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RESEARCH ARTICLE

Antitumor effect of the mTOR inhibitor everolimus in combination with trastuzumab on human breast cancer stem cells in vitro and in vivo

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Abstract This study evaluated the effects of a mammalian target of mTOR inhibitor everolimus alone or in combination with trastuzumab on stem cells from HER2-overexpressing primary breast cancer cells and the BT474 breast cancer cell line in vitro and in vivo. For the in vitro studies, we sorted $ESA^{+}CD44^{+}CD24^{-/low}$ cells as stem cells from primary breast cancer cells and BT474 cells using flow cytometry. The MTT assay was used to quantify the inhibitory effect of the drugs on total cells and stem cells specifically. Stem cell apoptosis, cell cycle distributions, and their tumorigenicity after treatment were investigated by flow cytometry or soft agar colony formation assays. For the in vivo studies, BALB/c mice were injected with BT474 stem cells, and the different treatments were administered. After necropsy, the expression of Ki67, CD31, AKT1, and phospho-AKT (Thr308) was analyzed by immunohistochemistry. For the in vitro studies, Treatment with everolimus resulted in stem cell growth inhibition in a dose-dependent manner. The combination of everolimus with trastuzumab was more effective at inhibiting cell growth ($P<0.001$) and tumorigenicity ($P<0.001$) compared with single-agent therapy. In addition, an increase in G1 cell cycle arrest and an increased population of cells in early apoptosis were seen in the combination treatment group compared with either of the single-agent groups ($P<0.01$). For the in vivo studies, everolimus plus trastuzumab therapy was much more effective at reducing tumor volume in mice compared with either single agent alone ($P<0.05$). Compared with everolimus alone, the combination of everolimus and trastuzumab reduced the expression of Ki67, AKT1, and phospho-AKT (Thr308) ($P<0.05$). We conclude that everolimus has effective inhibitory effects on HER2-overexpressing stem cells in vitro and vivo. Everolimus plus trastuzumab is a rational combination treatment that may be promising in human clinical trials.

Keywords Breast stem cells · mTOR · HER2-positive · Everolimus · Trastuzumab

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Introduction

Epidemiological studies of breast cancer have shown that approximately 20–30 % of breast cancer patients are positive for human epidermal growth factor receptor type 2 (HER2 and ErbB2/HER2) in immunohistochemical tests. Compared to HER2-negative breast cancer patients, HER2-positive patients have a shorter overall survival [1], which makes this type of cancer critical to treat especially. The discovery of the targeted therapeutic drug trastuzumab, an anti-HER2 receptor monoclonal antibody, was an important breakthrough in the treatment of HER-positive malignant tumors [2]. Trastuzumab has been routinely used in patients with HER2-positive breast cancer and has shown a distinct survival benefit [3]. However, trastuzumab was

found to be less effective in breast cancer patients with metastatic HER2-positive breast cancer, who exhibited drug resistance to the drug [4]. These studies indicated that trastuzumab has its limitations in the treatment of HER2-positive breast cancer, and combination therapy should be considered to reduce drug resistance during treatment.

As an important intracellular signal transduction pathway, the PI3K/AKT/mTOR signaling pathway plays pivotal roles in the proliferation, survival, and apoptosis of tumor cells [5]. The mammalian target of rapamycin, mTOR, is a downstream effector of the PI3K/AKT pathway. mTOR is a serine/threonine protein kinase with a molecular weight of 300 kDa and plays an important regulatory role in protein translation through regulating the phosphorylation of other kinases, such as the 40S ribosomal S6 kinase (S6K), cyclin-dependent kinase, and eukaryotic initiation factor (4EB) [6]. Although the transduction mechanism of this pathway has not been completely understood, it is certain that mTOR interacts with growth factors and their receptors and affects cell cycle progression and membrane transport processes besides regulating protein synthesis and regulation [7]. Due to the close relationship between tumorigenesis and abnormal mTOR pathway regulation, mTOR has become an important target in cancer treatment [8]. As a derivative of the mTOR inhibitor rapamycin, everolimus showed a therapeutic effect in the treatment of kidney cancer, breast cancer, and non-small cell lung cancer with some tolerable side effects in patients [9]. The activation of the AKT pathway in these cancer cells leads to the overactivation of mTOR, increase in S6K1 activity, and hyperphosphorylation of 4EBP1, which in turn accelerates cell cycle progression. This process is selectively blocked by the mTOR blocker everolimus. Because it selectively blocks mTOR signaling without negatively affecting normal cells, everolimus has mild side effects, which makes it unmatched by many other antitumor drugs [10]. With progress in targeted therapies, rational combinations of different antitumor mechanisms will render more benefit to patients. Using this as the theoretical basis, we studied the potential combinatorial effect of the mTOR-targeting therapeutic drug everolimus and the HER2-targeting drug trastuzumab to understand the effect of these two drugs on breast cancer stem cells.

Currently, an increasing number of studies have demonstrated the presence of cancer stem cells (CSCs) in extremely low numbers among cancer cells. These stem cells not only have the capability to differentiate into different types of cancer cells but also possess long-term self-renewal ability. These properties yield these stem cells crucial roles in the genesis and progression of malignant tumors [11, 12]. O'Brien et al. [12] obtained a $CD133^+$ cell subpopulation that contained CSCs through the screening of colon cancers. Compared with $CD133^-$ cells, these $CD133^+$ cells exhibited a significant difference in tumorigenicity in non-obese diabetic NOD/severe combined immunodeficiency mice. While 1×10^3 $CD133^+$ cells were sufficient to form tumors in 100 % of the mice, only 11 % of mice injected with 2.5×10^5 $CD133^-$ cells showed tumors. This result indicates that CSCs have a strong tumorigenic ability. Furthermore, it is believed that CSCs are closely associated with malignant tumor metastasis, relapse and treatment resistance. In fact, it was reported in various in vivo and in vitro studies that CSCs exhibited drug resistance to different extents against all routine cancer chemotherapies and radiotherapies [12–14]. In the related reports on breast cancers, it was shown that tumor-initiating cells (also called breast CSCs) displayed higher viability after chemotherapy among breast cancer cell lines [13, 15]. CSCs can mediate tumor recurrence due to their high tumor formation rate and resistance to radio- and chemotherapies, and therefore, effectively eradiating CSCs is necessary to achieve the desired antitumor effect.

CSCs have been identified in multiple primary tumor and cancer cell lines [11, 12]. For breast cancer, Al-Hajj et al. isolated $ESA^+Lin^-CD44^+CD24^{-/low}$ cells from breast cancer specimens of multiple different pathological types using different cancer cell surface antigen markers. The isolated ESA^+ Lin^- $CD44^+$ $CD24^{-/low}$ cells exhibited the characteristics of stem cells. A small number of CSCs with stem cell characteristics were found in the seemingly homogeneous cancer cells in long-term culture in vitro [16]. In this study, we sorted for $ESA^+CD44^+CD24^{-/low}$ cells from the BT474 HER2-positive breast cancer cell line and HER2-positive primary breast cancer cells. We then compared these phenotypic breast CSCs to total breast cancer cells in their response to the mTOR inhibitor everolimus alone or in combination with trastuzumab. The inhibitory effects of different therapeutic approaches on the in vitro and in vivo tumorigenicity of stem cells were compared to understand the effects of these therapies on HER2-positive breast cancer, which will provide a scientific basis for the rational clinical application of everolimus and trastuzumab.

Materials and methods

Cells and mice

The BT474 human breast cancer cell line was from the cell stock in our laboratory. Breast cancer specimens were collected with informed consent of the patients at the affiliated Cancer Hospital of Tianjin Medical University. The pathological diagnosis was invasive ductal carcinoma as determined by immunohistochemical test or positive FISH test. Female BALB/c nude mice, 4–6 weeks of age and with a

body weight of 20±2 g, were purchased from the Department of Laboratory Animal Science at Peking University Health Science Center [license number: SCXK (BJ) 2006–2008] and maintained under specific pathogen-free conditions.

Reagents

Everolimus (Certican®) was a gift from Novartis Pharma Stein AG, and trastuzumab (Herceptin®) was provided by Roche (Basel, Switzerland). Experimental consumables, including RPMI-1640, fetal bovine serum (FBS), Hank's solution, collagenase I, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypsin were purchased from Gibco (Life Technologies, Carlsbad, CA, USA). Fluorescently labeled antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and the annexin V-FITC apoptosis detection kit was from KeyGen (Nanjing, China). The soft agar cell cloning kit was the product of GenMed (Minneapolis, MN, USA). Anti-Ki67, anti-CD31, anti-AKT1, and anti-phospho-AKT (Thr308) primary antibodies, the streptavidin peroxidase (SP) ready-to-use goat anti-rabbit kit, and the DAB chromogenic kit were purchased from Beijing Biosynthesis Biotechnology Co. (Beijing, China), and all other reagents were provided by the Central Laboratory at Tianjin Cancer Hospital.

Experimental instruments

The flow cytometer (BD FACS Aria I), ELISA reader, tissue culture hood, incubator, centrifuge, and other experimental equipment were all provided by the Central Laboratory at Tianjin Cancer Hospital or the Institute of Hematology of the Chinese Academy of Medical Sciences.

Isolation of primary breast CSCs

Freshly collected breast cancer tissues (>1 cm^3) were cut into 1-mm^3 tissue blocks under sterile conditions and digested with freshly made collagenase I (1 mg/ml; filter-sterilized) at 37 °C for 1–3 h. The digested samples were filtered, and the cells were resuspended in RPMI-1640 media supplemented with 10 % FBS. The cells were then placed into collagen-coated tissue culture flasks and cultured with 5 % CO_2 at 37 °C overnight. The cells were sorted by flow cytometry on the following day. Before sorting, the cells in single-cell suspension were labeled with anti-CD44-APC, anti-CD24-PE, and anti-ESA-FITC antibodies. Four control groups were established for the first sorting: (1) cells labeled with the isotype antibodies of the above three antibodies, (2) cells labeled with the anti-CD44-APC antibody and the isotype control antibodies of the other two antibodies, (3) cells labeled with the anti-ESA-FITC antibody and the isotype control antibodies of the other two antibodies, and (4) cells labeled with the anti-CD24-PE antibody and the isotype control antibodies of the other two antibodies. Cells that were $CD44^+$, $CD24^{-/low}$, and ESA^+ were sorted by flow cytometry and placed into flasks or 96-well plates.

Isolation of BT474 stem cells

BT474 cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum, 2 mM glutamine, 10 μg/ml insulin, and penicillin/streptomycin dual antibiotics in 25-ml flasks at 37 °C with 5 % CO_2. The medium was changed and the cell morphology monitored daily. When the cells reached a density of 10^8, they were trypsinized and reduced to a single-cell suspension by vigorously pipetting the cells. The cells were labeled with anti-CD44-APC, anti-CD24-PE, and anti-ESA-FITC antibodies before sorting, with the control groups established as described above. Cells that were $CD44^+$, $CD24^{-/low}$, and ESA^+ were sorted by flow cytometry and placed into flasks or 96-well plates.

Stem cell culture

The isolated stem cells were cultured in serum-free stem cell culture medium [DMEM:F12 (1:1) medium containing 1:50 B27, 20 ng/ml EGF, 0.4 % bovine serum albumin, and 4 μg/ml insulin] to prevent the cultured breast CSCs from differentiating.

MTT assay

The 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay (MTT assay) was used to compare the effects of everolimus or trastuzumab on total breast cancer cells and breast CSCs. The total cells and the stem cells from the BT474 cell line and the primary breast cancer cells were respectively seeded into 96-well plates with different concentrations of the drugs, with five wells for each concentration, and the cells were cultured at 37 °C with 5 % CO_2 in an incubator for 24 h. The concentrations of everolimus were 1 nM, 10 nM, 100 nM, 1 μM and 10 μM, and the concentrations of trastuzumab were 0.5, 1, 10, 50, and 100 μg/ml. The combinatorial inhibitory effect of everolimus and trastuzumab on the in vitro growth of breast CSCs was examined by MTT assay as well using 10 μg/ml trastuzumab in combination of increasing concentrations of everolimus (1 nM, 10 nM, 100 nM and 1 μM).

After drug treatment for 24 h, 20 μl MTT [5 mg/ml in phosphate buffered saline (PBS)] was added to each well,

and the cells were incubated at 37 °C with 5 % CO_2 and saturated humidity for 4 h. Following the subsequent removal of the supernatant, 150 μl dimethyl sulfoxide (DMSO) was added to each well, and the cells were vortexed for 10 min. The light absorbance (OD value) was measured for each well using an ELISA reader. Each experiment was repeated in triplicate, and dose–response curves were plotted. The probit software of the statistical software package SPSS 17.0 for Windows (SPSS, Chicago, IL, USA) was used to calculate the inhibitory concentration (IC_{50}) of each drug.

Cell cycle and apoptosis examination by flow cytometry

BT474 stem cells that were sorted by flow cytometry were cultured in stem cell culture medium in 25-ml cell culture flasks. The cells were divided into four groups: (1) the control group (blank control), (2) the Ever group (100 nM everolimus), (3) the Tz group (10 μg/ml trastuzumab), and (4) the Ever+Tz group (100 nM everolimus and 10 μg/ml trastuzumab). Culture medium with 0.5 % DMSO was added to the blank control group. After treatment, the cells were cultured in an incubator at 37 °C with 5 % CO_2 for 24 h before the cells were collected.

For cell cycle determination, the cells in the different treatment groups were fixed in ice-cold ethanol for 24 h. The ethanol was removed, 500 μl RNase-containing propidium iodide (PI) and 1 mL PBS were added, and the cells were incubated at 4 °C for 30–60 min in the dark. The samples were then sorted by flow cytometry according to each cell cycle stage, and G0/G1 %, S%, and G2/M% were calculated to obtain the cell cycle distribution.

To understand the effect of drug treatment on stem cell apoptosis, annexin-FITC and PI were added to the single-cell suspension and mixed well before incubation at room temperature for 5–15 min in the dark. The cells were then sorted by flow cytometry within 1 h after incubation to measure the rate of apoptosis.

Soft agar colony formation assay

The bottom layer of soft agar (0.9 %) was prepared in a six-well plate, and the top layer (0.5 %) was prepared with 5×10^4/ml BT474 stem cells in single-cell suspension. The cells were divided into four groups: (1) the control group (blank control), (2) the Ever group (100 nM everolimus), (3) the Tz group (10 μg/ml trastuzumab), and (4) the Ever+Tz group (100 nM everolimus and 10 μg/ml trastuzumab). The cells were cultured in an incubator at 37 °C with 5 % CO_2 for 2 weeks and observed for colony formation by microscopy; colonies of >30 cells were counted under a microscope. The experiments were repeated in triplicate.

Establishment of experimental animal model and drug intervention

Cultured BT474 stem cells were collected and pelleted by centrifugation at 1,000 rpm for 5 min. The cells were then washed with serum- and antibiotics-free DMEM medium three times. After the cells were counted, aliquots of cells at 1×10^5/100 μl in serum- and antibiotic-free culture medium in (microcentrifuge tubes) were sent to the animal room under sterile conditions. A volume of 100 μl stem cell suspension was injected beneath the left breast pad of BALB/c nude mice. The injected mice were housed in clean cages under a constant temperature of 20–25 °C with free access to food and water. When the tumor volume was approximately 300 mm^3 (approximately 9 days after stem cell injection), the tumor-bearing mice were randomly divided into four groups (five animals/group): (1) the control group (normal saline), (2) the Ever group (2 mg/kg everolimus), (3) the Tz group (5 mg/kg trastuzumab), and (4) the Ever+Tz group (2 mg/kg everolimus and 5 mg/kg trastuzumab); this day was denoted as day 1. Thereafter, the greatest longitudinal diameter (L) and the greatest transverse diameter (W) of the xenograft tumors in mice were measured by caliper in the morning once every 3 days, and tumor volumes were calculated and recorded when the test articles were administered. After the last tumor volume measurement on day 16, the mice were euthanized by cervical dislocation, and tumor specimens were collected and fixed for histochemical assays.

Immunohistochemistry

Immunohistochemistry staining was performed in the method of streptavidin-perosidase (SP) using Ki67, CD31, AKT1, and pAKT antibody for all specimens. Serial sections were sliced from paraffin blocks, and the sections were dewaxed and treated with high temperature and pressure for 3.5 min for epitope retrieval. The sections were then incubated in 3 % hydrogen peroxide solution, blocked with serum, and incubated with anti-Ki67, anti-CD31, anti-AKT1, and anti-phosphor-AKT (Thr308) primary antibodies at dilutions of 1:100, 1:150, 1:100, and 1:100, respectively. After overnight incubation at 4 °C, the sections were incubated with secondary antibody at 37 °C for 40 min. The sections were then incubated with a horseradish peroxidase-labeled avidin working solution for 20 min, developed with DAB reagent and monitored by microscopy. The sections were counterstained with hematoxylin for 1–2 min, rinsed in 1 % hydrochloric acid in ethanol, and blued with ammonia for 2 min. The sections were dehydrated using an ethanol gradient, cleared, and mounted. Both positive and negative controls were included, and PBS was used to replace the primary antibody for the negative control. Cells with brown

or dark brown particles in the cytoplasm or nucleus were counted as positively stained cells. Five microscopic fields were randomly selected for observation at high magnification, and the percentage of positively stained cells in the fields was used for grading. Cells without a significant difference between the staining intensity and the background were considered negatively stained. An average positivity (average positive rate) was calculated for each section and was used to calculate the unit value of positive staining.

Statistical analysis

The measurement data were expressed as $\overline{X} \pm s$ and analyzed with statistical methods, including the Student's test and two-way ANOVA. The SPSS 17.0 software package was used for data processing, with α=0.05 as the significance level.

Results

Inhibitory effect of everolimus and trastuzumab alone and in combination on breast cancer cell growth

To understand the inhibitory effect of trastuzumab on the growth of breast CSCs compared to that of non-stem tumor cells, we used the MTT assay to generate cell growth inhibition curves of total BT474 and primary breast cancer cells and compared them to those of their respective stem cells (Fig. 1a). While the BT474 total and stem cells both showed a dose-dependent inhibitory response to trastuzumab that increased with the increase in trastuzumab concentration, the cell populations differed in their extent of the response. Compared with the total cells, the BT474 stem cells were significantly less sensitive to trastuzumab at the same concentration (P<0.001). As listed in Table 1, the IC_{50} of trastuzumab in the BT474 total cells was 2.095 μg/ml and that in the corresponding stem cells was 45.492 μg/ml, which was 19 times that of the total cells, indicating greater trastuzumab resistance in the stem cells. The same phenomenon was observed in the primary breast cancer cells. Compared with the total cells, the growth of the stem cells was significantly less affected by trastuzumab at all tested concentrations (P<0.001). The IC_{50} of trastuzumab in primary breast CSCs was 16 times that in the corresponding total cells (Table 1).

We also plotted the growth inhibition curve of everolimus in breast CSCs and calculated the IC_{50} for both the total cells and the stem cells. Figure 1b shows that everolimus exhibited a dose-dependent inhibition in both the total cells and the stem cells from the BT474 cell line and the primary breast cancer cells, albeit with different degrees of growth inhibition. Compared with the total cells, everolimus was less effective in growth inhibition in the stem cells at all tested concentrations (P<0.001). The IC_{50} values of everolimus for BT474 and the primary CSCs were 2,054 and

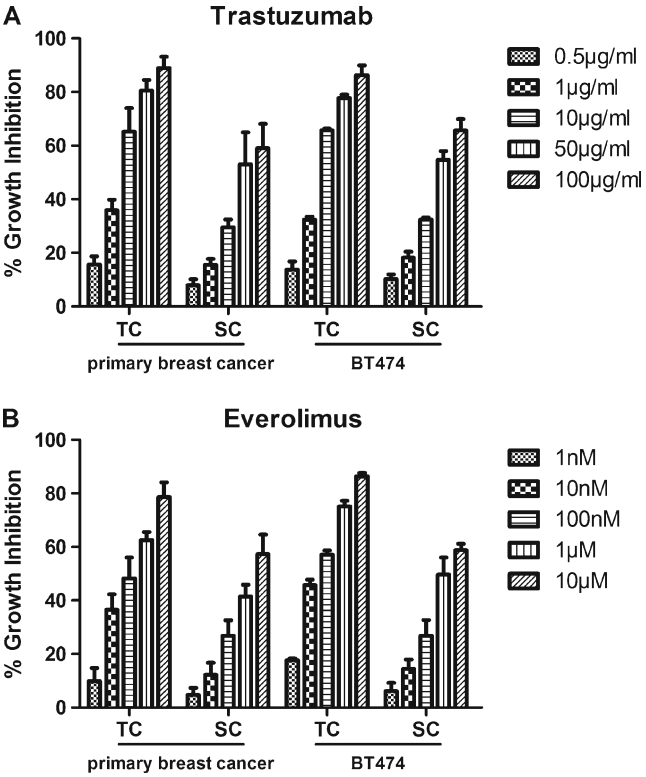

Fig. 1 Anti-proliferative effect of trastuzumab or everolimus alone in breast cancer total cells (TC) and stem cells (SC). **a** Compared with the total cells, the stem cells isolated from BT474 or primary breast cancer cells displayed a certain degree of resistance to treatment with trastuzumab(P<0.001). Dose–response curves were obtained by MTT assays after 24-h exposure to trastuzumab alone (0.5, 1, 10, 50, and 100 μg/ml). **b** Stem cells were more resistant to treatment with everolimus, compared with total cells in BT474 and primary breast cancer cells (P<0.001). The stem cells were exposed to everolimus (1 nM, 10 nM, 100 nM, 1 μM, 10 μM) for 24 h. Each column represents the mean ± SD (n=3). The statistical analysis was performed with two-way ANOVA using Tukey's test for pairwise comparisons

Table 1 Fifty percent inhibitory concentrations for trastuzumab and everolimus in breast cancer cells

Cell line		IC_{50} trastuzumab (μg/ml)	IC_{50} everolimus (nM)
Primary breast cancer	Total cells	2.095	156
	Stem cells	45.492	3,227
BT474	Total cells	2.52	71
	Stem cells	48.946	2,054

IC_{50} 50 % inhibitory concentration

3,227 nM, or 29 times and 21 times greater than the IC_{50} values for their corresponding total cells, respectively (Table 1).

Different concentrations of everolimus were combined with a fixed concentration of trastuzumab to understand the inhibitory effect of the two drugs combined on the growth of breast CSCs and to determine the most effective combinatorial concentrations. The growth inhibition curves were plotted after the 24-h culture of breast CSCs in the presence of the drugs at different concentrations (Fig. 2). The combination of everolimus with 10 μg/ml trastuzumab showed more effective inhibition of primary breast CSC growth compared with everolimus alone (Fig. 2a) ($P<0.001$). The IC_{50} of trastuzumab for the primary breast CSCs was approximately 45 μg/ml in this study (Table 1). When >100 nM everolimus was added, just 10 μg/ml trastuzumab could inhibit the growth of 50 % CSCs($P<0.01$). For the BT474 stem cells, the inhibitory rate of 10 μg/ml trastuzumab was significantly increased when 10–1,000 nM everolimus was added ($P<0.05$). While the IC_{50} of trastuzumab alone was approximately 49 μg/ml (Table 1), the inhibition rate increased by more than 50 % when >10 nm everolimus was added (Fig. 2b).

Inhibitory effect of everolimus and trastuzumab alone or in combination on the stem cell cycle

The cell cycle status of untreated BT474 stem cells and those treated with 100 nM everolimus alone, 10 μg/ml trastuzumab alone, or the two drugs combined was analyzed by flow cytometry to measure the cell distribution at each stage in the cell cycle. The BT474 stem cells treated with everolimus or trastuzumab alone showed a significantly increased number of cells arrested at G0–G1 phase when compared with the control group ($P<0.05$) (Fig. 3). However, an even higher percentage of BT474 stem cells was arrested at G0–G1 phase when the two drugs were combined. Compared with everolimus or trastuzumab alone, these two drugs in combination exhibited significantly enhanced effects on stem cell cycle arrest at G0–G1 phase ($P=0.002$ or $P=0.004$, respectively).

Inhibitory effect of everolimus and trastuzumab alone or in combination on stem cell apoptosis

To study the effects of individual drugs or the two drugs in combination on early apoptosis in BT474 stem cells, we detected early apoptotic cells by flow cytometry with annexin-FITC and PI double staining. The BT474 stem cells were cultured in stem cell medium for 24 h alone or in the presence of 100 nM everolimus, 10 μg/ml trastuzumab, or the two drugs together. The early apoptosis rate of the BT474 stem cell control group was 3.13±0.29 %, while those of the everolimus, trastuzumab, and Ever + Tz groups were 5.26±0.35 %, 6.30±0.19 %, and 10.13±1.20 %, respectively ($P<0.05$). Moreover, when compared with the everolimus or trastuzumab groups, the group treated with the two drugs in combination displayed a significantly increased apoptotic rate ($P<0.001$ or $P=0.001$ respectively; Fig. 4).

Inhibitory effect of everolimus and trastuzumab alone or in combination on stem cell clonogenicity

The effects of everolimus, trastuzumab, and the two drugs combined on BT474 stem cell in vitro colony formation were evaluated using the soft agar cloning assay (Fig. 5). In this experiment, the BT474 stem cells were cultured in medium with different concentrations of the drugs for 2 weeks, and colony formation was observed by microscopy.

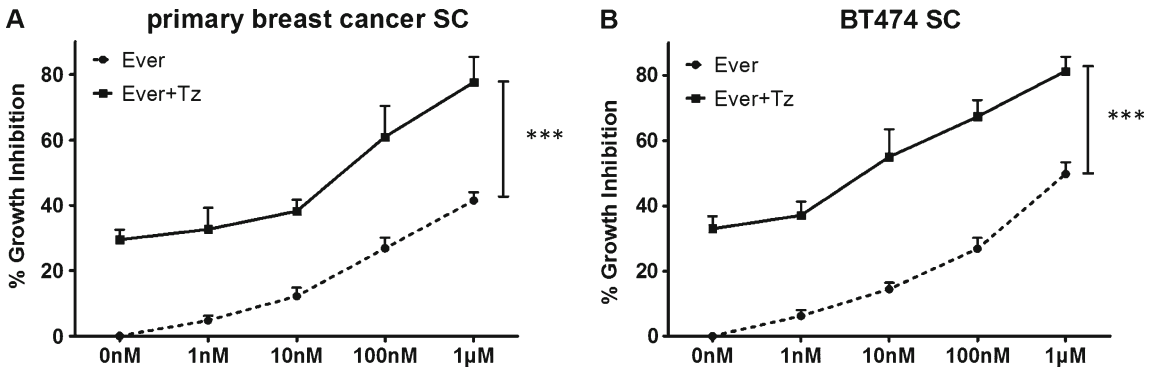

Fig. 2 The inhibitory effects of combination treatment with everolimus and trastuzumab on breast cancer stem cells. The stem cells were treated with everolimus at concentrations of 1 nM, 10 nM, 100 nM, and 1 μM alone or with trastuzumab at10 μg/ml. After 24 h, MTT assays were performed to measure cell proliferations. We found that the combination treatment had an additive growth inhibitory effect on the stem cells in vitro compared with treatment with everolimus alone ($P<0.001$). Each column represents the mean ± SD ($n=3$). The statistical analysis was performed with two-way ANOVA using Tukey's test for pairwise comparisons. *NS* not significant; *$P\leq0.05$, **$P<0.01$, ***$P<0.001$ compared with the everolimus group

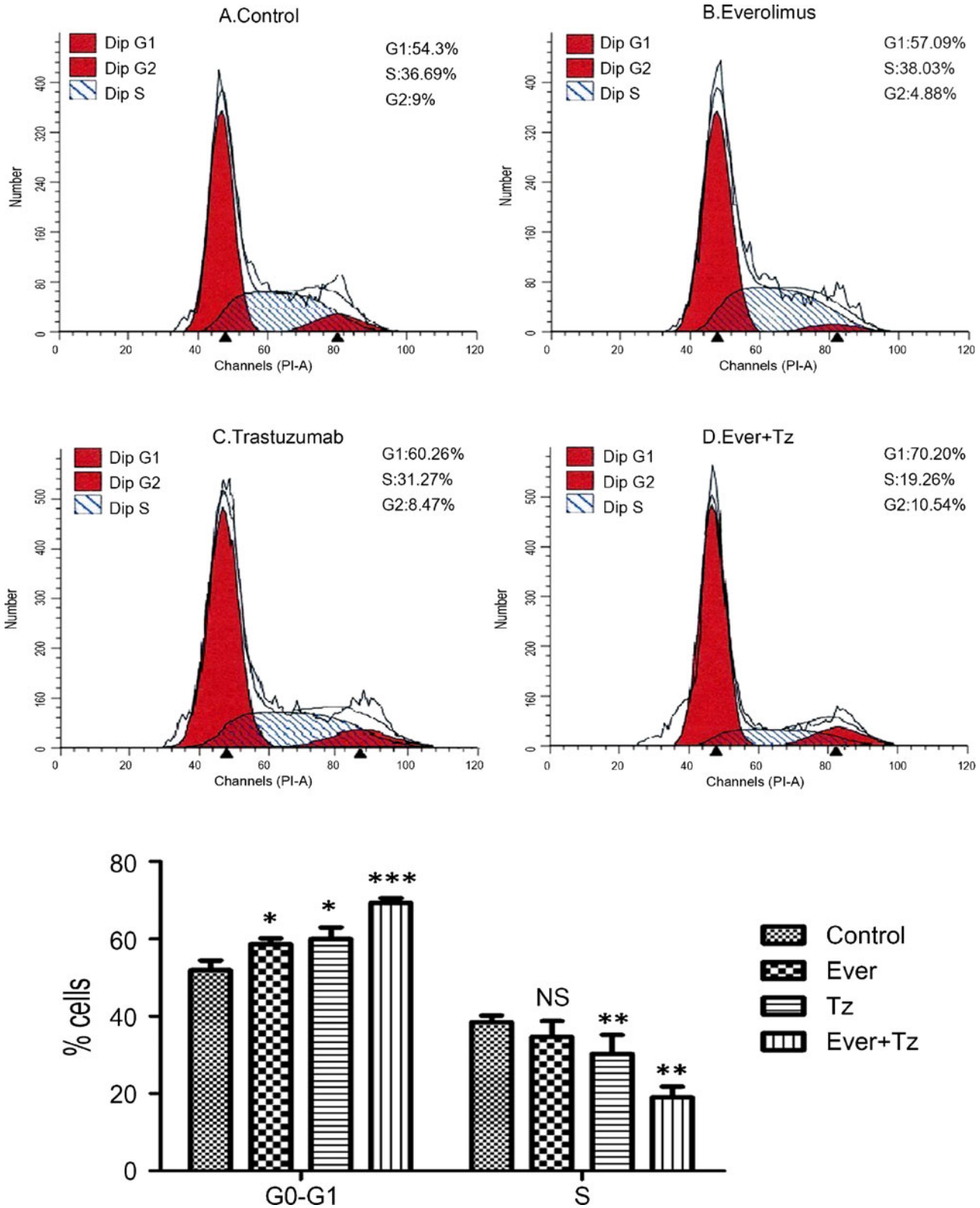

Fig. 3 The effect of the combination of everolimus and trastuzumab on the cell cycle distribution of BT474 stem cells measured by flow cytometry. **a** Control. **b** 100 nM everolimus. **c** 10 μg/ml trastuzumab. **d** 100 nM everolimus + 10 μg/ml trastuzumab. After exposure to the treatments for 24 h, the fraction of cells in the G0–G1 phase of the cell cycle was significantly higher in the combination group than in either single-agent group($P<0.01$). Each column represents the mean ± SD ($n=3$). The statistical analysis was performed with Student's *t* test. *NS* not significant; *$P\leq0.05$, **$P<0.01$, ***$P<0.001$ compared with the control group

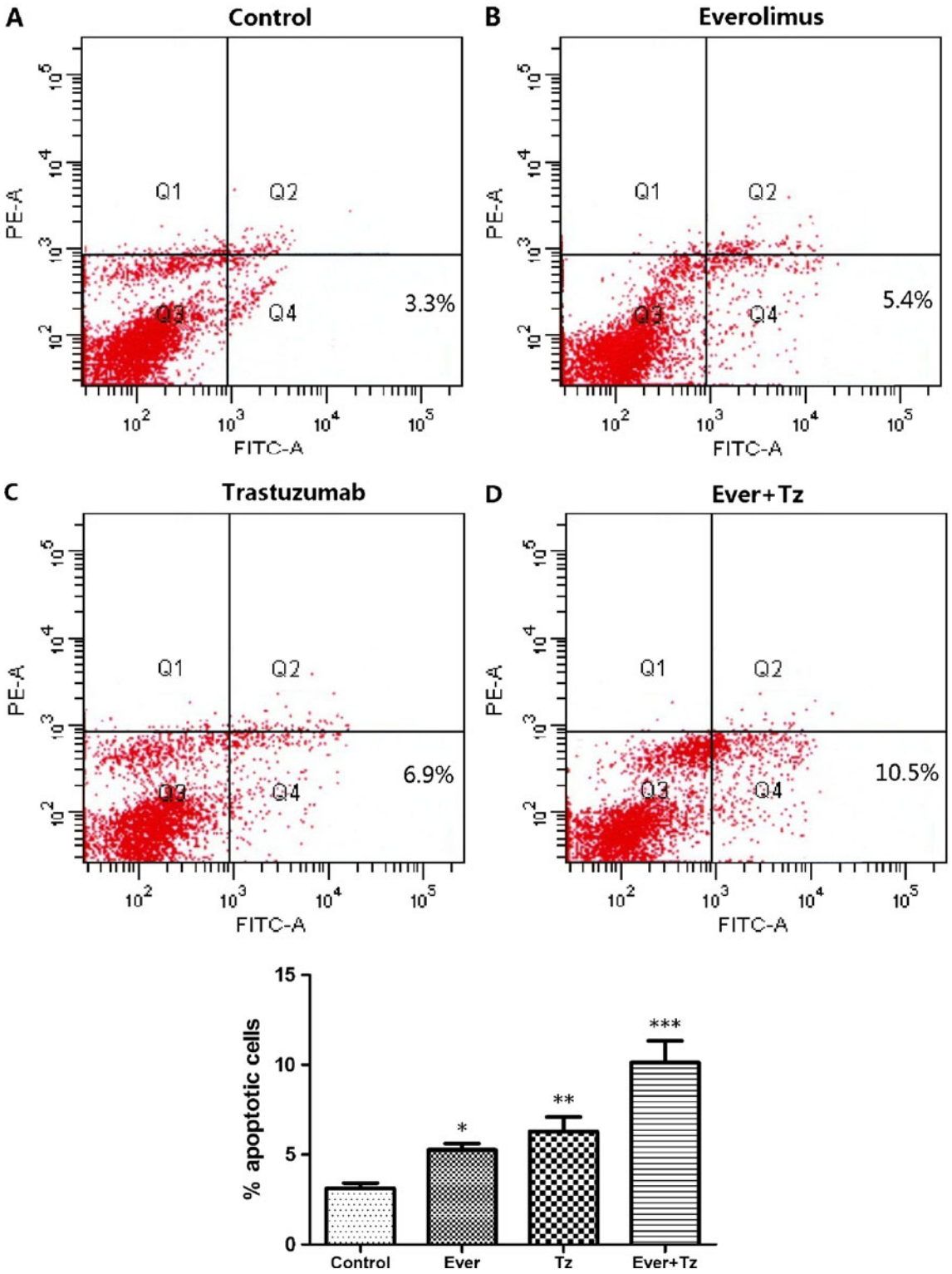

Fig. 4 Effect of the combination of everolimus and trastuzumab on the induction of early apoptosis in BT474 stem cells measured by flow cytometry. **a** Control. **b** 100 nM everolimus. **c** 10 μg/ml trastuzumab. **d** 100 nM everolimus + 10 μg/ml trastuzumab. After exposure to the different treatments for 24 h, the proportion of early apoptotic cells was analyzed using annexin V-FITC and PI. Compared with the everolimus or trastuzumab alone group, combination treatment induced an increase in the population of cells undergoing early apoptosis ($P<0.01$). Each column represents the mean ± SD ($n=3$). The statistical analysis was performed with Student's t test. *NS* not significant; $*P\leq0.05$, $**P<0.01$, $***P<0.001$ compared with the control group

The number of colonies in four randomly selected microscopic fields was then counted and averaged. The number of colonies in the everolimus group showed a slight decrease compared to that of the control group (81.77 vs. 100 %, respectively; $P<0.001$). Trastuzumab caused a greater decrease (61.00 %; $P<0.001$), while cells in the Ever + Tz group showed only 37.86 % colony formation compared to the control group ($P<0.001$). Therefore, the combination treatment was significantly more effective at inhibiting BT474 stem cell colony formation than any of the drugs alone ($P<0.001$).

Effect of everolimus and trastuzumab alone and in combination on tumor growth in a xenograft animal model

The construction of the xenograft tumor model was described in "Materials and methods." The tumor size and mouse body weight were measured every 3 days after dosing, and the tumor volume was calculated using the following formula: volume (cm^3)=length×width^2×0.5. Compared to the control group with a tumor size of 698±136.36 mm^3, the everolimus, trastuzumab, and drug combination groups

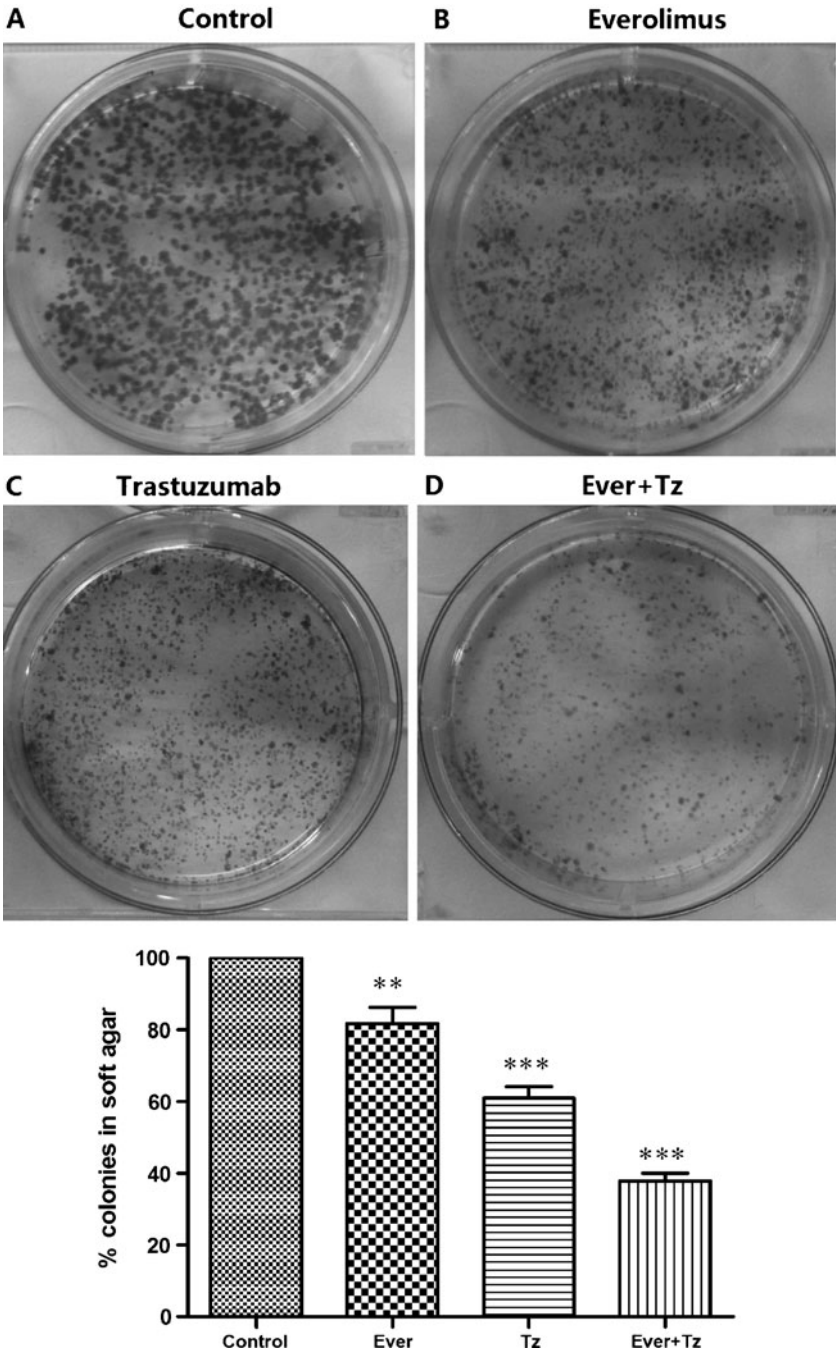

Fig. 5 BT474 stem cells were plated for soft agar assays and treated with everolimus alone, trastuzumab alone, the combination of the two drugs, or left untreated. **a** Control. **b** 100 nM everolimus. **c** 10 μg/ml trastuzumab. **d** 100 nM everolimus + 10 μg/ml trastuzumab. Compared control group, 2 weeks of culture with the drug single or combination significantly reduced the colony formation ability of the stem cells ($P<0.01$). Each data point represents the mean ± S.D. ($n=3$). The statistical analysis was performed with Student's t test. *NS* not significant; *$P\leq0.05$, **$P<0.01$, ***$P<0.001$ compared with the control group

showed significant reductions in mean tumor sizes (590.6± 49.1, 508.6±35.6, and 410.8±75.1 mm^3, respectively; $P<0.01$; Fig. 6a). Compared to the mean xenograft tumor size in the trastuzumab group, the mean tumor size in the everolimus group was larger, which suggests that the inhibitory effect of everolimus at the tested concentration was relatively weaker on xenograft tumor size ($P=0.03$). When the two drugs were combined, the xenograft tumor size was smaller than those of the groups treated with everolimus or trastuzumab alone, indicating that the combination of the drugs significantly decreased the xenograft tumor size ($P<0.001$ or $P=0.018$, respectively). To understand whether the drugs affected the body weight of the animals while they exerted their antitumor effects, the body weight of the xenograft tumor mouse model was monitored. No significant difference in body weight was observed in xenograft tumor-bearing mice between the three treatment groups during the 16-day treatment (Fig. 6b).

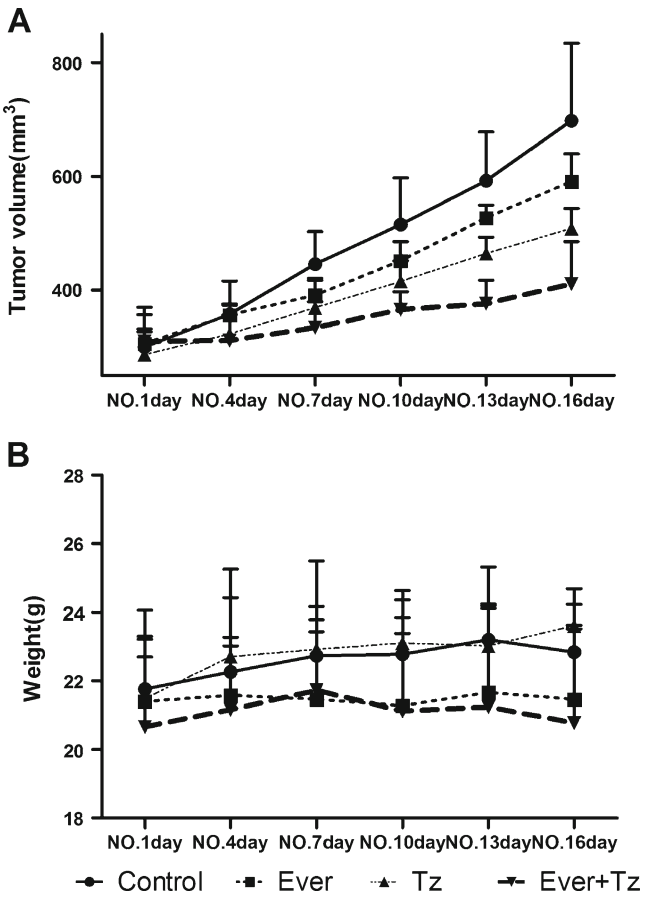

Fig. 6 Stem cells from the BT474 cell line were inoculated in the left inguinal mammary fat pad of syngeneic Balb/c mice, and the mice were divided into four groups. Animals were treated with normal saline, 2 mg/kg everolimus, 5 mg/kg trastuzumab, or 2 mg/kg everolimus + 5 mg/kg trastuzumab. The tumor sizes were measured every 3 days with a caliper, and the tumor volumes were calculated with the equation $V = (L \times W^2) \times 0.5$, in which L is the length and W is the width of the tumor. **a** Effects of the different treatments on tumor volume. **b** Effects of different treatments on mouse weight. Each point represents the mean ± SD ($n=5$)

Effect of everolimus and trastuzumab alone or in combination on the expression of Ki67, CD31, AKT1, and phospho-AKT (Thr308)

The expression of Ki67, CD31, AKT1, and phospho-AKT (Thr308) in the xenograft tumor tissues was detected by immunohistochemistry after the different drug treatments. We found that treatment with everolimus alone caused the upregulation of AKT1 and phospho-AKT (Thr308) ($P=0.024$ and $P=0.041$, respectively) and the downregulation of Ki67 ($P=0.015$) and CD31. However, the downregulation of CD31 expression was not statistically significant ($P>0.05$).

Compared with the control group, trastuzumab alone did not change the number of Ki67-positive cells ($P>0.05$), but it significantly reduced the proportions of the cells with positive expression of AKT1 or phospho-AKT (Thr308) ($P=0.006$ or $P<0.001$, respectively). Similar to everolimus, trastuzumab did not have a significant effect on CD31 [Fig. 7 (3)].

Trastuzumab alone reduced the number of Ki67-positive cells by 6.4 %, although this reduction was not significant, while the Ever + Tz combinatorial treatment significantly reduced Ki67 expression by 21.6 % compared to the control or trastuzumab group ($P=0.004$ or $P=0.03$; Fig. 7a). However, neither the individual drugs nor the two-drug combination significantly changed the percentage of CD31-positive cells (Fig. 7b). The study on AKT1 expression found that combination of the two drugs reversed the upregulation of AKT1 by everolimus alone and significantly decreased the number of AKT1-positive cells by 12.83 % ($P=0.044$; Fig. 7c). Similar to the effect on AKT1 expression, everolimus alone increased phospho-AKT (Thr308) expression level by 11.7 % compared to the control group, while the combinatorial treatment decreased phospho-AKT (Thr308) expression ($P=0.026$; Fig. 7d).

Discussion

Only a limited number of studies have directly reported on breast CSCs. This study is the first report on HER2-positive breast CSCs, which emphasizes the originality of the study. This study aimed to determine the effect of the combinatorial treatment of everolimus and trastuzumab on HER-positive breast CSCs.

This study first examined the effect of the anti-HER2 monoclonal antibody trastuzumab on total HER2-positive breast cancer cells and the isolated CSCs, and it was found that trastuzumab had an inhibitory effect on breast CSCs.

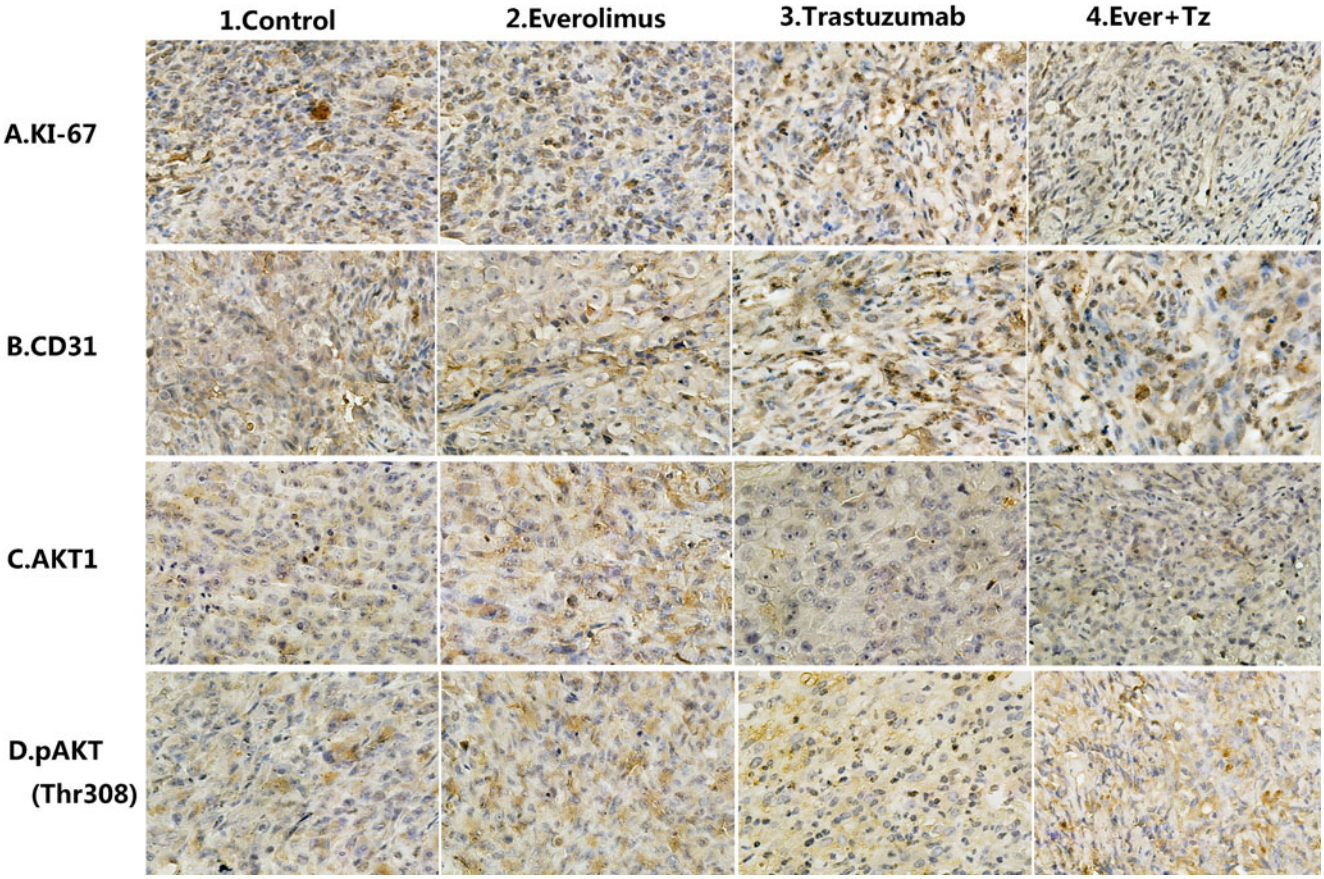

Fig. 7 Ki67, CD31, AKT1, and phospho-Akt (Thr308) expression in breast cancer BT474 stem cells xenograft tumors. **a** Ki67 expression. **b** CD31 expression. **c** AKT1 expression. **d** Phospho-AKT (Thr308) expression. *1* Control: normal saline; *2* 2 mg/kg everolimus; *3* 5 mg/kg trastuzumab; *4* 2 mg/kg everolimus + 5 mg/kg trastuzumab. Nuclei were counterstained with hematoxylin (*blue*) (magnification, ×400)

The study by Magnifico et al. demonstrated that HER2-positive breast cancer cell lines had a higher percentage of breast CSCs and could be effectively inhibited by trastuzumab[17]. The mechanism underlying the inhibitory effect of trastuzumab on HER2-positive stem cells has not been fully understood, although a widely accepted hypothesis is that it is related to Fcγ receptor III-mediated antibody dependent cytotoxicity [17]. In this study, through examination of the cell cycle, apoptosis and the protein expression levels of CD31, Ki67, AKT1, and phospho-AKT (Thr308), we found that, similar to its effect on total cells [18], trastuzumab induced cell cycle arrest of HER2-positive breast CSCs at G0–G1 phase. Trastuzumab also induced early stem cell apoptosis, which is similar to its effects on total cells or patient tissues [19]. The study on the expression of Ki67 and CD31 found that trastuzumab alone had no effect on the protein expression of Ki67 and CD31 in HER2-positive stem cells, which is consistent with the results from previous study that trastuzumab displayed no effects on Ki67 and CD31 expression in total cells [20]. In this study, we found that breast CSCs showed a certain degree of drug resistance against conventional therapeutic doses of trastuzumab. The IC_{50} of trastuzumab for BT474 total cells was 2.25 μg/ml, while the concentration had to be 49 μg/ml to reach 50 % inhibition of cell growth for the stem cells, which was 19 times the IC_{50} for total cells. This drug resistance was also found in primary HER2-positive breast cancer cells. These results may be related to the mechanism underlying the drug resistance of breast CSCs. Recent studies have shown that CSCs can pump out chemotherapy drugs because of their high expression of ABCG2 and other ABC transporters, which has been considered the mechanism for chemotherapy resistance [21]. Liu et al. differentially compared gene expression between $ESA^{+}CD44^{+}CD24^{-/low}Lin^{-}$ breast CSCs and normal breast epithelial cells and identified 186 genes with significant differences in expression, which were characterized as an invasive gene signature (IGS) [22]. The state of the IGS is a significant reference for distant metastasis, disease-free survival, and overall survival of breast cancer patients.

The close relationship between dysregulation of the mTOR kinase pathway and tumorigenesis makes mTOR an important target for cancer therapy. Everolimus exerts its antitumor function to a certain extent as an mTOR

inhibitor [23]. We showed in this study that the inhibitory effect of everolimus on the growth of HER2-positive breast CSCs was enhanced in a dose-dependent manner. In addition, in vitro colony formation experiments showed that everolimus reduced the in vitro tumorigenicity of this type of breast CSCs. The mechanistic studies showed that, as a central regulator for protein synthesis at the G1 phase of a cell cycle, mTOR not only plays major roles in the growth and proliferation of normal cells but is also closely related to the transformation of normal cells to tumor cells and tumor cell growth and proliferation. Therefore, mTOR is one of the central cell growth regulators [10, 24]. Previous studies also confirmed that, as an mTOR inhibitor, everolimus could induce cell cycle arrest of HER2-positive breast cancer cell lines at G1 phase [25] and early apoptosis of breast cancer cells [26]. The in vivo experiments found that treatment with everolimus inhibited the growth of xenograft tumors formed by BT474 stem cells and significantly decreased Ki67 expression. Similar results were reported in the study by Mosley et al. [27], in which rapamycin inhibited tumor growth in MMTV/Ne transgenic mice that overexpressed an HER2 homolog. We studied the effects of everolimus on AKT1 and pAKT and found that everolimus induced the increased expression of AKT1 and pAKT in the xenograft tumors formed by stem cells. The possible mechanism underlying this effect can be explained by a negative feedback effect, in which the specific inhibitory effect of everolimus on mTOR, downstream of the PI3K/AKT pathway, could activate a negative feedback to induce the overexpression of AKT in xenograft tumor tissues. A number of previous studies have reported this negative feedback mechanism of mTOR inhibitors on AKT [28–30], in a phase I clinical trial in which everolimus induced the overexpression of pAKT in the breast cancer tissues of patients after oral dosing of everolimus for 4 weeks [28]. In cancer therapy, the activation of AKT is considered to be an adverse factor [31], and thus, the anti-tumor effect of everolimus is compromising because of its induction of pAKT activation. These results also encourage the search for new combination therapy to improve the inhibitory effect of everolimus on cancer cells [28].

The preliminary tests showed that both trastuzumab and everolimus have some limitations in targeting breast CSCs. Breast CSCs exhibited drug resistance to the treatment with trastuzumab, and trastuzumab treatment did not change the expression of the cell proliferation regulator Ki67 in stem cells, which indicated that trastuzumab did not affect the proliferation of tumor cells in vivo, consistent with previous findings [20]. In everolimus treatment, everolimus induced the overexpression of pAKT and AKT1, which is detrimental to cancer treatment. Moreover, the targets of the two drugs are related. On the one hand, mTOR inhibitors have certain inhibitory effects on drug resistance to trastuzumab treatment. Through the dysfunction of the tumor suppressor PTEN or activation by other signals, the PI3K/AKT/mTOR signal transduction pathway inhibits apoptosis and promotes cell cycle progression, which leads to cell proliferation, angiogenesis, and malignant tumor invasion and metastasis, while the dysfunction of the tumor suppressor gene PTEN is a known regulatory factor for trastuzumab resistance [32]. On the other hand, the application of anti-HER2 antibodies can also affect the expression of mTOR. phosphatidylinositol-3-hydroxy kinase (PI3K) is an important signaling transducer of tyrosine kinase receptors such as HER2. After being stimulated by growth factors, HER2 in the cell membrane activates intracellular PI3K to phosphorylate its substrate PIP2 into PIP3. AKT (also called protein kinase B; PKB) binds to PIP3 and is activated by phosphorylation at Thr308 and Ser474 [33]. Activated AKT in turn activates its downstream molecule mTOR. These correlations suggest that combinatorial treatment with mTOR and HER2 as the targets can enhance the inhibitory effect of these drugs on tumor cells to a certain extent.

Recent experiments have confirmed that an appropriate concentration of everolimus can enhance the inhibitory effect of trastuzumab on tumor cells both in vivo and in vitro. By studying the effect of trastuzumab combined with rapamycin, Miller et al. found that the combination of the two drugs was more effective than any of the drugs alone in vivo [20], making all of the 26 HER2-positive metastatic tumors shrink. Moreover, trastuzumab reduced the overexpression of pHER3 and pAKT caused by the negative feedback due to everolimus. The effect of the combination of these two drugs was also confirmed in clinical studies. In a multicenter phase I clinical study on HER2-positive trastuzumab-resistant metastatic breast cancer [34], the combination of these two drugs with different specific targets showed a synergistic effect with good tolerance among the patients. Similar to these results, we found that the combination of these two drugs was more effective at inhibiting the growth of breast CSCs and in vitro tumorigenicity. In addition, compared with everolimus and trastuzumab alone, combined Ever + Tz significantly increased the percentage of BT474 stem cells at G0–G1 phase. Furthermore, the combination of these two drugs showed a greatly enhanced capability of inducing early apoptosis of stem cells. In the in vivo experiments, compared to individual drugs, the combination of these two drugs was more effective at inhibiting the growth of the breast CSC xenograft tumors and reducing the expression of Ki67, AKT1, and pAKT. Compared with previous results [20], we used the stem cells as a more targeted study of trastuzumab resistance. Combined with preliminary clinical studies, these data showed that the combination of the mTOR blocker everolimus and the targeted therapeutic drug trastuzumab may become a new therapeutic option for patients with HER2-positive trastuzumab-resistant metastatic breast cancer.

In summary, breast CSCs were sorted using flow cytometry for the experiments in this study. The mTOR antagonist everolimus showed an inhibitory effect on CSCs and effectively enhanced the sensitivity of HER2-positive breast CSCs to trastuzumab and affected the proliferation, adhesion, and metastasis of breast CSCs to prevent tumor recurrence and metastasis. This study provides a theoretical basis for the combination of everolimus with other target-specific treatments. However, the specific underlying antitumor mechanism needs to be further investigated.

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Conflicts of interest None

References

1. Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nat Clin Pract Oncol. 2006;3(5):269–80.
2. Hudis CA. Trastuzumab–mechanism of action and use in clinical practice. N Engl J Med. 2007;357(1):39–51.
3. Xu Y, Sun Q. Headway in resistance to endocrine therapy in breast cancer. J Thorac Dis. 2010;2(3):171–7.
4. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, Gianni L, Baselga J, Bell R, Jackisch C, Cameron D, Dowsett M, Barrios CH, Steger G, Huang CS, Andersson M, Inbar M, Lichinitser M, Láng I, Nitz U, Iwata H, Thomssen C, Lohrisch C, Suter TM, Rüschoff J, Suto T, Greatorex V, Ward C, Straehle C, McFadden E, Dolci MS, Gelber RD, Herceptin Adjuvant (HERA) Trial Study Team. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005;353(16):1659–72.
5. Lv YG, Yu F, Yao Q, Chen JH, Wang L. The role of survivin in diagnosis, prognosis and treatment of breast cancer. J Thorac Dis. 2010;2(2):100–10.
6. Sabatini DM. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer. 2006;6(9):729–34.
7. Schmelzle T, Hall MN. TOR, a central controller of cell growth. Cell. 2000;103(2):253–62.
8. Baldo P, Cecco S, Giacomin E, Lazzarini R, Ros B, Marastoni S. mTOR pathway and mTOR inhibitors as agents for cancer therapy. Curr Cancer Drug Targets. 2008;8(8):647–65.
9. Rowinsky EK. Targeting the molecular target of rapamycin (mTOR). Curr Opin Oncol. 2004;16(6):564–75.
10. Gera JF, Mellinghoff IK, Shi Y, Rettig MB, Tran C, Hsu JH, Sawyers CL, Lichtenstein AK. AKT activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-myc expression. J Biol Chem. 2004;279(4):2737–46.
11. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003;100(7):3983–8.
12. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature. 2007;445(7123):106–10.
13. Shafee N, Smith CR, Wei S, Kim Y, Mills GB, Hortobagyi GN, Stanbridge EJ, Lee EY. Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. Cancer Res. 2008;68(9):3243–50.
14. Hambardzumyan D, Squatrito M, Holland EC. Radiation resistance and stem-like cells in brain tumors. Cancer Cell. 2006;10(6):454–6.
15. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, Song E. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell. 2007;131(6):1109–23.
16. Setoguchi T, Taga T, Kondo T. Cancer stem cells persist in many cancer cell lines. Cell Cycle. 2004;3(4):414–5.
17. Magnifico A, Albano L, Campaner S, Delia D, Castiglioni F, Gasparini P, Sozzi G, Fontanella E, Menard S, Tagliabue E. Tumor-initiating cells of HER2-positive carcinoma cell lines express the highest oncoprotein levels and are sensitive to trastuzumab. Clin Cancer Res. 2009;15(6):2010–21.
18. Gijsen M, King P, Perera T, Parker PJ, Harris AL, Larijani B, Kong A. HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 herceptin in breast cancer. PLoS Biol. 2010;8(12):e1000563.
19. Shah C, Miller TW, Wyatt SK, McKinley ET, Olivares MG, Sanchez V, Nolting DD, Buck JR, Zhao P, Ansari MS, Baldwin RM, Gore JC, Schiff R, Arteaga CL, Manning HC. Imaging biomarkers predict response to anti-HER2 (ErbB2) therapy in preclinical models of breast cancer. Clin Cancer Res. 2009;15(14):4712–21.
20. Miller TW, Forbes JT, Shah C, Wyatt SK, Manning HC, Olivares MG, Sanchez V, Dugger TC, de Matos Granja N, Narasanna A, Cook RS, Kennedy JP, Lindsley CW, Arteaga CL. Inhibition of mammalian target of rapamycin is required for optimal antitumor effect of HER2 inhibitors against HER2-overexpressing cancer cells. Clin Cancer Res. 2009;15(23):7266–76.
21. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414(6859):105–11.
22. Liu R, Wang X, Chen GY, Dalerba P, Gurney A, Hoey T, Sherlock G, Lewicki J, Shedden K, Clarke MF. The prognostic role of a gene signature from tumorigenic breast-cancer cells. N Engl J Med. 2007;356(3):217–26.
23. Xu CX, Li Y, Yue P, Owonikoko TK, Ramalingam SS, Khuri FR, Sun SY. The combination of RAD001 and NVP-BEZ235 exerts synergistic anticancer activity against non-small cell lung cancer in vitro and in vivo. PLoS One. 2011;6(6):e20899.
24. Advani SH. Targeting mTOR pathway: a new concept in cancer therapy. Indian J Med Paediatr Oncol. 2010;31(4):132–6.
25. Noh WC, Mondesire WH, Peng J, Jian W, Zhang H, Dong J, Mills GB, Hung MC, Meric-Bernstam F. Determinants of rapamycin sensitivity in breast cancer cells. Clin Cancer Res. 2004;10(3):1013–23.
26. Dragowska WH, Weppler SA, Qadir MA, Wong LY, Franssen Y, Baker JH, Kapanen AI, Kierkels GJ, Masin D, Minchinton AI, Gelmon KA, Bally MB. The combination of gefitinib and RAD001 inhibits growth of HER2 overexpressing breast cancer cells and tumors irrespective of trastuzumab sensitivity. BMC Cancer. 2011;11:420.
27. Mosley JD, Poirier JT, Seachrist DD, Landis MD, Keri RA. Rapamycin inhibits multiple stages of c-Neu/ErbB2 induced tumor progression in a transgenic mouse model of HER2-positive breast cancer. Mol Cancer Ther. 2007;6(8):2188–97.
28. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J, Rosen N. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res. 2006;66(3):1500–8.

29. Wan X, Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. Oncogene. 2007;26(13):1932–40.
30. Okuzumi T, Fiedler D, Zhang C, Gray DC, Aizenstein B, Hoffman R, Shokat KM. Inhibitor hijacking of Akt activation. Nat Chem Biol. 2009;5(7):484–93.
31. Papademetriou K, Ardavanis A, Kountourakis P. Neoadjuvant therapy for locally advanced breast cancer: focus on chemotherapy and biological targeted treatments' armamentarium. J Thorac Dis. 2010;2(3):160–70.
32. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, Linn SC, Gonzalez-Angulo AM, Stemke-Hale K, Hauptmann M, Beijersbergen RL, Mills GB, van de Vijver MJ, Bernards R. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell. 2007;12(4):395–402.
33. Hill MM, Hemmings BA. Inhibition of protein kinase B/Akt. implications for cancer therapy. Pharmacol Ther. 2002;93(2–3):243–51.
34. Andre F, Campone M, O'Regan R, Manlius C, Massacesi C, Sahmoud T, Mukhopadhyay P, Soria JC, Naughton M, Hurvitz SA. Phase I study of everolimus plus weekly paclitaxel and trastuzumab in patients with metastatic breast cancer pretreated with trastuzumab. J Clin Oncol. 2010;28(34):5110–5.