billerica, MA, USA). DMEM supplemented with 10% FBS was added to the lower wells. Following incubation for 24 h, cells on the upper surface of the inserts were scraped off, and cells on the lower surface were fixed with formaldehyde for 5 min at 37°C, stained with 4% crystal violet for 15 min at 37°C and counted under a light microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan). Cell invasion assays were performed using a Cell Invasion Assay kit (ECM550; EMD Millipore), according to the manufacturer's protocol.

Analysis of the cell cycle. To analyze the effects of Tunicamycin (5 mg/ml) on the cell cycle stage of MCF-7 or SKBR-3 cells, flow cytometry was performed. MCF-7 or SKBR-3 cells in the exponential phase of growth, or Akt-overexpressing MCF-7 or SKBR-3 cells, were treated with Tunicamycin (5 mg/ml) for 48 h at 37°C. Cells were then washed, trypsinized for 5 min at 37°C, and rinsed with PBS. All cells were fixed in 75% ice-cold ethanol for 15 min and then washed with PBS three times. The fixed cells were washed with RNaseA (20 μ g ml/l; Fermentas; Thermo Fisher Scientific, Inc.) and stained using PI (20 μ g ml/l; Sigma-Aldrich; Merck KGaA) for 10 min at 37°C. The percentages of cells in the G1/G1 and S phase were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences). Cells were analyzed using the FCS Express IVD software (version 4).

Western blotting. MCF-7 or SKBR-3 cells were treated using Tunicamycin (5 mg/ml), harvested by scraping and lysed using radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich; Merck KGaA) at 37°C for 2 h, followed by homogenization at 4°C for 10 min. Protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Proteins (20 μ g) were analyzed via SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane (EMD Millipore). Following blocking with 5% bull serum albumin in PBS (Sigma-Aldrich; Merck KGaA) at 37°C for 2 h, proteins were incubated with rabbit anti-human Akt (dilution, 1:500; cat no. ab38449), NF- κ B (dilution, 1:400; cat no. ab7204) or β -actin (dilution, 1:500; cat no. ab32572; all Abcam, Cambridge, UK) antibodies for 12 h at 4°C. A horseradish peroxidase-conjugated goat anti-rabbit IgG (cat no. PV-6001; ZSGB-BIO, Beijing, China) was used at a dilution factor of 1:5,000 and used for analyzing protein expression (Olympus BX51, Olympus; Tokyo, Japan). The results were visualized using a chemiluminescence detection system (GE Healthcare Life Sciences, Chicago, IL, USA). The density of the bands was analyzed by Quantity One software version 4.62 (Bio-Rad Laboratories, Inc.) detected using a Western Blotting Luminol Reagent (GE Healthcare Life Sciences, Chicago, IL, USA).

Animal studies. A total of 80 specific pathogen-free male Balb/c (6-8 weeks old; body weight, 25-32 g) mice were purchased from Slack Co., Ltd. (Shanghai, China, <http://qiye20447702.xinlimaoyi.com/>). Mice were maintained in a 12/12 h light/dark cycle with *ad libitum* food and water. Each mouse was housed individually. Experimental mice were implanted with either MCF-7 cells (5x10⁷) or SKBR-3 cells (5x10⁷) in the groin, and divided into two groups (n=40 in each group). Treatments were initiated on the fifth day following tumor

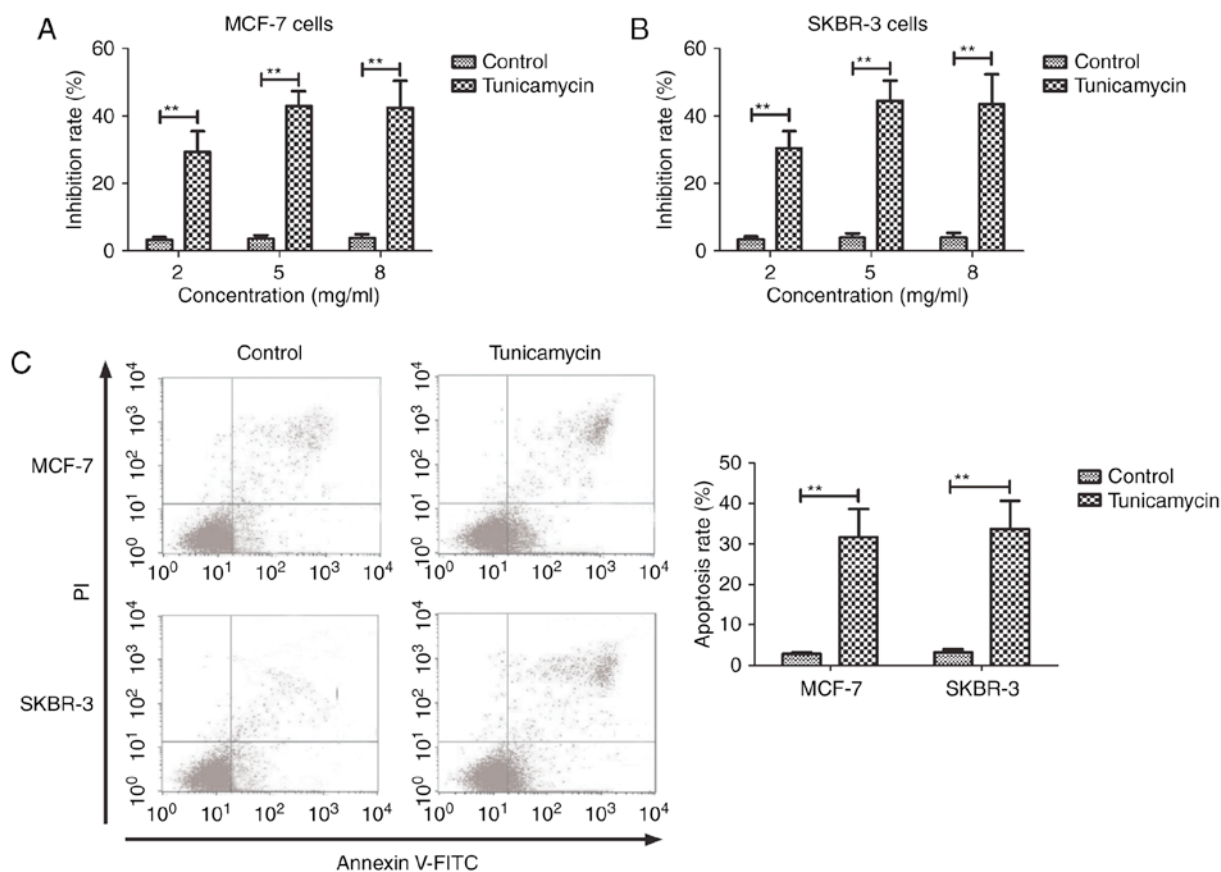


Figure 1. Tunicamycin treatment inhibits the growth of and promotes the apoptosis of breast cancer cells. Tunicamycin significantly inhibits (A) MCF-7 and (B) SKBR-3 cell growth in a dose-dependent manner. (C) Tunicamycin induces the apoptosis of MCF-7 and SKBR-3 cells *in vitro*. **P<0.01 vs. the control.

implantation (diameter: 5-8 mm). Tumor-bearing mice were intravenously injected with Tunicamycin (5 mg/kg), or with PBS as the control. The treatment was performed once a day for a total of 10 days. The tumor volumes were calculated as detailed in a previous study (16). Mice were sacrificed once the tumor diameter had reached 18 mm.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis. For the analysis of myocardial cell apoptosis, a TUNEL assay (Bimake, Houston, TX, USA) was used to detect TUNEL-positive cells. Tumor cells sourced from experimental mice following treatment with Tunicamycin or PBS were fixed using 4% paraformaldehyde solution for 60 min at 4°C. The tumor sections were washed with PBS three times, and then permeabilized by immersing cells slides in 0.2% Triton X-100 solution in PBS for 30 min at 4°C. Subsequently, cells were incubated with equilibration buffer (Sigma-Aldrich; Merck KGaA) for 30 min at 4°C. The tumor sections were then incubated with 50 μ l of the reaction mixture at 37°C for 60 min, and washed 3 times with PBS. The cells nuclei were stained with 4',6-diamidino-2-phenylindole for 60 min at 4°C. Finally, tumor tissue images were captured with a ZEISS LSM 510 confocal microscope at 488 nm (magnification, x40).

Statistical analysis. Software used for statistical analysis included the SPSS 19.0 statistical package (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad

Software, Inc., La Jolla, CA, USA). Data were analyzed using an unpaired Student's t-test, and comparisons between the data of multiple groups were performed using one-way analysis of variance, followed by Dunnett's test. Data are presented as the mean \pm the standard error of the mean obtained from three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Tunicamycin treatment inhibits the growth of and promotes the apoptosis of breast cancer cells. The inhibitory effects of Tunicamycin on breast cancer cells were investigated in the present study. As presented in Fig. 1A and B, Tunicamycin significantly inhibited the growth of MCF-7 and SKBR-3 cells in a dose-dependent manner (2, 5 and 8 mg/ml), compared with the control cells (P<0.01). A dose of 5 mg/ml maximally inhibited the growth of MCF-7 and SKBR-3 cells. It was revealed that Tunicamycin significantly induced the apoptosis of MCF-7 and SKBR-3 cells *in vitro* compared with control cells (P<0.01; Fig. 1C). These data suggest that Tunicamycin treatment may inhibit the growth and promote the apoptosis of breast cancer cells.

Tunicamycin treatment inhibits the aggressiveness of and arrests the cell cycle of breast cancer cells. The efficacy of Tunicamycin on the aggressiveness of breast cancer cells was analyzed. As presented in Fig. 2A and B, it was observed that

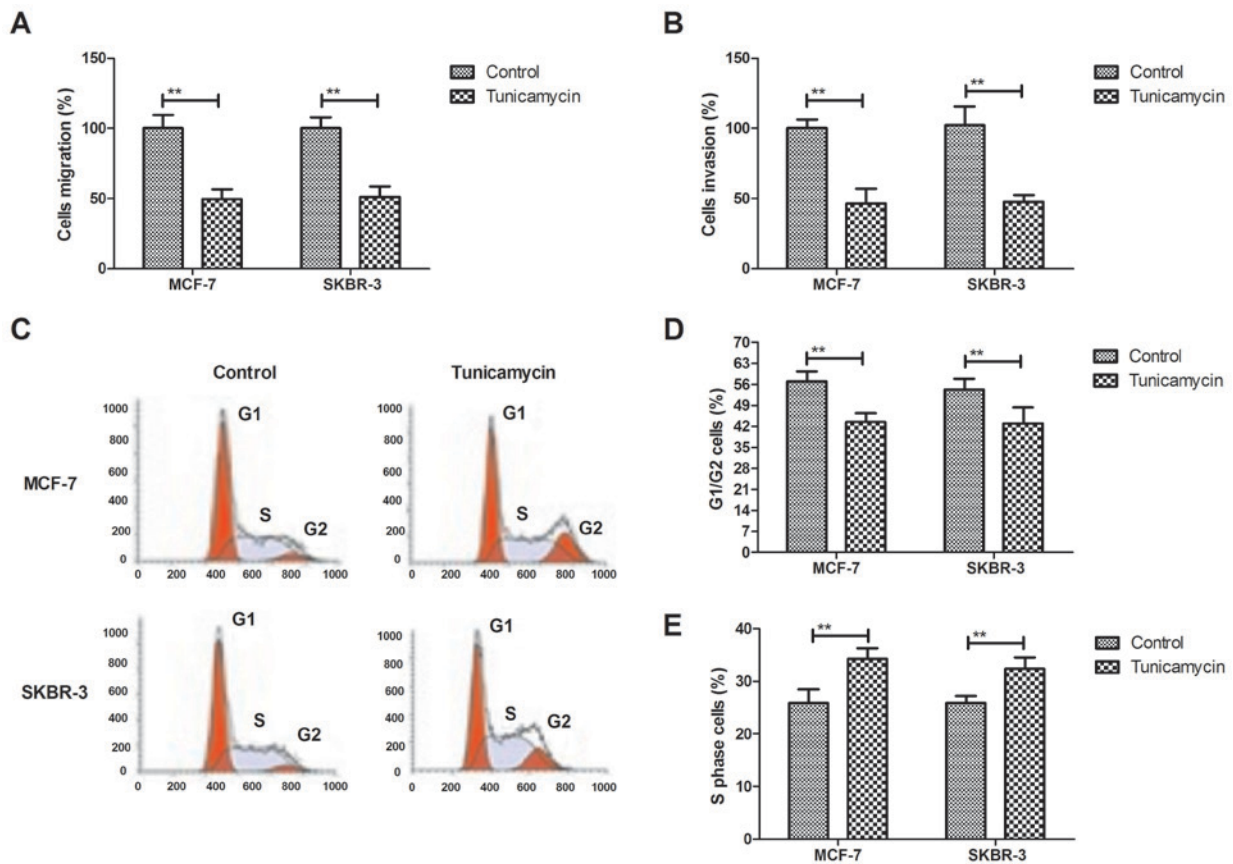


Figure 2. Tunicamycin suppresses the aggressiveness of and arrests the cell cycle of breast cancer cells. Tunicamycin (5 mg/ml) inhibits the (A) migration and (B) invasion of MCF-7 and SKBR-3 cells after 48 h of incubation. (C) Representative flow cytometry images of the cell cycle of MCF-7 and SKBR-3 cells following treatment with Tunicamycin. Quantification of Tunicamycin-arrested cell cycles of MCF-7 and SKBR-3 cells at the (D) G1/G2 and (E) S phases. ** $P < 0.01$ vs. the control.

Tunicamycin (5 mg/ml) significantly inhibited the migration and invasion of MCF-7 and SKBR-3 cells after 48 h of incubation, as compared with the control cells ($P < 0.01$). Representative flow cytometry images revealed that Tunicamycin treatment affected the cell cycle of MCF-7 and SKBR-3 cells (Fig. 2C). It was additionally revealed that Tunicamycin significantly arrested the cell cycle of MCF-7 and SKBR-3 cells at the G1/G2 and S phase, compared with the control cells ($P < 0.01$; Fig. 2D and E). These results suggest that Tunicamycin treatment may inhibit the aggressiveness of and arrest the cell cycle of breast cancer cells.

Tunicamycin regulates the growth and aggressiveness of breast cancer cells through the Akt/NF- κ B signaling pathway.

The potential molecular mechanism mediated by Tunicamycin and involved in the inhibition of breast cancer cell growth and aggressiveness was further analyzed. As presented in Fig. 3A, Tunicamycin treatment suppressed Akt and NF- κ B expression in MCF-7 and SKBR-3 cells, relative to in the control cells. OPAKT increased NF- κ B expression and cancelled out the effect of Tunicamycin-inhibited (OPAKT/TC) NF- κ B expression in MCF-7 and SKBR-3 cells (Fig. 3B). Overexpression of Akt significantly cancelled out the effect of Tunicamycin-inhibited growth on MCF-7 and SKBR-3 cells compared with cells treated with Tunicamycin ($P < 0.01$; Fig. 3C). Tunicamycin-inhibited migration and invasion in MCF-7 and SKBR-3 cells was also significantly abolished

by the overexpression of Akt, compared with in cells treated Tunicamycin ($P < 0.01$; Fig. 3D and E). These results suggest that Tunicamycin regulates the growth and aggressiveness of breast cancer cells through the Akt/NF- κ B signaling pathway.

In vivo efficacy of Tunicamycin in tumor-bearing mice. The *in vivo* effects of Tunicamycin were investigated in MCF-7- and SKBR-3-bearing mice. As presented in Fig. 4A and B, Tunicamycin treatment (5 mg/kg) significantly inhibited MCF-7 ($P < 0.01$), and non-significantly inhibited SKBR-3 tumor growth compared with the control. It was additionally observed that Tunicamycin treatment significantly increased the number of apoptotic cells in MCF-7 and SKBR-3 tumor sections compared with tumor sections from PBS-treated mice ($P < 0.01$; Fig. 4C and D). Observations revealed that Tunicamycin treatment significantly prolonged the survival of MCF-7- and SKBR-3-bearing mice, compared with PBS-treated mice ($P < 0.01$; Fig. 4E and F). Western blot analysis revealed that Tunicamycin downregulated Akt expression and NF- κ B protein expression in MCF-7 and SKBR-3 tumor sections (Fig. 4G and H). These results suggest that Tunicamycin treatment may significantly inhibit breast cancer growth *in vivo*.

Discussion

Breast cancer is one of the most common female malignant tumor types to occur in the mammary gland epithelial

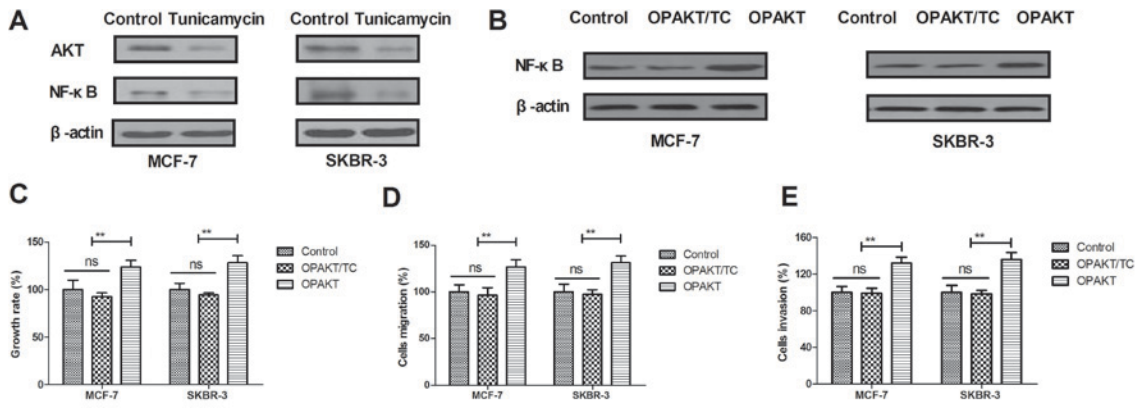


Figure 3. Tunicamycin treatment regulates the growth and aggressiveness of breast cancer cells through the Akt/NF-κB signaling pathway. (A) Tunicamycin suppresses Akt and NF-κB expression in MCF-7 and SKBR-3 cells. (B) OPAKT increases NF-κB expression and abrogated Tunicamycin-inhibited (OPAKT/TC) NF-κB expression in MCF-7 and SKBR-3 cells. (C) OPAKT abolishes the Tunicamycin-inhibited growth of MCF-7 and SKBR-3 cells. OPAKT abolishes Tunicamycin-inhibited (D) migration and (E) invasion in MCF-7 and SKBR-3 cells. **P<0.01 vs. Tunicamycin-treated cells. Akt, protein kinase B; NF-κB, nuclear factor-κB; OPAKT, overexpression of Akt, OPAKT/TC, overexpression of Akt+ Tunicamycin; ns, not significant.

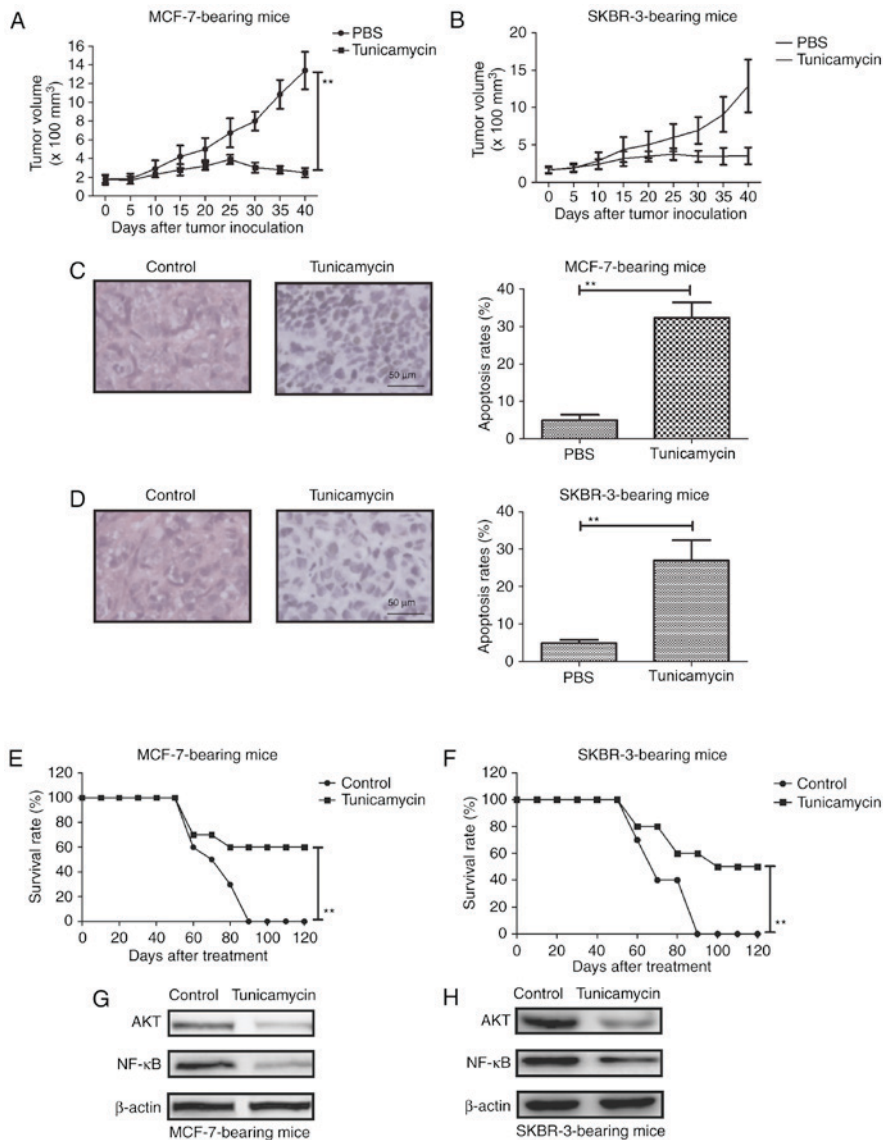


Figure 4. *In vivo* therapeutic efficacy of Tunicamycin in tumor-bearing mice. Tunicamycin treatment (5 mg/kg) inhibits (A) MCF-7 and (B) SKBR-3 tumor growth compared with the control. Tunicamycin treatment increases the number of apoptotic cells in (C) MCF-7 and (D) SKBR-3 tumor sections, compared with in the PBS-group. Tunicamycin treatment prolongs the survival of (E) MCF-7- and (F) SKBR-3-bearing mice compared with PBS-treated mice. Tunicamycin downregulates Akt and NF-κB protein expression in (G) MCF-7 and (H) SKBR-3 tumor sections. **P<0.01 vs. PBS-treated mice. Akt, protein kinase B; NF-κB, nuclear factor-κB.

tissue (17). Tunicamycin has been widely used to enhance the antitumor activity of trastuzumab on breast cancer *in vitro* and *in vivo* (18). In the present study, the efficacy and potential mechanism of Tunicamycin against breast cancer growth, apoptosis and aggressiveness *in vitro* and *in vivo* were explored. Results revealed that Tunicamycin treatment significantly inhibited the growth, migration and invasion of MCF-7 and SKBR-3 cells. In addition, Tunicamycin treatment induced the apoptosis of MCF-7 and SKBR-3 cells, and arrested the cell cycle. Notably, it was observed that Tunicamycin treatment decreased Akt and NF- κ B expression, whilst the overexpression of Akt cancelled out the effect of Tunicamycin-inhibited cell growth and aggressiveness, suggesting that Tunicamycin regulates cell growth and aggressiveness through the Akt/NF- κ B signaling pathway.

A previous study had demonstrated that Tunicamycin-induced ER stress may regulate C-C motif chemokine ligand 5 expression and secretion via signal transducer and activator of transcription 3, followed by the decreased transmigration of MCF-7 breast cancer cells (19). The results of the present study indicate that Tunicamycin inhibited growth and aggressiveness through the Akt/NF- κ B signaling pathway. Kim *et al* (20) demonstrated that Tunicamycin may induce paraptosis potentiated by inhibition of the BRAF^{V600E} mutation in the FRO anaplastic thyroid carcinoma cell line. However, it was revealed in the present study that Tunicamycin may induce apoptosis and arrest the cell cycle at the G1/G1 and S phase in MCF-7 and SKBR-3 cells. Han *et al* (18) indicated that Tunicamycin enhances the antitumor activity of trastuzumab against breast cancer *in vitro* and *in vivo*, and the results revealed that combinations of trastuzumab with the N-glycosylation inhibitor Tunicamycin may be a promising approach for improving the clinical efficacy of trastuzumab. Furthermore, Tunicamycin-induced endoplasmic reticulum stress resulted in reduction of the *in vitro* subpopulation and invasion of CD44⁺/CD24⁻ phenotype breast cancer stem cells (21). Findings of the present study indicate that Tunicamycin inhibits the growth of breast cancer cells through increasing the number of apoptotic bodies in tumor tissues in tumor-bearing mice.

A systematic review and new research directions have highlighted the association between cell migration and female breast cancer across various ethnicities (22). In the present study, the potential signaling pathway underlying the Tunicamycin-mediated growth and aggressiveness of MCF-7 and SKBR-3 cells was explored. Findings indicated that Tunicamycin is an efficient anti-cancer agent, suggesting that the Akt/NF- κ B signaling pathway may be a potential target for the treatment of breast cancer.

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