



Original Article

Inhibitory effects of everolimus in combination with paclitaxel on adriamycin-resistant breast cancer cell line MDA-MB-231

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ABSTRACT

Objective: We aimed to evaluate the therapeutic effects of paclitaxel in combination with mTOR inhibitor everolimus on adriamycin-resistant breast cancer cell line MDA-MB-231 (MDA-MB-231/ADR).**Materials and methods:** MDA-MB-231/ADR cells were treated with different concentrations of paclitaxel and everolimus. The IC₅₀ values after 48 h of treatment were measured by the MTT assay. The apoptosis rate and cell cycle were detected by flow cytometry. The protein expressions of Akt, PI3K, mTOR, p-PI3K, p-AKT and p-mTOR were detected by Western blot.**Results:** When paclitaxel at $\geq 1.56 \mu\text{g/ml}$ was used, the growth of MDA-MB-231/ADR cells was inhibited more significantly than that of control group ($P < 0.05$). After treatment with $\geq 6.25 \mu\text{g/ml}$ everolimus, the cell growth was also suppressed more significantly ($P < 0.05$). The IC₅₀ values of everolimus and paclitaxel were $32.50 \mu\text{g/ml}$ and $7.80 \mu\text{g/ml}$, respectively. The inhibition rate of paclitaxel plus everolimus was significantly enhanced with increasing paclitaxel concentration ($P < 0.001$). After treatment with $7.80 \mu\text{g/ml}$ paclitaxel, the two drugs had best synergistic inhibitory effects on proliferation. Compared with drugs alone, the combination significantly promoted apoptosis ($P < 0.001$). The paclitaxel + everolimus group had significantly more cells in the G0-G1 phase than those of control and individual drug groups ($P < 0.001$). Everolimus significantly decreased mTOR and p-mTOR expressions compared with those of control group ($P < 0.001$). Compared with everolimus alone, the combination reduced the expressions more significantly ($P < 0.05$). Paclitaxel decreased the expression levels of PI3K, p-PI3K and p-AKT. Compared with paclitaxel alone, the combination significantly promoted the reduction of PI3K, p-PI3K and p-AKT expressions ($P < 0.05$).**Conclusion:** Everolimus can enhance the effect of paclitaxel on MDA-MB-231/ADR cells, inhibit cell proliferation, induce apoptosis and arrest cell cycle in the G1 phase mainly by down-regulating the expressions of key proteins in the mTOR signaling pathway.© 2020 Taiwan Association of Obstetrics & Gynecology. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Breast cancer is one of the most malignant tumors that affect women's health, with the incidence rate increasing annually. There are about 1.4 million new cases worldwide each year [1]. Breast cancer remains the leading cause for female deaths at present [2]. Currently, breast cancer is mainly treated by chemotherapy, radiotherapy, surgery, endocrine therapy and traditional medicine. It is well-established that chemotherapy plays an important role in the treatment of breast cancer. Preoperative or postoperative

chemotherapy can effectively relieve the symptoms of patients [3]. However, the multi-drug resistance of breast cancer cells to chemotherapeutic drugs often causes failure in clinical practice.

Adriamycin is a broad-spectrum anticancer drug that can intercalate DNA double strands to form stable complexes, thereby inhibiting DNA replication and RNA synthesis [4]. Nowadays, the mortality rate of breast cancer patient has been significantly reduced due to development of early detection methods and new drugs [5]. The therapeutic effect of adriamycin on breast cancer is evidently affected by resistance. Adriamycin often induces adverse reactions such as phlebitis, nausea, hyperpyrexia, hair loss and vomiting, especially cardiotoxicity at high doses, so its clinical use is largely limited.

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Kovalchuk et al. and Filkowski et al. reported that the PTEN protein expressions in adriamycin-resistant (ADR) breast cancer cell lines significantly reduced [6,7]. PTEN is the negative regulator at the upstream of mammalian target of rapamycin (mTOR). When PTEN is deleted, PI3K protein and AKT activity increase, which can up-regulate mTOR expression, facilitate tumor cell proliferation and inhibit apoptosis, indicating that the mTOR signaling pathway is activated upon drug resistance during breast cancer treatment. Therefore, studies on the mTOR signaling pathway and drugs targeting this pathway are crucial for the selection of other drugs after drug resistance.

Everolimus is an oral immunosuppressive agent and mTOR-specific inhibitor that suppresses cell growth, proliferation and G1-S phase transition as well as induces apoptosis by blocking the mTOR signaling pathway [8]. In 2012, everolimus was approved by FDA to be combined with exemestane to treat hormone receptor-positive human epidermal growth factor receptor type 2-negative breast cancer (HR+, HER2-) [9]. As a tetracyclic diterpenoid, paclitaxel is one of the most effective drugs for treating various solid tumors [10]. It induces apoptosis by interfering with cancer cell tubulin, terminating mitosis and interrupting cell growth to promote atrophy. Accumulating evidence has verified that paclitaxel combined with rapamycin exerted stronger inhibitory effects on cell proliferation than paclitaxel alone, and they worked synergistically by regulating the mTOR pathway [11].

Based on the close relationship between mTOR and tumor cell apoptosis and drug resistance, we here intended to explore whether everolimus enhanced the effect of paclitaxel on MDA-MB-231/ADR cell line, and to provide a potential therapy for overcoming breast cancer resistance.

Materials and methods

Cells and reagents

Human MDA-MB-231/ADR cell line was purchased from Shanghai Institute of Cell Research, Chinese Academy of Sciences (China). MTT assay kit, annexin V-FITC apoptosis detection kit, cell cycle and apoptosis detection kit, BCA protein quantification kit and ECL reagent were all bought from Beyotime Institute of Biotechnology Co., Ltd. (China). Adriamycin, paclitaxel and everolimus were obtained from Dalian Meilun Biotechnology Co., Ltd. (China).

Cell culture

MDA-MB-231/ADR cells were inoculated into 8–10 ml of complete medium containing 90% RPMI1640 medium and 10% fetal bovine serum (FBS) and cultured in a 37 °C incubator with 5% CO₂.

Measurement of IC₅₀ values by MTT assay

A single cell suspension was prepared using complete medium containing RPMI 1640 medium and 10% FBS. Then the cells were seeded into 96-well plates at the density of 5000–10,000/well and cultured overnight. After adhesion, the cells were treated with 10 µl of different concentrations of drugs (paclitaxel: 1.56 µg/ml, 3.12 µg/ml, 6.25 µg/ml, 12.50 µg/ml, 25.00 µg/ml, 50.00 µg/ml, 100.00 µg/ml; everolimus: 1.56 µg/ml, 3.12 µg/ml, 6.25 µg/ml, 12.50 µg/ml, 25.00 µg/ml, 50.00 µg/ml, 100.00 µg/ml). The experiment for each drug at each concentration was performed four times. The wells without drugs were used as controls, and those without cells or drugs were used as blank controls. The cells were incubated for 2–3 days at 37 °C with 5% CO₂. After culture, 20 µl of MTT solution was added to each well for 4 h of incubation, and then the supernatant was discarded. Subsequently, 150 µl of DMSO was added to each

well, and the mixture was incubated for 10 min to dissolve crystals. The optical density of each well was measured with a microplate reader at 570 nm, and the cell growth curve was plotted by software. The IC₅₀ values of chemotherapeutic drugs against MDA-MB-231/ADR cells were calculated.

Analysis of combined treatment outcomes

Everolimus and paclitaxel were combined at various proportions. The cells were treated with a combination of everolimus at its IC₅₀ and paclitaxel at 1/64, 1/32, 1/16, 1/8, 1/4, 1/2 and 1 of IC₅₀, and the inhibition rate was calculated. The combination index (CI) was calculated by Compu Syn software (Combo Syn Inc., USA) using the Chou-Talalay method. CI values of >1.1, 0.9–1.1 and < 0.9 corresponded to antagonistic, additive and synergistic effects, respectively [12].

Experimental grouping

MDA-MB-231/ADR cells in the logarithmic growth phase were seeded into 6-well plates at the density of 2×10^5 /well and cultured at 37 °C with 5% CO₂. After adherence, the cells were added 2 ml of medium containing IC₅₀ values of everolimus and paclitaxel or 7.80 µg/ml paclitaxel in combination with 32.50 µg/ml everolimus, cultured for 48 h, and collected for subsequent experiments.

Detection of cell apoptosis by flow cytometry

The cell culture medium was transferred to a 15 ml centrifuge tube. The adherent cells were washed twice with PBS and digested with 1 ml of trypsin containing EDTA at room temperature for 2 min. Digestion was terminated by adding 2–4 ml of complete culture medium. The cell culture medium was centrifuged at 1000×g for 5 min to discard the supernatant. Then the cells were collected, resuspended in PBS and counted. The resuspended cells were centrifuged at 1000×g for 5 min to discard the supernatant, added 195 µl of annexin V-FITC buffer for resuspension, mixed with 5 µl of annexin V-FITC solution, and added 10 µl of propidium iodide (PI) staining solution. The cells were incubated for 15 min at room temperature in dark, during which they were resuspended twice. Afterwards, they were placed in an ice bath. This experiment was repeated three times.

Detection of cell cycle by flow cytometry

The cell culture medium was collected into a 15 ml centrifuge tube prior to use. The cells were digested with trypsin as mentioned above. The culture medium was thereafter added to a 15 ml centrifuge tube and centrifuged at 1000×g for 5 min to discard the supernatant. The cells were resuspended with 1 ml of pre-cooled PBS, placed in a 1.5 ml centrifuge tube, and centrifuged at 1000×g for 5 min to discard the supernatant. Then the cells were added 1 ml of pre-cooled 70% ethanol, mixed by pipetting, fixed at 24 °C for 24 h, and centrifuged for 5 min at about 1000×g for precipitation. After the supernatant was discarded, the cells were resuspended with 1 ml of pre-cooled PBS. Subsequently, 0.5 ml of PI staining solution was added to each tube, and the cell precipitate was resuspended, placed in a water bath at 37 °C for 30 min in dark and then stored at 4 °C in dark. Flow cytometry was completed within 24 h after staining. The red fluorescence was detected at the excitation wavelength of 488 nm. The distribution of cell cycle was determined using software in the system. The test was repeated three times and the results were averaged.

Detection of protein expression changes by Western blot

The cells were digested with trypsin, centrifuged, diluted into a cell suspension, inoculated into a six-well plate at 1×10^5 /well and cultured in a 37 °C incubator with 5% CO₂. After adherence, drugs were added. DMSO and blank group were used as control groups. After 30 min of culture, the supernatant was removed, and the residue was washed twice with pre-cooled PBS, added an appropriate amount of RIPA lysate, lysed for 30 min, centrifuged at 12,000/min for 4 min at 4 °C and collected. After protein concentration was quantified by BCA kit, 5% SDS-PAGE was conducted. Afterwards, the product was transferred onto a PVDF membrane, blocked with 5% skim milk for 2 h, incubated with primary antibodies overnight at 4 °C, washed by TBST, incubated with HRP-labeled goat anti-rabbit IgG antibody for 2 h at 37 °C, and developed using ECL reagent. Images were collected by an automatic gel imaging system. GAPDH was used as the internal reference to detect protein levels.

Statistical analysis

All data were analyzed by SPSS16.0 software. The categorical data conforming to normal distribution were represented as mean \pm standard deviation. Intergroup comparisons were performed by the independent t test. $P < 0.05$ was considered statistically significant.

Results

Effects of paclitaxel and everolimus alone on MDA-MB-231/ADR cell proliferation

The MTT assay showed that after treatment with paclitaxel and everolimus alone, the proliferation of MDA-MB-231/ADR cells was evidently inhibited dose-dependently compared with that of the control group (Fig. 1). When paclitaxel at ≥ 1.56 μ g/ml was used, the growth of MDA-MB-231/ADR cells was inhibited more significantly than that of the control group ($P < 0.05$). After treatment with ≥ 6.25 μ g/ml everolimus, the cell growth was also suppressed more

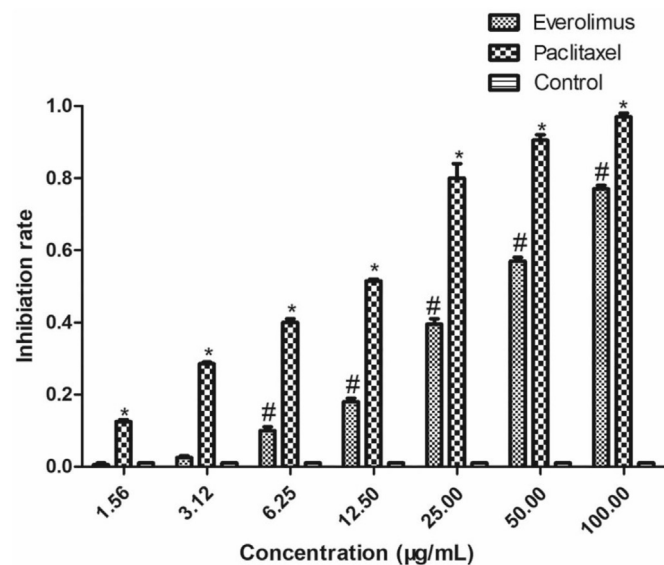


Fig. 1. Inhibitory effects of paclitaxel and everolimus alone on MDA-MB-231/ADR cell proliferation. $x \pm s$, $n = 6$. *,#Compared with Control, $P < 0.05$.

significantly ($P < 0.05$). The IC₅₀ values of everolimus and paclitaxel were 32.50 μ g/ml and 7.80 μ g/ml, respectively.

Therapeutic effects of paclitaxel in combination with everolimus

As summarized in Table 1 and Fig. 2, the CI values are all lower than 0.9 after treatment by 0.13–7.80 μ g/ml paclitaxel in combination with 32.50 μ g/ml everolimus, indicating that the two drugs synergistically suppressed MDA-MB-231/ADR cell proliferation. The synergistic effect was attenuated with decreasing paclitaxel dose. The inhibition rate of paclitaxel plus everolimus was significantly enhanced with increasing paclitaxel concentration ($P < 0.001$). After treatment with 7.80 μ g/ml paclitaxel, the two drugs had the best synergistic inhibitory effects on cell proliferation.

Effects of paclitaxel and everolimus alone or in combination on MDA-MB-231/ADR cell apoptosis

Compared with drugs alone, the combination significantly promoted apoptosis ($P < 0.001$) (Fig. 3).

Effects of paclitaxel and everolimus alone or in combination on MDA-MB-231/ADR cell cycle

The paclitaxel + everolimus group had significantly more cells in the G₀-G₁ phase than those of control and individual drug groups ($P < 0.001$) (Fig. 4). Thus, paclitaxel in combination with everolimus mainly arrested the cell cycle in the G₀-G₁ phase.

Effects of paclitaxel and everolimus alone or in combination on expressions of proteins related to the PI3K/AKT/mTOR signaling pathway

Everolimus significantly decreased the expressions of mTOR and p-mTOR compared with those of the control group ($P < 0.001$) (Fig. 5). Compared with everolimus alone, the combination reduced the expressions of mTOR and p-mTOR more significantly ($P < 0.05$). Paclitaxel decreased the expression levels of PI3K, p-PI3K and p-AKT. Compared with paclitaxel alone, the combination significantly promoted the reduction of PI3K, p-PI3K and p-AKT expressions ($P < 0.05$).

Discussion

Breast cancer is the most common invasive cancer and the second leading cause of death among women worldwide. Many chemotherapy drugs, including hormones, cytotoxic drugs and immunosuppressive agents, have been used for neoadjuvant chemotherapy and adjuvant chemotherapy. Initially, they were effective in treating most early breast cancer patients [13]. However, resistance develops over time, leading to unsatisfactory or failed chemotherapy. Adriamycin is a broad-spectrum anticancer

Table 1
Therapeutic effects of paclitaxel in combination with everolimus.

Paclitaxel (μ g/mL)	Everolimus (μ g/mL)	CI
0.13	32.50	0.86 ± 0.03
0.25	32.50	0.79 ± 0.05
0.49	32.50	0.71 ± 0.03
0.98	32.50	0.62 ± 0.04
1.95	32.50	0.51 ± 0.05
3.90	32.50	0.41 ± 0.04
7.80	32.50	0.35 ± 0.05

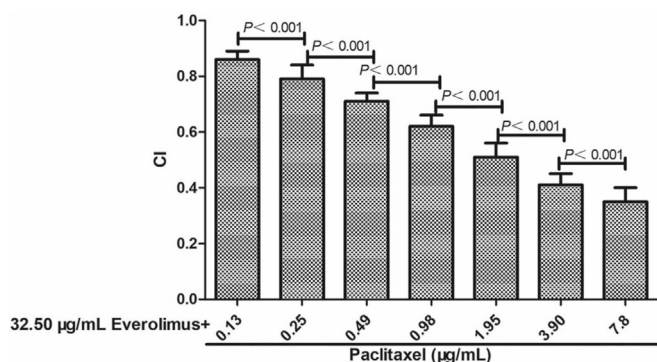


Fig. 2. Therapeutic effects of paclitaxel in combination with everolimus.

drug that has been widely used clinically. Long-term use of adriamycin, many patients will develop resistance and lead to treatment failure. Drug resistance is caused by the activation of the mTOR signaling pathway [14]. Everolimus, an inhibitor of mTOR, is an aromatase inhibitor and the first drug approved by the FDA for the treatment of advanced postmenopausal breast cancer. This study evaluated the anti-proliferative effects of everolimus and paclitaxel on MDA-MB-231/ADR cells and its related molecular mechanism. Everolimus enhanced the inhibitory effects of paclitaxel on cell proliferation. In other words, these two drugs showed synergistic inhibitory effects on MDA-MB-231/ADR cells.

When the cells were treated with different concentrations of paclitaxel and everolimus, the CI values were all lower than 0.9, indicating that the two drugs had synergistic inhibitory effects on MDA-MB-231/ADR cell proliferation. The inhibitory rate of paclitaxel combined with everolimus increased with rising paclitaxel dose. When the concentration of paclitaxel reached 7.80 µg/ml, the two drugs had the best synergistic effect on cell proliferation inhibition. Meanwhile, the synergistic effect was dose-dependent.

The cell cycle refers to the cycle of eukaryotic cells that can continue to divide from the end of one mitosis to the end of the next division [15]. Herein, the number of G0-G1 phase cells in the paclitaxel plus everolimus group increased significantly, which was significantly higher than those control and two individual drug groups ($P < 0.001$). Therefore, the two drugs synergistically blocked the cell cycle, thereby inhibiting cell growth and proliferation. We postulated that changes in the cell cycle distribution can be attributed to those in cell properties, but further studies are still needed to explore the underlying mechanisms.

Apoptosis plays a key role in the onset and progression of tumors [16]. In this study, flow cytometry was used to detect the effect of everolimus and paclitaxel alone or in combination on the apoptosis rate of MDA-MB-231/ADR cells. Treatment with everolimus or paclitaxel alone significantly induced MDA-MB-231/ADR cell apoptosis. Compared with the single drug alone, the combined group significantly increased the apoptotic rate, and the difference was statistically significant compared with the single drug group. Western blot showed that paclitaxel can down-regulate the expression of PI3K and inhibit the phosphorylation of PI3K and AKT. As an mTOR inhibitor, everolimus treatment significantly down-regulated the expression levels of various proteins and their phosphorylation in the mTOR signaling pathway. Compared with everolimus alone, when the two drugs were used in combination, except for the AKT protein, the levels of other proteins were significantly down-regulated, and the difference was statistically significant. Herein, the level of mTOR expression in everolimus was reduced, suggesting that everolimus can reverse the effects of paclitaxel. Consistently, Beeram et al. found that fulvestrant reversed AKT-mediated antagonism and anti-estrogen treatment of reduction [17]. De Graffenried reported that inhibiting mTOR restored the activity of tamoxifen towards abnormal AKT in breast cancer cells [18]. Hurvitz et al. evaluated the therapeutic effects and safety of combining trastuzumab and paclitaxel with everolimus as the first-line treatment for patients with HER2+ advanced breast cancer. They found that although all groups had similar

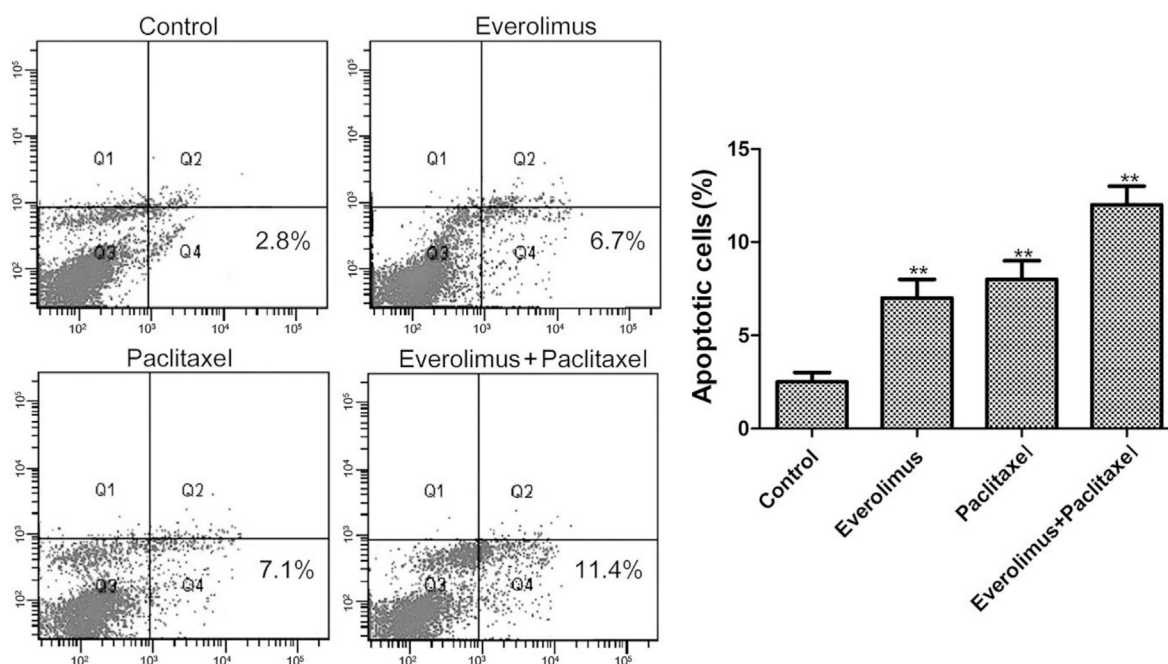


Fig. 3. Effects of paclitaxel and everolimus alone or in combination on MDA-MB-231/ADR cell apoptosis. **Compared with Control, $P < 0.001$.

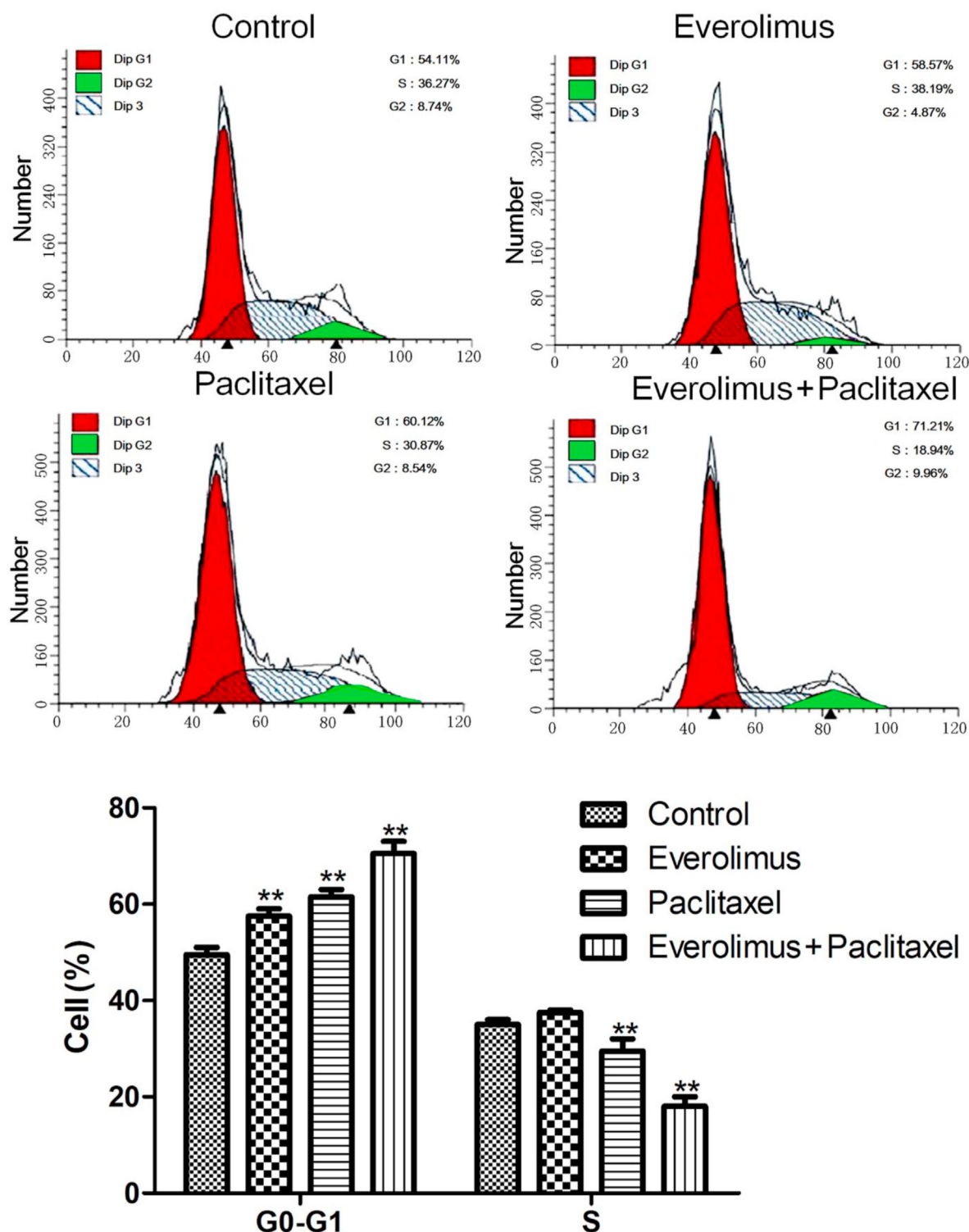


Fig. 4. Effects of paclitaxel and everolimus alone or in combination on MDA-MB-231/ADR cell cycle. **Compared with Control, $P < 0.001$.

progression-free survival, adding everolimus prolonged the survival of the HR-, HER2+ population by (7 ± 2) months [19].

PI3K, an upstream regulator of mTOR, plays a central role in the activation of mTOR and is thought to be synchronized with the behavior of mTOR. Lung adenocarcinoma resistant to cisplatin [20], breast cancer cytotoxic drugs [21] and other chemotherapeutic drugs are associated with increased and overexpressed AKT.

However, the expression levels of AKT, PI3K, and p-PI3K did not change significantly after treatment with everolimus, an mTOR inhibitor. It is well-established that mTOR is regulated by both the PI3K/AKT and RAF/MEK/ERK signaling pathways, and that the interconnected PI3K/AKT/mTOR and Raf/MEK/ERK cascades work synergistically [22,23]. Therefore, we hypothesized that paclitaxel and everolimus exerted synergistic effects on MDA-MB-231/ADR

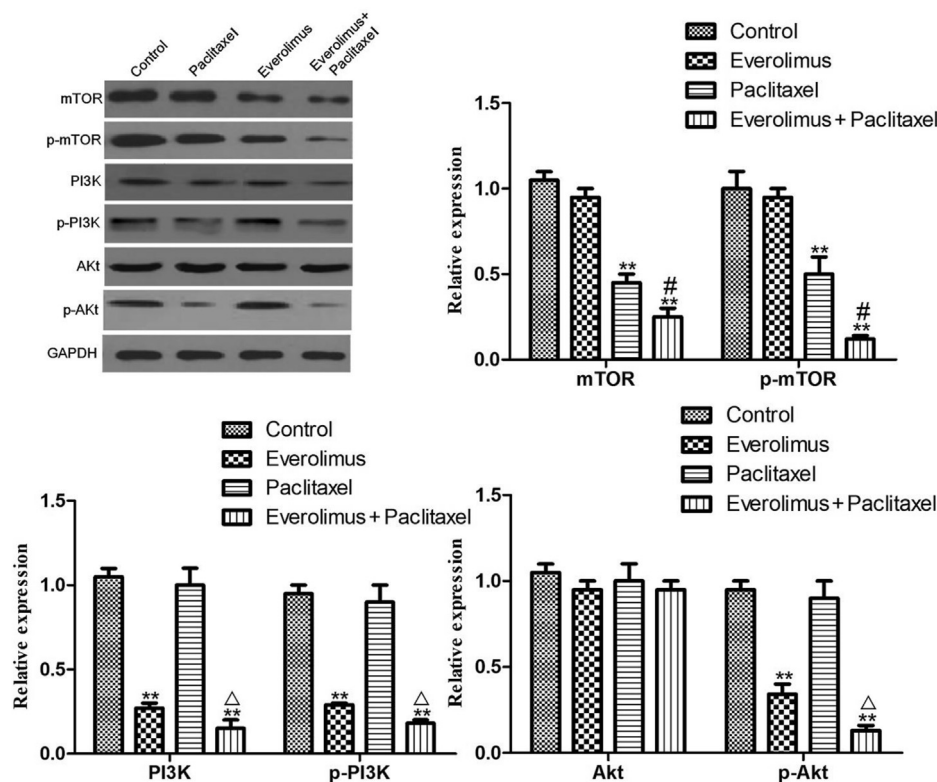


Fig. 5. Effects of paclitaxel and everolimus alone or in combination on expressions of proteins related to the PI3K/AKT/mTOR signaling pathway. **Compared with Control, $P < 0.001$; #compared with paclitaxel group, $P < 0.05$; Δcompared with everolimus group, $P < 0.05$.

cells through regulation via the PI3K/AKT signaling pathway as well as activation of mTOR via the RAF/MEK/ERK pathway. Further in-depth studies regarding the key genes in the RAF/MEK/ERK pathway are ongoing in our group.

In summary, everolimus can enhance the effects of paclitaxel on MDA-MB-231/ADR cells, induce apoptosis and inhibit cell proliferation. These findings further suggest that traditional cytotoxic chemotherapeutic drugs combined with mTOR inhibitors have great potential for the treatment of tumors. The combination of these two drugs may provide a potential method for the treatment of drug-resistant breast cancer women and further clinical trials.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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