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Original Article

Econazole nitrate reversed the resistance of breast cancer cells to Adriamycin through inhibiting the PI3K/AKT signaling pathway

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Abstract: Activation of the phosphoinositide 3 kinase (PI3K)/AKT pathway is frequently implicated in resistance to anticancer therapies. PI3K inhibitors can restore sensitivity to standard breast cancer therapies, including endocrine therapy, HER2-targeted agents, and chemotherapy. Our previous research showed that econazole, a novel PI3Ka inhibitor, inhibits the PI3K/AKT pathway and induces apoptosis in lung cancer cells. In this study, econazole showed significant cytotoxic activity against Adriamycin-resistant breast cancer cells *in vitro* and *in vivo*. Additionally, econazole significantly sensitized MDA-MB-231 and MCF-7 cells to Adriamycin via inhibiting the PI3K/AKT pathway. Overexpression of constitutively active AKT1 abolished the function of econazole. The combination of econazole and Adriamycin exerted synergistic inhibitory effects in breast cancer cells *in vitro* and *in vivo*. Taken together, the PI3K inhibitor econazole could effectively overcome Adriamycin resistance and showed synergistic effects with chemotherapy on breast cancer.

Keywords: Econazole, PI3K, AKT, Adriamycin, drug resistance, breast cancer

Introduction

In recent decades, advances in cytotoxic chemotherapy and targeted therapies have improved survival rates for breast cancer. However, nearly all initially responsive breast tumors eventually develop resistance, leading to therapeutic failure and cancer-related death [1]. The biological causes of drug resistance have been extensively studied and attributed to diverse molecular mechanisms, including reduced drug accumulation, metabolic detoxification, anti-apoptosis, autophagy, and other mechanisms. Breast cancer multidrug resistance (MDR) is associated with P-glycoprotein (P-gp), breast cancer resistance protein, multidrug resistance-associated protein, and apoptosis-related proteins [2]. Recent studies have suggested that the phosphoinositide 3 kinase (PI3K)/AKT pathway is associated with resistance to endocrine therapy, human epidermal growth factor receptor 2 (HER2)-directed therapy, and cytotoxic therapy in breast cancer [3]. When the PI3K/AKT pathway is activated in breast cancer, AKT can phosphorylate multiple substrates, which promote cell proliferation, survival, metastasis, and chemoresistance [4]. The upregulation of AKT can promote the anti-apoptotic protein Bcl-2 and P-gp to induce multidrug resistance [5].

PI3K inhibitors have been successfully used to enhance the sensitivity of breast cancer cells to drug-induced apoptosis [5]. NVP-BKM120, a panclass inhibitor of PI3K, showed significant cytotoxic activity against MDR breast cancer cells and a strong synergistic anti-proliferative effect in combination with doxorubicin [6]. A PI3K inhibitor Alpelisib (BYL719) in combination with letrozole achieved an acceptable safety profile and limited efficacy in endocrine therapy-resistant HR+HER2-metastatic breast cancer in Phase I/II clinical trials [6]. BYL 719 has been approved by the FDA to be marketed in combination with fulvestrant for the treatment of advanced metastatic breast cancer with PIK3CA mutation in postmenopausal females with HR+/HER2-tumors [7]. The addition of the selective PI3K inhibitor to HER-2-targeted therapy could restore sensitivity to trastuzumab and represents a superior treatment strategy [8]. Therefore, the PI3K/AKT pathway has a central role in development of novel strategies to overcome breast cancer drug resistance.

Econazole nitrate, an anti-fungal agent, exhibited cytotoxic activities in cancer cell lines [9] and inhibited the proliferation of MCF-7 breast cancer cells *in vitro* and *in vivo* [10]. We previously reported that econazole is a new PI3K inhibitor that promotes lung cancer cell apoptosis by inhibiting the PI3K/AKT pathway [11]. In the present study, we analyzed the efficacy of econazole in Adriamycin-resistant (ADR) MDA-MB-231 and MCF-7 and their parental breast cancer cell lines. We confirmed that econazole nitrate had significant anti-tumor effects and potent activity in overcoming Adriamycin resistance in breast cancer. In the mechanism, econazole inhibited the PI3K/AKT pathway because overexpression of catalytic constitutively active AKT1 significantly abolished these phenotypes. These findings suggest that econazole could be used for breast cancer second-line therapy in combination with Adriamycin.

Materials and methods

Chemicals, antibodies, cell lines, and cell culture

Econazole and Adriamycin were purchased from Selleck Chemicals (Houston, TX, USA). Antibodies against P-gp, PI3Kp110α, total-AKT, phospho-AKT (S473), phospho-AKT (T308), Bcl-2, IKKα, phospho-IKKα, GSK-3β, phospho-GSK-3β, and β-actin were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Breast cancer cell lines (MDA-MB-231 and MCF-7) and MDR breast cancer cell lines (MDA-MB-231/ADR and MCF-7/ADR) were obtained from the Cell Bank of Kunming Institute of Zoology, Chinese Academy of Sciences and were respectively cultured in RPMI 1640 and DMEM medium at 37°C in 5% CO_2. Both media contained 10% fetal bovine serum (FBS) (Invitrogen, Rockville, MD, USA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

AKT1ca overexpression in breast cancer cell lines

The pCDH-AKT1ca-IRES-GFP retroviral vector was constructed and used to prepare lentivirus and to infect MDA-MB-231/ADR, MDA-MB-231, MCF-7/ADR, and MCF-7 cells. The plasmid DNA was obtained from Prof. Binhui Li at the Capital Medical University. Lentiviruses were prepared according to our previous methods [12]. AKT1ca expression was confirmed by Western blotting.

MTT assays

The breast cancer cells were seeded in 96-well plates at a density of 7×10^3 cells per well for 24 hours and treated with 1, 3, 10 and 30 μM concentration gradients of the tested agents for 24, 48 and 72 hours. The growth inhibitory effects of the tested agents were evaluated by MTT assays. After treatment, 10 μl of 5 mg/ml 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and further incubated for 4 hours. The medium was then discarded, and the precipitate was dissolved in DMSO. Absorbance was measured at 570 nm using a Synergy 2 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) according to the standard protocol. The IC_{50} values were calculated using GraphPad Prism 5 (San Diego, CA, USA).

Apoptosis measurement by flow cytometry

The breast cancer cells were seeded in 24-well plates at a density of 6×10^4 cells per well for 24 hours and exposed to drugs for 24 hours. Then, the cells were harvested and stained with Annexin V-FITC/propidium iodide (PI) according to the manufacturer's instructions (Beijing 4A Biotech Co., Ltd, Beijing, China). Apoptotic cells were analyzed by flow cytometry (CyFlow Space/Partec, Germany).

Western blotting

Cells were harvested and lysed with RIPA buffer containing 1 mM PMSF and protease inhibitor cocktail at 4°C for 30 minutes and then centrifuged at 13,000 rpm for 15 minutes. The supernatants were recovered, and the protein concentrations were measured using the BCA Protein Assay Kit (ThermoScientifc, MA, USA). The same amounts of cell lysates were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Sigma, Shanghai, China). After blocking with skim milk, the membranes were incubated sequentially with appropriately diluted primary and secondary antibodies. Proteins were detected using the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA). An anti-β-actin antibody (Cell Signaling Technologies) was used to monitor loading.

PI3Kα kinase activity assays

Inhibition of PI3Kα by the econazole and BYL719 (J&K Scientific Ltd., Beijing, China) was examined in a cell-free system by assessing the phosphorylation of a poly-EY (4:1 Glu, Tyr) peptide substrate with recombinant kinases PI3Kα (Upstate Biotechnology). Inhibition of the recombinant kinases was evaluated by using the ADP-Glo Kinase assay kit according to the manufacturer's instruction (Promega, Madison, WI, USA). Briefly, the econazole and BYL719 in a range of different concentration (1-300 nM) were incubated with 4 ng of the recombinant kinases and 0.2 μg/mL of the poly-EY substrate at room temperature for 60 min. Then, 5 μL of ADP-Glo reagent was added and incubation continued at room temperature for another 40 min. Finally, 10 μL of kinase detection reagent was added and the mixture was allowed to incubate at room temperature for 30 min before the measurement of luminescence by GloMax 20/20 Luminometer (Promega).

Tumorigenesis and treatment in nude mice

Female BALB/C nude mice of 5-6 weeks old were purchased from Department of Animal Experiment, Kunming Medical University and raised under pathogen-free conditions. MDA-MB-231/ADR cells (1×10^6/0.2 ml PBS per mice) were injected subcutaneously into the right flank of the mice. Fourteen days after inoculation, the tumors grew to a volume of 80-100 mm^3. The mice were randomly divided into four groups (six mice per group) and injected by intraperitoneal injection (i.p.) every day for 21 days with 10% DMSO + 13% castor oil + 77% PBS (control group), econazole (40 mg/kg), Adriamycin (40 mg/kg), econazole (40 mg/kg) plus Adriamycin (40 mg/kg). Tumor volumes were measured every 3-4 days after tumor appearance and calculated by the equation $V=ab^2/2$ (a= longest axis; b= shortest axis). The mice were sacrificed on day 21 after treatment, and tumors were isolated and weighted. The study was approved by the laboratory animal ethics committee of Kunming Medical University.

Statistical analysis

The results were obtained from at least three different experiments and expressed as the mean ± SEM (the standard error of mean). Statistical analysis was performed using one-way ANOVA (Analysis Of Variance) and factorial analysis, and the difference was considered significant if $P<0.05$. Statistically significant results were marked with asterisks (*) in the figures.

Results

Econazole shows potent cytotoxicity in ADR breast cancer cell lines

To confirm whether MDA-MB-231/ADR and MCF-7/ADR cells are indeed resistant to Adriamycin, we performed the MTT assay. Adriamycin (1-10 μM) significantly reduced cell viability in a time- and dose-dependent manner in MDA-MB-231 and MCF-7 parental cells but showed little cytotoxicity against MDA-MB-231/ADR and MCF-7/ADR cells (**Figure 1A**). Then, we examined the cytotoxic activity of econazole in sensitive and ADR breast cancer cell lines using the MTT assay. Interestingly, econazole (1-10 μM) exhibited stronger inhibitory effects in ADR breast cancer cell lines than in parental cell lines in a dose-dependent manner (**Figure 1B**).

To evaluate whether the combination of econazole and Adriamycin synergistically kill ADR breast cancer cells, we treated breast cancer cells with increasing concentrations of econ-

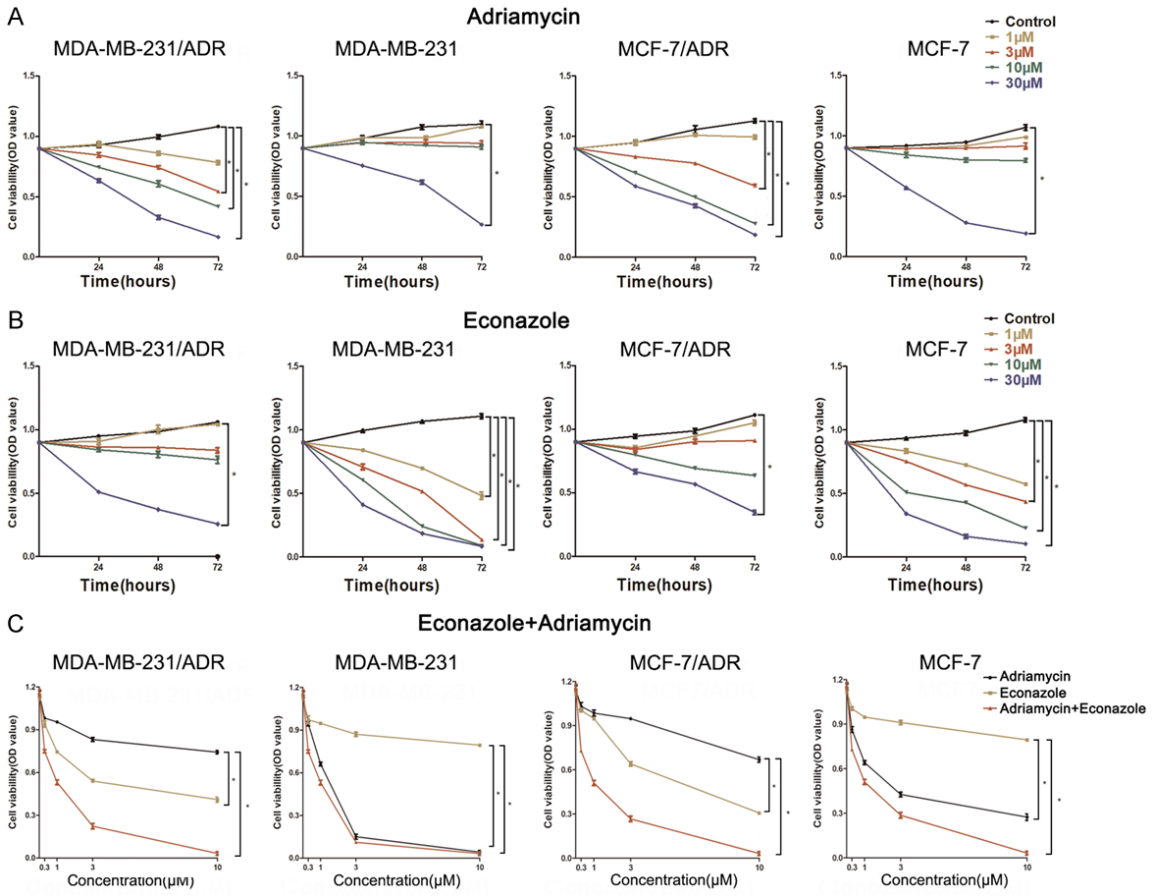


Figure 1. The cytotoxic effects of econazole and Adriamycin in two ADR and sensitive breast cancer cell lines. A. Adriamycin (1, 3, 10, and 30 μM) efficiently decreased the viability of sensitive breast cancer cells (MDA-MB-231 and MCF-7) at 24, 48, and 72 hours, as measured by MTT assays. MDA-MB-231/ADR and MCF-7/ADR cells showed resistance to low concentrations (<10 μM) of Adriamycin. B. Econazole (1, 3, 10, and 30 μM) efficiently decreased the viability of ADR breast cancer cells (MDA-MB-231/ADR and MCF-7/ADR) at 24, 48, and 72 hours, as measured by MTT assays. Interestingly, MDA-MB-231 and MCF-7 cells were not sensitive to low concentrations (<10 μM) of econazole. C. The combination of econazole (0.3-10 μM) and Adriamycin (0.3-10 μM) synergistically decreased the viability of ADR breast cancer cells, as measured by MTT assays at 72 hours. Statistical analysis was performed by one-way ANOVA (Analysis Of Variance), and the difference was considered significant if $P<0.05$, which is marked with an asterisk (*) in the figures; if $P<0.01$, the results are marked with two asterisks (**).

Table 1. IC_{50} value of econazole and Adriamycin in breast cancer cells

	Econazole (μM) N=5	Adriamycin (μM) N=5	Econazole + Adriamycin (μM) N=5	*P* value
MB-231/ADR	5.6±0.23	15.9±1.88	1.2±0.08	<0.05
MB-231	17.8±1.31	1.6±0.09	1.4±0.06	<0.05
MCF-7/ADR	4.2±0.18	23.7±2.98	0.9±0.04	<0.05
MCF-7	15.7±1.91	2.8±0.31	1.5±0.07	<0.05

azole and Adriamycin either alone or in combination for 24 hours. As shown in **Figure 1C**, the addition of econazole only slightly sensitized MDA-MB-231 and MCF-7 parental breast cancer cell lines to Adriamycin. However, econazole dramatically sensitized MDA-MB-231/ADR and MCF-7/ADR to Adriamycin. The combination treatment exhibited synergistic inhibitory effects in the two ADR breast cancer cell lines (**Table 1**). These results demonstrate that econazole is a potent cytotoxic agent to ADR breast cancer cells and has synergistic inhibitory

effect in combination with Adriamycin on ADR breast cancer cells.

Econazole induces ADR breast cancer cell apoptosis

We evaluated whether the cytotoxic effect of econazole on breast cancer cells is mediated by apoptosis. We treated breast cancer cells (MDA-MB-231/ADR, MDA-MB-231, MCF-7/ADR and MCF-7) with econazole and Adriamycin either alone or in combination for 24 hours and detected apoptosis using Annexin V/PI analysis. As illustrated in **Figure 2A**, **2B**, Adriamycin (3 μM) induced little apoptosis in MDA-MB-231/ADR breast cancer cells. In contrast, econazole (3 μM) induced more apoptosis in MDA-MB-231/ADR cells than in MDA-MB-231 cells. Combination therapy significantly induced over 30% of Annexin V-positive apoptotic cells. Similar results were observed in MCF-7/ADR and MCF-7 cell lines (**Figure 2C**, **2D**). These results suggested that econazole induced apoptosis in both ADR and parental breast cancer cells in combination with Adriamycin.

Econazole decreases pAKT, P-gp, and Bcl-2 protein expression levels

Accumulating evidence has indicated that the activation of the PI3K/AKT pathway is involved in the acquisition of resistance to chemotherapeutic drugs. The overexpression of AKT downstream genes, such as P-gp and Bcl-2, contribute to the development of MDR in many drug-resistant tumor cells [5, 13]. Therefore, we assessed the effect of econazole on the expression of the PI3K/AKT signaling pathway, P-gp, and Bcl-2 proteins in MDA-MB-231, MDA-MB-231/ADR, MCF-7, and MCF-7/ADR breast cancer cells by Western blotting. As expected, phosphorylated AKT (both T308 and S473), P-gp, and Bcl-2 protein expression levels were significantly decreased by econazole, whereas PI3Kα and total AKT protein levels were not affected (**Figure 3**). Interestingly, econazole decreased P-gp and Bcl-2 levels more efficiently in MDA-MB-231/ADR and MCF-7/ADR cells than in MDA-MB-231 and MCF-7 cells. These results demonstrated that econazole may overcome Adriamycin resistance through blocking PI3K/AKT signaling in breast cancer cells.

Econazole inhibits PI3Kα kinase activity in vitro

The inhibitory effects of econazole and BYL719 on the activity of PI3Kα kinase was evaluated in a cell-free system as described in the method section. The experimental results showed that econazole and BYL719 inhibited PI3Kα kinase activity in a dose dependent manner (IC_{50}: 79.29±6.97 nM and 8.68±2.13) (**Figure 3C**).

Overexpression of constitutively active AKT1 abolished the sensitization effect of econazole in MDR breast cancer cell lines

Because econazole functions possibly through inhibiting PI3Kα/AKT in breast cancer cells, we questioned whether constitutively active AKT1 could rescue the phenotypes. We used lentiviruses carrying the AKT1ca gene to infect MDA-MB-231/ADR and MCF-7/ADR cells. We first confirmed that AKT1ca was successfully expressed by Western blotting (**Figure 4A**). As expected, AKT1ca overexpression partially blocked the decrease of pGSK3β (S9), P-gp, and Bcl-2 that was induced by econazole in MDA-MB-231/ADR and MCF-7/ADR cells. Consistently, AKT1ca overexpression significantly increased the survival of MDA-MB-231/ADR and MCF-7/ADR cells in the presence of econazole (**Figure 4B**, **4C**). Finally, we demonstrated that AKT1ca overexpression significantly decreased the apoptosis induced by econazole in MDA-MB-231/ADR and MCF-7/ADR cells (**Figure 4D**, **4E**).

Econazole and combination with Adriamycin suppressed MDA-MB-231/ADR tumor growth in nude mice

To investigate whether econazole sensitizes ADR breast cancer cells to Adriamycin *in vivo*, we used MDA-MB-231/ADR cells to generate xenografts in BALB/C nude mice. Fourteen days after inoculation, the tumors grew to a volume of 80-100 m^3. The mice were randomly divided into four groups (six mice per group) and treated with econazole (40 mg/kg), Adriamycin (40 mg/kg), econazole (40 mg/kg) plus Adriamycin (40 mg/kg), and control (10% DMSO + 13% castor oil + 77% PBS). As expected, the tumors continued to grow in the Adriamycin-treated groups, indicating resistance, whereas econazole substantially suppressed tumor growth (**Figure 5**). Moreover, the mice treated with both econazole and Adriamycin exhibited an even more dramatic reduction of MDA-MB-231/ADR xenograft growth (**Figure 5**), with no significant change in body weight (**Figure 5C**). These results suggest-

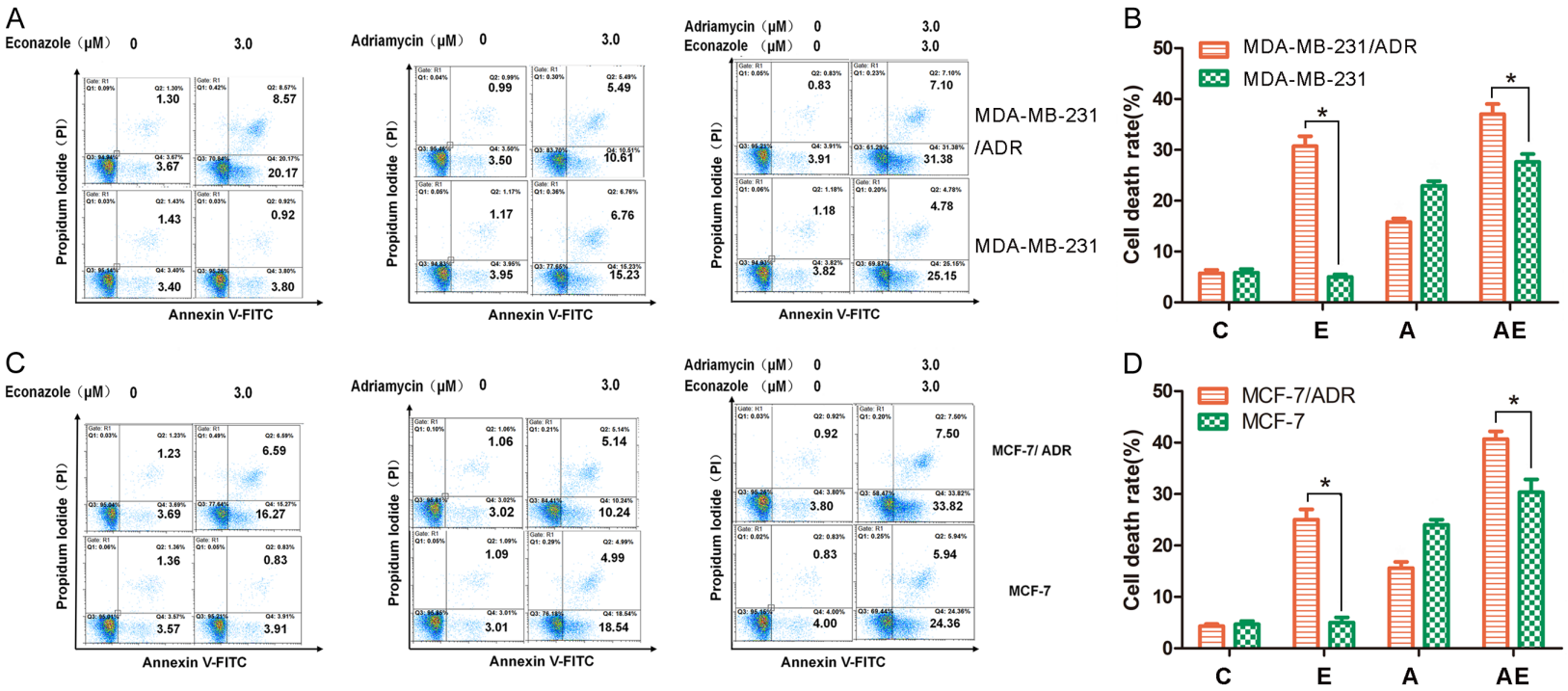


Figure 2. Econazole and Adriamycin induced apoptosis in ADR and sensitive breast cancer cell lines. (A) Econazole (3 μM), Adriamycin (3 μM), and the combination of econazole (3 μM) with Adriamycin (3 μM) treatment (24 hours) induced apoptosis in MDA-MB-231/ADR and MDA-MB-231 breast cancer cells. Apoptosis was measured by Annexin V/PI staining and flow cytometry ($P<0.05$). (B) The quantitative results of (A). (C) Econazole (3 μM), Adriamycin (3 μM), and the combination of econazole (3 μM) with Adriamycin (3 μM) treatment (24 hours) induced apoptosis in MCF-7/ADR and MCF-7 breast cancer cells. Apoptosis was measured by Annexin V/PI staining and flow cytometry ($P<0.05$). (D) The quantitative results of (C).

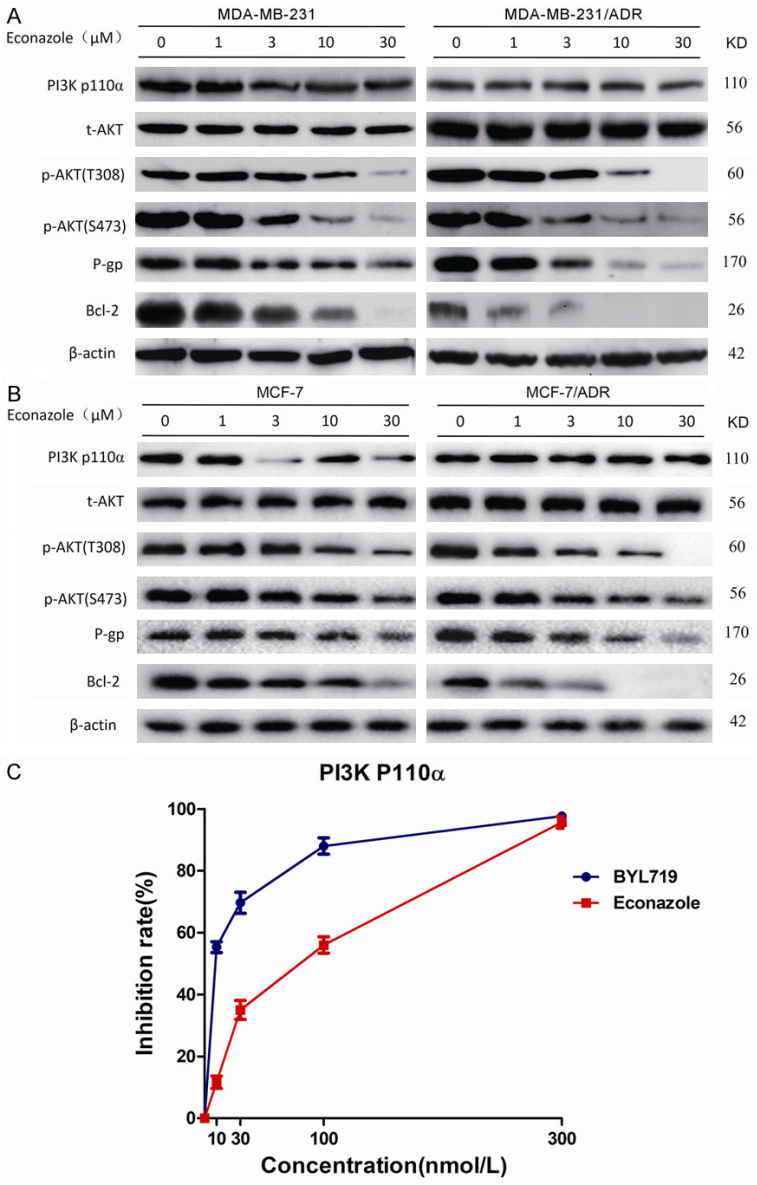


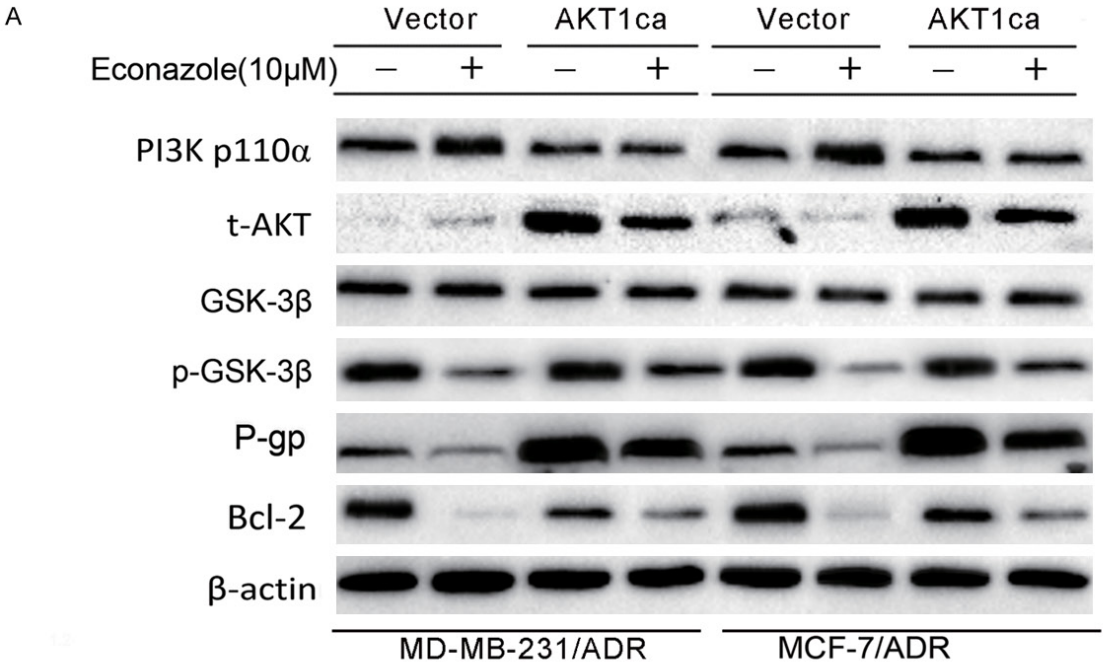
Figure 3. Econazole inhibited the PI3K/AKT pathway in ADR and sensitive breast cancer cell lines. A. The expression levels of pAKT (S473 and T308), P-gp, and Bcl-2 were decreased by econazole in MDA-MB-231/ADR and MDA-MB-231 cells in a dose-dependent manner. The cells were treated with increasing concentrations of econazole (0, 1, 3, 10, and 30 μM) for 24 hours. Econazole had no effect on PI3K p110α or t-AKT. b-actin was used as a loading control. B. The expression levels of pAKT (S473 and T308), P-gp, and Bcl-2 were decreased by econazole in MCF-7/ADR and MCF-7 cells in a dose-dependent manner. The cells were treated with increasing concentrations of econazole (0, 1, 3, 10, and 30 μM) for 24 hours. Econazole had no effect on PI3K p110α or t-AKT. b-actin was used as a loading control. C. Econazole and BYL719 significantly inhibited PI3Kα kinase activity in a dose dependent manner (IC_{50}: 79.29±6.97 nM and 8.68±2.13).

ed that econazole is a potentially safe and effective anti-cancer drug to overcome chemotherapy resistance and synergizes with Adriamycin chemotherapy in breast cancer.

Discussion

Resistance to chemotherapy is a major cause of treatment failure in breast cancer. Accumulating evidence indicates that the activation of the PI3K/AKT signaling pathway contributes to the intrinsic insensitivity of cancer cells to chemotherapy. In addition, PI3K/AKT pathway activation frequently occurs in breast cancer and leads to resistance to chemotherapy, endocrine therapy, and anti-HER2 therapies [13, 14]. PI3K may be a potential therapeutic target for MDR breast cancer [15, 16], and PI3K inhibitors could overcome resistance in MDR breast cancer cells. Multiple clinical studies of PI3K inhibitors in combination with traditional drugs in breast cancer are ongoing [6, 17]. Recently, the PI3Kα inhibitor Alpelisib/BYL719 was approved for treatment in PIK-3CA-mutant HR+HER2-metastatic breast cancer patients in combination with fulvestrant [7]. In this study, we investigated the therapeutic value of econazole, an azole anti-fungal agent that inhibits the PI3K/AKT pathway in ADR breast cancer cells.

In this study, we demonstrated that Adriamycin, a traditional chemotherapeutic agent, had little inhibitory effect on two ADR breast cancer cell lines (MDA-MB-231/ADR and MCF-7/ADR) but significantly affected the sensitive parental cell lines (MDA-MB-231 and MCF-7) at the same concentration. Importantly, econazole reduced cell viability more effectively in ADR breast cancer cells compared with the parental breast cancer cell lines, indicating that the ADR cell lines are more addicted to PI3K/AKT activation. Moreover, the combina-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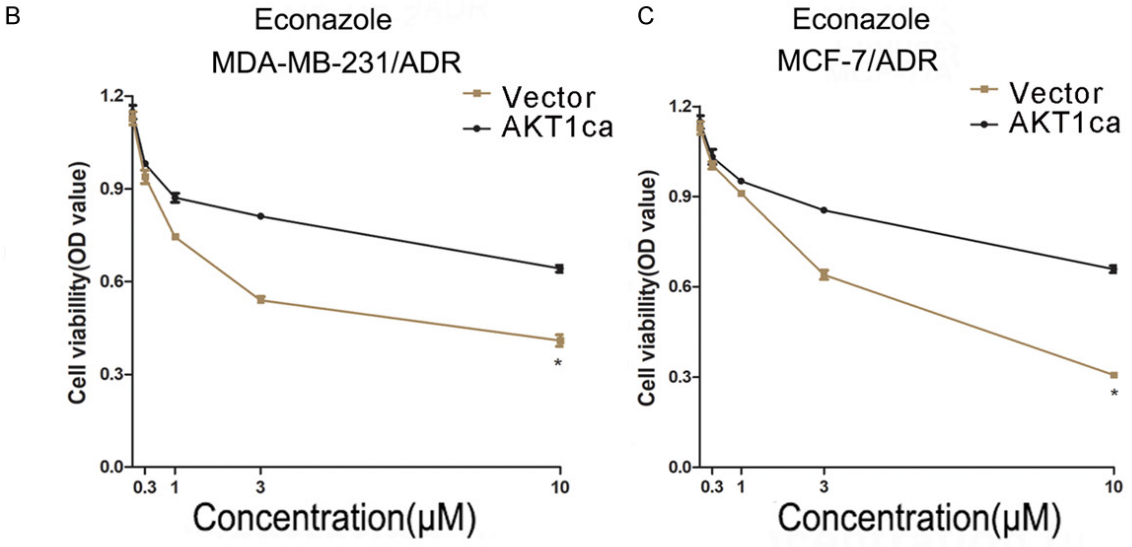
MCF-7/ADR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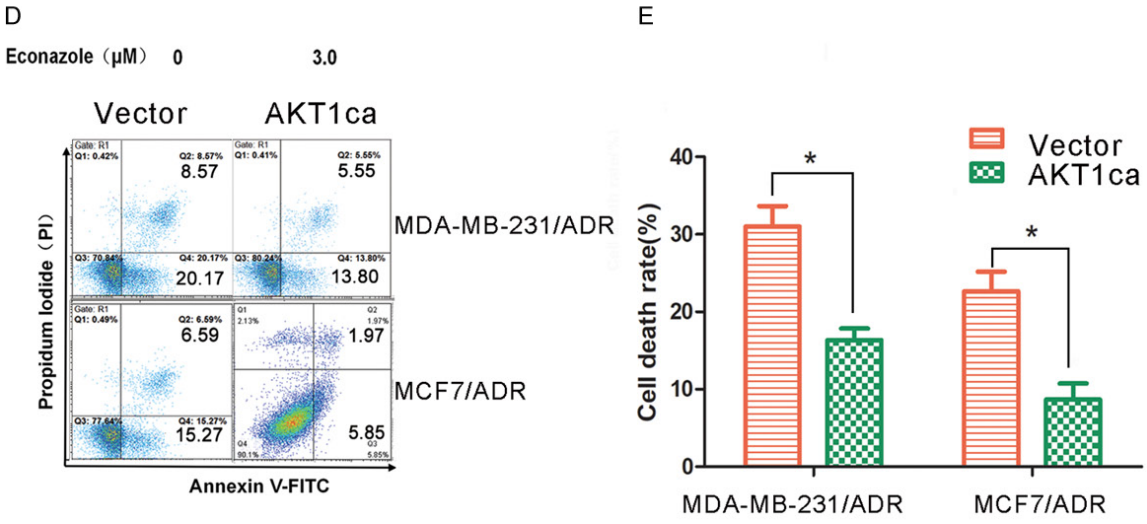
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MDA-MB-231/ADR
MCF7/ADR

Figure 4. The overexpression of AKT1ca gene decreased econazole-induced apoptosis in MDR breast cancer cell lines. (A) AKT1ca overexpression decreased econazole-induced decreases of P-gp and Bcl-2 protein levels in two ADR breast cancer cell lines (MDA-MB-231/ADR and MCF-7/ADR), as measured by Western blotting. pGSK3β (S9) was used as a positive control because it is phosphorylated by AKT. β-actin was used as a loading control. (B) AKT1ca overexpression significantly decreased the econazole-induced loss of cell viability in MDA-MB-231/ADR, as measured by MTT assays at 72 hours ($P<0.05$). (C) AKT1ca overexpression significantly decreased the econazole-induced loss of cell viability in MCF-7/ADR, as measured by MTT assays at 72 hours ($P<0.05$). (D) AKT1ca overexpression significantly decreased econazole (3 μM, 24 hours)-induced apoptosis in both MDA-MB-231/ADR and MCF-7/ADR cells. Apoptosis was measured by Annexin V/PI staining and flow cytometry. (E) Quantitative results of (D) ($P<0.05$).

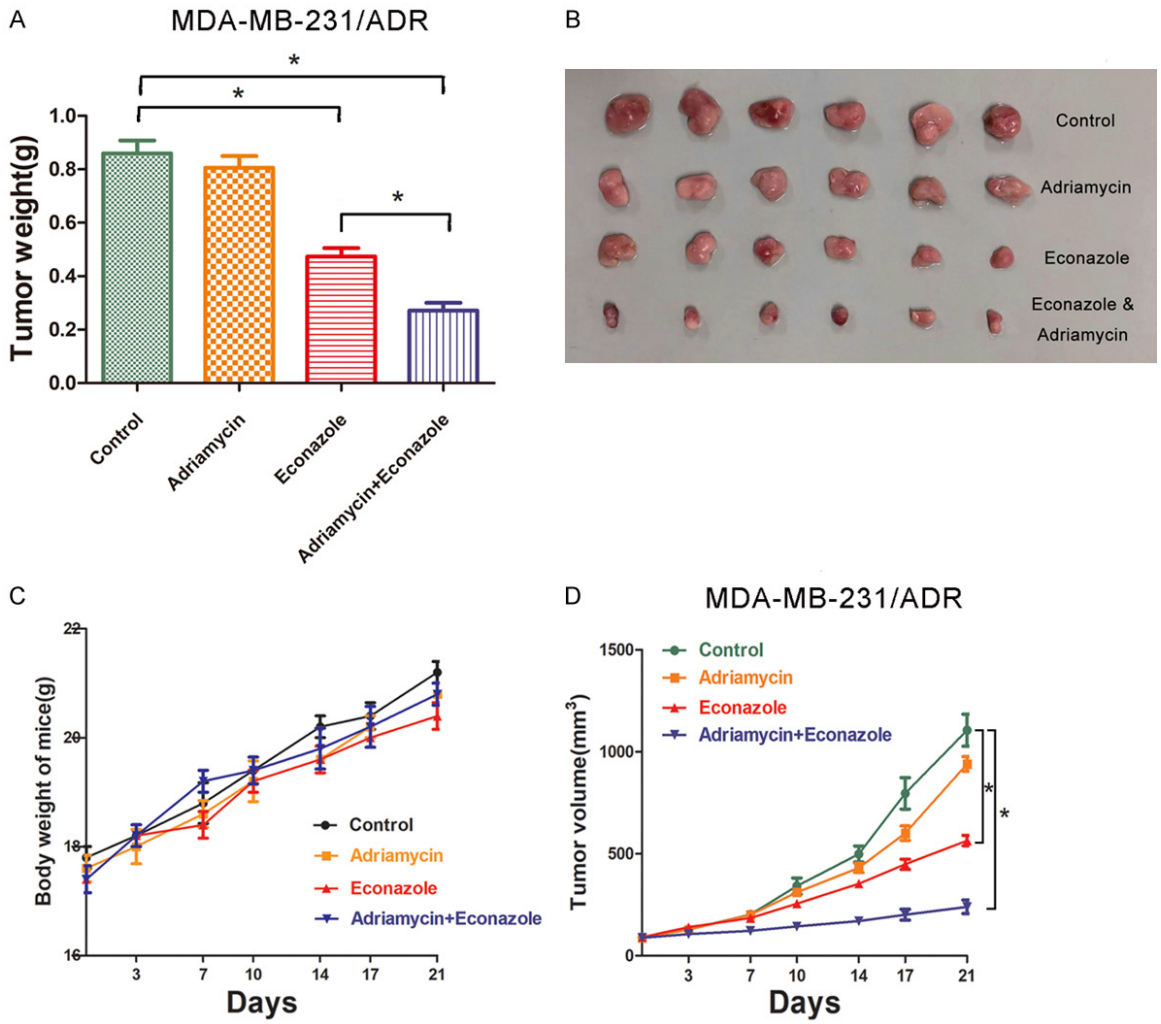

Figure 5. Econazole significantly suppressed MDA-MB-231/ADR tumor growth in nude mice and sensitized cancer cells to Adriamycin *in vivo*. A. Econazole (40 mg/kg) significantly suppressed MDA-MB-231/ADR tumor growth in nude mice, as measured by tumor weights. The combination of econazole (40 mg/kg) and Adriamycin (40 mg/kg) further decreased tumor weights. Adriamycin (40 mg/kg) treatment for 21 days had no effect on tumor weights. B. Econazole alone or in combination with Adriamycin reduced MDA-MB-231/ADR tumor masses in nude mice. C. Econazole alone or in combination with Adriamycin had little effect on mouse body weights at different time points. D. Econazole alone or in combination with Adriamycin significantly suppressed MDA-MB-231/ADR tumor growth in nude mice, as measured by tumor volume over the course of 21 days ($P<0.05$).

tion of econazole and Adriamycin exhibited synergistic effects in ADR breast cancer cells *in vitro* and *in vivo*. Previous studies showed that econazole had an inhibitory effect in MCF-7 breast cancer xenografts [18]. Our study confirmed for the first time that econazole also has an inhibitory effect on MDA-MB-231/ADR breast cancer xenografts.

Econazole is an azole antifungal with anticancer activity that can induce apoptosis in a wide range of cancers, such as breast cancer [9], prostate cancer [19], colon cancer [20], and lung cancer [11]. However, the effect of econazole in drug-resistant cancer cells has not been investigated. In this study, we showed that econazole is a PI3Kα inhibitor although its inhibitory efficacy is lower than BYL719 (**Figure 3C**). Econazole exhibited a more potent effect on inducing apoptosis in ADR breast cancer cells than in Adriamycin-sensitive cells. Moreover, the combination of econazole and Adriamycin exhibited a synergistic effect in ADR cells. A new PI3Kα-specific inhibitor, BYL719, was recently approved by the USA FDA in combination with fulvestrant in the treatment of advanced metastatic breast cancer with a PIK3CA mutation in postmenopausal females with HR+/HER2-tumors. A PIK3CA mutation is the best positive predictor of BYL719 sensitivity [21]. The small molecule PI3K inhibitor PI103 cooperated with doxorubicin to synergistically induce apoptosis and reduce the growth of neuroblastoma cells *in vitro* and *in vivo* [22]. Similarly, the combination of the PI3K inhibitor BKM120 with doxorubicin also suppressed breast cancer growth [23]. The addition of BAY 80-6946 (the selective alpha/delta isoform dominant PI3K inhibitor) to HER2-targeted therapy could restore sensitivity to trastuzumab and lapatinib [17].

AKT, as a key downstream factor of PI3K, can phosphorylate multiple substrates, which promotes acquired resistance to treatment [24]. A high level of pAKT has a crucial role in MDR. P-gp-mediated MDR is the major clinical impediment to chemotherapy in breast cancers [13]. LY294002, the first selective PI3K inhibitor, can partially reverse MDR by downregulating the expression levels of pAKT and P-gp [25]. In our study, econazole significantly inhibited the expression levels of pAKT and P-gp expression in two ADR breast cancer cell lines. Econazole may sensitize breast cancer cells to other chemotherapy agents besides Adriamycin, although we did not evaluate this possibility in this study. Additionally, Bcl-2, a potent inhibitor of apoptosis, is a crucial PI3K/AKT downstream protein promoting cell survival [22]. Econazole dramatically decreased Bcl-2 protein expression levels in two ADR breast cancer cell lines. Consistently, econazole induced a high level of apoptosis in two ADR breast cancer cell lines. After AKT1ca was overexpressed, econazole reduced P-gp and Bcl-2 protein expression and apoptosis. These results clearly indicate that econazole induces apoptosis in ADR breast cancer cells through inhibiting the PI3K/AKT pathway.

Activated pAKT promoted the downstream protein phosphorylation of BAD, dissociated the pro-apoptosis protein BAD and the anti-apoptotic protein Bcl-2, and upregulated the expression of Bcl-2 [5]. A high level of pAKT has a crucial role in MDR and upregulates the expression of P-gp, but the mechanism is unclear. The AKT inhibitor Ipatasertib in combination with paclitaxel significantly extended the progression-free survival of metastatic TNBC patients in a phase II clinical trial [21].

Econazole exerted a synergistic effect with Adriamycin *in vitro* and *in vivo*. Econazole showed significant anti-tumor effects in MDA-MB-231/ADR breast cancer xenografts in nude mice by daily intraperitoneal (I.P.) injection for 21 days. Moreover, the mice receiving econazole together with Adriamycin exhibited an even more dramatic reduction of tumor growth than those that received either econazole or Adriamycin alone. Importantly, the combination therapies were well tolerated and did not affect body weights. Thus, the combination of econazole with Adriamycin or other chemotherapeutic agents may be used for breast cancer patients who are resistant to traditional chemotherapies. Clinical studies will be required to test this hypothesis in the future.

In summary, we demonstrated that econazole has a strong cytotoxic effect in ADR breast cancer cell lines through inhibiting the PI3K/AKT pathway. The combination of econazole with Adriamycin showed synergistic anti-tumor activity in a preclinical animal model. These results provide a new potential second-line therapeutic strategy for breast cancer patients who fail first-line therapy.

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Disclosure of conflict of interest

None.

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