Dual Inhibition of PI3K and mTOR Mitigates Compensatory AKT Activation and Improves Tamoxifen Response in Breast Cancer

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Abstract
Everolimus, an mTOR inhibitor, showed great clinical efficacy in combination with tamoxifen, letrozole, or exemestane for the treatment of estrogen receptor-positive (ER+) breast cancer. However, its antitumor activity was shown to be compromised by a compensatory process involving AKT activation. Here, it was determined whether combining an additional PI3K inhibitor can reverse this phenomenon and improve treatment efficacy. In breast cancer cells (MCF-7 and BT474), everolimus inhibited the mTOR downstream activity by limiting phosphorylation of p70S6K and 4EBP1, which resulted in p-Ser473-AKT activation. However, addition of a LY294002, a PI3K inhibitor, to tamoxifen and everolimus treatment improved the antitumor effect compared with tamoxifen alone or the other two agents in combination. Moreover, LY294002 suppressed the activity of the PI3K/AKT/mTOR axis and mitigated the p-Ser473-AKT activation feedback loop in both cell lines. Critically, this combination scheme also significantly inhibited the expression of HIF-1a, an angiogenesis marker, under hypoxic conditions and reduced blood vessel sprout formation in vitro. Finally, it was shown that the three-agent cocktail had the greatest efficacy in inhibiting MCF-7 xenograft tumor growth and angiogenesis. Taken together, these results suggest that inhibition of PI3K and mTOR may further improve therapy in ER+ breast cancer cells.

Implications: Combinatorial inhibition of the PI3K/AKT/mTOR signaling axis may enhance endocrine-based therapy in breast cancer. Mol Cancer Res; 11(10); 1269–78. ©2013 AACR.
alone in patient with metastatic breast cancer refractory to previous antiestrogen therapies (11). In neoadjuvant setting, combination of everolimus and letrozole had a better response rate and greater Ki67 expression inhibition compared with letrozole alone (12). Furthermore, everolimus also enhanced the treatment efficacy of tamoxifen in patients with ER-positive metastatic breast cancer (TAMRAD study; ref. 13).

However, the response rate to endocrine and everolimus treatment was relatively low, 9.5% in BOLERO-2 and 14% in TAMRAD study, which may be due to a negative feedback signaling pathways activation, including Akt phosphorylation, and downstream signaling activation (11, 13). Baselga and colleagues showed that pAkt expression was upregulated in 50% of solid tumors treated with everolimus alone in a phase I study, which may attenuate the response with mTOR inhibitor (14). Among all the upstream signaling pathways in the mTOR complex, PI3K pathway is the most relevant in breast cancer. An estimated 10% to 50% of breast cancers are associated with PI3K mutations affecting this pathway (15). In addition, the traditional growth factors and hormone receptors (HR) have been also shown to funnel through the PI3K pathway. BEZ235, a dual PI3K and mTOR inhibitor, has been shown to be able to inhibit the growth of breast cancer cell with PI3K mutations (16). Thus, novel PI3K inhibitors in combination with mTOR antagonists might be a better treatment regimen in ER-positive breast cancer (17).

Here, we aimed at investigating whether addition of PI3K inhibitor to tamoxifen and everolimus can increase the antitumor activity in a preclinical breast cancer model compared with single PI3K/Akt/mTOR pathway inhibitor, which will gain more evidence to support the hypothesis that concurrent PI3K and mTOR inhibitors treatment may not only block everolimus-induced pAkt activation but also improve the mTOR complex (mTORC)-1 signaling inhibition. These findings may lead to a new endocrine treatment strategy by dual targeting PI3K/Akt/mTOR signaling pathway with antiestrogen agents in ER-positive breast cancer.

Materials and Methods

Cell culture and reagents

Human ER expression breast cancer cell lines MCF-7 and BT474 were purchased from the American Type Culture Collection, which were cultured in Dulbecco's modified Eagle medium (DMEM), RPMI-1640 medium containing 10% FBS with 1% penicillin-streptomycin (Invitrogen Corp). MCF-7 and BT474 were maintained at 37°C in humidified atmosphere of 5% CO₂.

Everolimus and matched placebo control agents were kindly provided by Novartis Institutes for BioMedical Research-Oncology (Basel, Switzerland); tamoxifen and LY294002 (a PI3K inhibitor) were purchased from Sigma and stored as aliquots at −20°C. BKM120 and BEZ-235 were purchased from Selleck Chemicals and dissolved in dimethyl sulfoxide (DMSO) and stored as aliquots at −20°C. Antibodies against p-Akt (Ser473), Akt, p-p70S6K (Ser2448), p70S6K, p-4E-BP1 (Thr37/46), 4E-BP1, and hypoxia-inducible factor-1α (HIF-1α) were purchased from Cell Signaling Technology. Human VEGF and VEGF₁₆₅ Biotinylated antibody were purchase from R&D Systems.

Cell proliferation assay

After a 24-hour serum withdrawal, MCF-7 and BT474 were digested and plated at 3,000 cells/well into 96-well flat-bottomed cell culture plates in 0.1 mL complete culture medium for a night. Media were exchanged for DMEM or RPMI-1640 with 1% FBS, and the cells were treated for 1 to 5 days with vehicle (DMSO) or various combination of tamoxifen (20 μmol/L), everolimus (20 nmol/L), PI3K inhibitors (LY294002 10 μmol/L or BKM120 500 μmol/L), and BEZ-235 (100 nmol/L). After drug treatment, 10 μL of cell counting kit-8 (CCK-8) reagent was added into each well, and the plates were incubated in a 37°C incubator with 5% CO₂ for 3 hours. Cell proliferation was determined by CCK-8 assay and read on multifwell scanning spectrophotometer (Thermo) at A450 for wavelength correction. All experiments were done with 5 wells per experiment and repeated at least 3 times.

Cell-cycle and apoptosis analysis

MCF-7 and BT474 cells were treated with experimental agent single or combination in medium containing 10% FBS for 48 hours. Then cells were digested and washed with PBS for 2 times. Five microliter Annexin-V (Roche) was incubated with suspended MCF-7 cells in 100 μL PBS and kept in dark for 10 minutes, then 5 μL Propidium Iodide (PI) (Roche) was added and kept in dark for 10 minutes. Ten thousand cells were analyzed for Annexin-V/PI staining by fluorescence detection using FACSscan (BD Biosciences), and the data were analyzed with Cell-Quest software (BD Biosciences). The apoptosis of BT474 treated with experimental agents was done as mentioned above.

MCF-7 cells were treated with experimental agent single or combination in medium containing 10% FBS for 48 hours. Cells were digested and washed with PBS for 2 times, then fixed with 70% ethanol at 4°C for at least 24 hours, and then the cell pellet was suspended in 1 mL of PI staining solution and kept in the dark at room temperature for 30 minutes. Five thousand cells were analyzed for PI staining by fluorescence detection using FACSscan (BD Biosciences), and the data were analyzed with Cell-Quest software (BD Biosciences). Cell-cycle status of BT474 was also done similar to MCF7.

Colony formation assay

Cells (MCF-7 and BT474; single-cell suspension) were cultured in 6-well plate at a density of 1,000 cells per well. After 24 hours, MCF-7 and BT474 cells were treated with tamoxifen (20 μmol/L), everolimus (20 nmol/L), PI3K inhibitors (LY294002 10 μmol/L), and DMSO as vehicle control, and incubated for 15 days to allow colony formation. Cell culture medium was changed with the corresponding concentration of the agents every 3 day. Colonies containing more than 50 cells were counted and evaluated.

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Western blot analyses

MCF-7 and BT474 cells were cultured in 3.5 cm diameter plates (80%-90% confluence), and washed by PBS, and then serum-starved for overnight. Cells were then treated with single or combination agents for 10 minutes, 30 minutes, 60 minutes, 24 hours, and 48 hours. Procedures for protein extraction and analysis were described previously (18).

Cells were lysed in radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl/PH 7.4; 1% NP-40; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl-fluoride; 1 mmol/L Na3VO4; 1 mmol/L NaF; 1 mmol/L okadaic acid; and 1 mmol/L phosphatase inhibitor). Proteins (20–50 μg) were separated on 8% to 12% SDS/PAGE gel and then transferred onto polyvinylidene difluoride membranes (Millipore). After blocking the membranes with 5% fat-free milk in TBST for 2 hours at room temperature, the membranes were incubated with appropriate dilution of fat-free milk in TBST for 2 hours at room temperature, the membranes (Millipore). After blocking the membranes with 5% fat-free milk in TBST for 2 hours at room temperature, the membranes were incubated with appropriate dilution of specific primary antibodies overnight at 4°C. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 hours and visualized using the enhanced chemiluminescent system (Thermo Fisher Scientific).

Endothelial sprout formation assay

Growth factor-reduced basement membranes were placed into 6-chamber slides (Matrigel; 125 μL/chamber; BD Biosciences), and 8 × 10³ endothelial cells were added on top with conditioned medium from MCF-7 or BT474 cells after 24-hour treatment with DMSO as vehicle control, or tamoxifen, everolimus, LY294002 alone or combination, and the chambers were incubated at 37°C for 24 hours. After incubation, the slides were fixed with methanol and stained with Diff-Quick solution II (Sigma). The slides were examined, and the sprouts were counted from 5 random fields using a microscope (×200). Further methods for endothelial sprout formation assay analysis were described in our previous report (18).

ELISA

The equal number of breast cancer cells (200,000/well) were plated in 6-well plates for 24 hours and then treated with various agents for 24 hours in a hypoxia and normoxia condition, and the conditioned medium was collected. Quantification of VEGF level in the supernatants was calculated by ELISA (QuantiKine ELISA kit; R&D Systems) as shown in the protocol.

Tumor xenografts in nude mice

All experimental animal procedures were conducted in compliance of the institutional requirements and were approved by the Shanghai Jiao Tong University School of Medicine Committee for the Use and Care of Animals (Shanghai, China). Six- to 8weeks old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Slaccas Laboratory Animal) were housed in air-filtered laminar flow cabinets with a 12-hour light cycle and food and water ad libitum. Mice were handled with aseptic procedures and allowed to aclimatize to local conditions for 1 week before the experimental manipulations. A 17β-estradiol pellet (Innovative Research of America) was implanted subcutaneously into each mouse 1 day before injection of MCF-7 cells. Cells (2 × 10⁶) were resuspended in PBS with Matrigel (1:1; BD Biosciences) and injected subcutaneously into the mammary fat pad of each mouse in 200 μL of final volume. When tumors reached a volume of 300 mm³ (termed day 0 for our experiment), we treated mice with experimental agents (tamoxifen daily; everolimus twice per week; LY294002 twice per week). Tumor xenografts were measured with calipers every 2 days, and tumor volume was determined using the formula: (length × width²) × (π/6). After 14 days of treatment, animals were anesthetized and killed by cervical dislocation.

Immunohistochemistry

Tumor xenografts were fixed immediately after removal in a 10% buffered formalin solution for a maximum of 48 hours at room temperature before being dehydrated and paraffin embedded. Slides were deparaffinized, and endogenous peroxidase activity was blocked by incubation in 0.3% H₂O₂ in methanol for 10 minutes at room temperature. Sections were then microwaved in PBS for 4 minutes for antigen retrieval and incubated with avidin and then with biotin for 15 minutes each to block nonspecific binding. An immunoperoxidase technique was then conducted using a commercial kit (Vectastain ABC Elite, Vector Laboratories). p-AKT(Ser473) and CD31 monoclonal antibodies’ staining was done according to the manufacturer’s instructions (Cell Signaling Technology). All sections had a negative control slide (no primary antibody) of an adjacent section to preclude nonspecific staining. All images were generated on Zeiss LSM 510 meta confocal laser scanning microscope.

Statistical analysis

Numerical data are expressed as mean ± SD. Statistical significance of differences in tumor growth in the single-agent or combination treatment group was analyzed by ANOVA or paired t test using the SPSS statistical software package (version 13.0; SPSS Company). Where indicated, a Kruskal–Wallis test and Dunn multiple comparisons tests were used. In all statistical analyses, two-sided P values < 0.05 were considered statistically significant.

Results

Growth effects and cell-cycle status of tamoxifen, everolimus, and PI3K inhibitors on breast cancer cells

The antitumor effect of single tamoxifen, everolimus, or PI3K inhibitor (LY294002 or BKM120, BEZ-235) on ER-positive breast cancer cell lines (MCF-7, BT474) was firstly examined after 48-hour and 72-hour treatment (Supplementary Fig. S1A and S1B). A significant dose-dependent reduction of viable cells number was observed in both 2 cell lines.

To determine the sensitivity of tamoxifen (10 μmol/L) alone or combination with everolimus (20 nmol/L) or PI3K inhibitor LY294002 (20 μmol/L), CCK-8 assay was used to
assess the growth-inhibitory effect. Compared with the control and tamoxifen alone treatment group, the combination of tamoxifen and everolimus or tamoxifen and LY294002 significantly inhibited the growth in both cell lines, even on the fifth day. Moreover, combination of 3 drugs treatment had the best antitumor effect compared with tamoxifen alone or with combination of 2 agents (Fig. 1A and B). In addition, this effect was also shown similarly in combination therapy with other PI3K inhibitors, including BKM120 (100 μmol/L) and BEZ-235 (100 nmol/L) on the basis of tamoxifen and everolimus treatment (Supplementary Fig. S2A and S2B), which suggested that a synergistic interaction in efficient antitumor was shown among tamoxifen, everolimus, and PI3K inhibitors in ER-positive breast cancer cells. Besides, colony formation assay also showed that 3 drugs combination treatment achieved the lowest number of colony formation (data were not shown). To explore the status of cell cycle affected by adding PI3K pathway inhibitor, PI staining and flow cytometry were used to assay its phase. As shown in Fig. 1C and D, after 48-hour treatment, the proportion of MCF-7 and BT474 cells in G₁ phase was increased to more than 90% in tamoxifen plus everolimus or LY294002 or both drugs group compared with control and tamoxifen alone. These findings suggested that the combining scheme was able to induce G₁ phase cell-cycle arrest in ER-positive breast cancer cells, which may make a contribution to inhibiting cell proliferation.

Effect of tamoxifen, everolimus, and PI3K inhibitors on signaling pathways in breast cancer cells

To determine the potential signaling pathway regulated by the treatment, MCF-7 and BT474 cells were treated with various concentrations of everolimus for 24 hours. Figure 2 showed that single everolimus treatment can reduce p-p70S6K and p-4E-BP1 expression in a dose-dependence style, both of which were recognized as the critical downstream effectors of PI3K/Akt/mTOR signaling pathway. Moreover, single everolimus treatment led to pAkt feedback-loop activation, which was associated with a dose-dependent everolimus treatment in BT474 cells. However, tamoxifen alone cannot gain the effect in both cell lines, even after 24-hour treatment (Fig. 3). After tamoxifen plus PI3K...
pathway inhibitors treatment from 30 minutes to 24 hours, both phosphorylation levels of p70S6K and 4E-BP1 were apparently inhibited (Fig. 3).

Increasing evidence shows that everolimus treatment alone can cause the activation of Akt level at 473 site, which was associated with the concentration of everolimus and may impair its antitumor effect (7), consistent with our above results. In this investigation, the results shown in Fig. 3B and C indicated that tamoxifen and everolimus treatment from 30 minutes to 24 hours can also induce pAkt473 activation in MCF-7 and BT474 cells. However, when tamoxifen and everolimus were combined with LY294002, pAkt473 activation induced by everolimus through feedback loop was apparently abolished in both cell lines (Fig. 3A–D), and still presented even after 24-hour treatment. Moreover, similar effects were also observed in combination of other PI3K inhibitors, like BKM120 or BEZ-235 (Supplementary Fig. S3).

To better elucidate the effect of these 3 agents combination, MCF-7 and BT474 cells were then treated in a different period of time. Figure 4 showed that the phosphorylation of p70S6K and 4E-BP1 were both significantly decreased in a time-dependent style. In this case, activation of pAkt473 was almost absent in MCF-7 cells and was gradually decreased followed by time increasing in BT474 cells.

Effect of PI3K/Akt/mTOR pathway inhibitors on antiangiogenesis in vitro

PI3K/Akt/mTOR pathway was associated with tumor angiogenesis through VEGF and HIF-1α gene transcriptional control in breast cancer (19). HIF-1α expression can be significantly induced in a hypoxia environment. In this current study, MCF-7 and BT474 cells treated with various combination of tamoxifen, everolimus, or LY294002 were incubated in a normoxia (20% O2) or a hypoxia condition (1% O2) for 24 hours. As expected, HIF-1α expression could not be detected in any group in a normoxia condition (Fig. 5A). However, in a hypoxia condition, a higher HIF-1α expression in both cells was detected in the control or tamoxifen treatment group, which significantly decreased by the addition of everolimus, LY294002, or both agents treatment. The previous evidence showed that HIF-1α expression may lead to angiogenesis (19). Therefore, our results suggest that combining everolimus with LY294002 may be responsible for antiangiogenesis efficacy in breast cancer cells.

For this purpose, VEGF, a critical angiogenesis biomarker in breast cancer, was analyzed by ELISA. In a hypoxia condition, tamoxifen, everolimus, and LY294002 combination treatment had the apparent efficacy in decreasing VEGF level (Fig. 5B and C) in MCF-7 and BT474 cells. Similarly, in a normoxia condition, VEGF levels in both cells generally followed a similar pattern of expression with the exception that much less VEGF was secreted than in a hypoxia condition (data not shown). Moreover, endothelial sprout formation assays also showed that combination of 3 agents treatment dramatically decreased blood vessel sprout formation both in MCF-7 (Fig. 5D and E) and BT474 (Fig. 5F and G) cells as compared with control, tamoxifen group, or tamoxifen plus everolimus group, or tamoxifen plus LY294002 group. Quantification assays confirmed the results as shown in Fig. 5E and G.

Figure 2. The effect of everolimus on PI3K/Akt/mTOR pathway status in MCF-7 (A) and BT474 (B) cells with a range of concentrations for 24 hours. Western blot analyses were used to evaluate PI3K/Akt/mTOR signaling pathway. Whole-cell lysates were separated on SDS-PAGE and immunoblotted with antibodies to p-P70S6K, T-P70S6K, p-Akt(Thr473), T-Akt, p-4EBP1, and T-4EBP1. The blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody to confirm equal protein loading. The results were repeated independently at least 3 times.
LY294002 enhances the antitumor and antiangiogenesis activity of tamoxifen and everolimus in vivo

To determine whether addition of PI3K inhibitor enhances antitumor efficacy of tamoxifen and everolimus in vivo, NOD/SCID mice were implanted subcutaneously with MCF-7 cells. After tumor is presented, mice were then randomized into 3 treatment groups: tamoxifen, tamoxifen plus everolimus, and tamoxifen, everolimus plus LY294002. As shown in Fig. 6A and B, at the end of 14-day treatment, smaller tumor volumes were found in mice treated with additional LY294002 (547 mm³) than those treated with single tamoxifen (1,578 mm³) or tamoxifen plus everolimus (1,067 mm³), respectively.

Our previous data showed that continuous everolimus treatment led to the activation of pAkt in breast cancer cells. Herein, we investigated the ability of LY294002 to reverse pAkt473 activation as well as enhance antiangiogenesis effects in MCF-7 xenograft models. Immunohistochemical analyses showed that combination of tamoxifen and everolimus treatment upregulated pAkt expression as compared with tamoxifen treatment. However, addition of LY294002 treatment apparently reversed the activation of pAkt (Fig. 6C). Furthermore, tumor tissues stained with an antibody to CD31, a specific marker for vascular endothelial cells, indicated that tumors treated with tamoxifen alone had more and larger blood vessel formations than those treated with addition of LY294002 (Fig. 6C). Moreover, combination of 3 agents treatment had much better antiangiogenesis activity than the tamoxifen plus everolimus treatment without increasing short-term toxicity.

Discussion

Endocrine therapy is the cornerstone treatment for patients with HR-positive breast cancer (4). However, not
Dual Inhibition of PI3K and mTOR in Breast Cancer

Figure 4. PI3K/Akt/mTOR pathway status after tamoxifen, everolimus, and LY294002 combination therapy for a different period of time in MCF7 (left) and BT474 (right). Cell extracts were prepared from MCF-7 and BT474 cells with the pretreatment of tamoxifen (10 μmol/L), mTOR inhibitor everolimus (20 nmol/L), and PI3K inhibitor LY294002 (10 μmol/L) combination for 10 minutes, 30 minutes, 60 minutes, 24 hours, and 48 hours. Then whole-cell protein was used to detect the expressions of total p-P70S6K, T-P70S6K, p-Akt(Thr473), T-Akt, p-4EBP1, T-4EBP1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by Western blot assays. The results repeated were independently at least 3 times. TAM, tamoxifen; Eve, everolimus; LY, LY294002.

all of these patients will respond to endocrine therapy due to primary or secondary endocrine therapy resistance, including receptor tyrosine kinase signaling activation (5). PI3K/Akt/mTOR pathway was activated in many solid tumors, which was associated with tamoxifen treatment resistance and worse outcome in patients with breast cancer (7). Activation of ER has been shown to occur in a ligand-independent manner, including direct activation of ER by mTOR pathway, which played a central role in regulating cell growth, proliferation, survival, and angiogenesis through phosphorylation of downstream substrates p70S6K and 4E-BP1. Targeting mTOR therapy combined with endocrine therapy agents achieved superior outcome than endocrine therapy alone (8–10). In this manuscript, we have shown that everolimus plus tamoxifen had better antitumor activity and greater p70S6K and 4E-BP1 expression inhibition than tamoxifen alone in breast cancer cell lines. In MCF-7 xenograft models, everolimus plus tamoxifen was more effective in delaying tumor growth than tamoxifen treatment alone. Besides, LY294002, a PI3K inhibitor, also showed synergistic antitumor efficacy with tamoxifen treatment, which suggested that ER-positive breast cancer patients may restore sensitivity of endocrine therapy by concurrent treatment with PI3K/AKT/mTOR signaling pathway inhibitors. Both everolimus and LY294002 acted mainly by induction of cell-cycle arrest, particularly in G1 phase, which was similar with other study using PI3K and mTOR dual inhibitors (17).

Target mTOR therapy alone can cause a multiple feedback loops and cross-talk with other signaling pathways, which may lead to pAkt activation and attenuate the responses to mTOR inhibition (14). Here, everolimus alone or combination with tamoxifen treatment can inhibit mTOR activity, but pAkt expression was upregulated in ER-positive breast cancer cell lines and in vivo tumor model, which may explain the relative low response rate in metastatic breast cancer therapy (11). Theoretically, inhibition of the alterations in PI3K/Akt/mTOR pathway nodal points may reduce the feedback pAkt activation and restore treatment sensitivity (18). He and colleagues reported that resveratrol, a natural phytoalexin, enhanced the antitumor activity of rapamycin in multiple breast cancer cell lines mainly by suppressing rapamycin-induced Akt signaling (20). PI3K was the upstream kinase of mTOR signaling and regulated mTOR and Akt activity. Therefore, cotargeting mTOR and PI3K may enhance mTOR inhibition as well as prevent pAKT feedback-loop activation. PI3K inhibitors (LY294002, BKM120) were selected to concurrent with everolimus and tamoxifen therapy in ER-positive breast cancer cells. In our study, we found that addition of PI3K inhibitor to tamoxifen and everolimus had the best antitumor efficacy and can significantly decrease p-p70S6K, p-4E-BP1 as well as p-AKT expression, indicating that dual PI3K and mTOR inhibition may restore the sensitivity of everolimus and also augment the effects of endocrine therapy in ER-positive breast cancer cell lines.

mTOR has 2 complexes, mTORC1 and mTORC2, in PI3K pathway. mTORC1 can regulate translation of proteins involved in cell growth, proliferation, and survival, which can be activated by ligand binding to tyrosine kinase receptors such as insulin-like growth factor-1 receptor, EGF receptor, and HER2 (10). Besides, mTORC1 also regulate the tumor angiogenesis activity via VEGF and HIF-1α gene transcriptional control (19). In breast cancer, studies have shown that VEGF receptor and mTOR inhibitors show similar but also distinct effects on tumor vascular biology, which has implications for their clinical activity alone or in combination. Preclinical and clinical data showed that VEGF levels in tumor tissue were related with worse response to endocrine therapy (21, 22). Retrospective clinical data showed that decreasing VEGF level may restore sensitivity and improve efficacy to.
After 24 hours of treatment with tamoxifen, everolimus, LY294002 alone or combination, medium free of serum were changed after 3 times washing with PBS, then supernatant were collected 24 hours later, and were analyzed by ELISA. Results represent 3 independent experiments carried out in triplicate.

Figure 5. Effects of PI3K pathway inhibitors on angiogenesis in MCF-7 and BT474 breast cancer cell lines in vitro. A, HIF-1α expression profile of MCF-7 and BT474 cells after tamoxifen alone, or combined with everolimus or LY294002 treatment in a normoxia or hypoxia conditions for 24 hours. Cell extracts were prepared from MCF-7 and BT474 cells with the pretreatment tamoxifen (10 µmol/L), mTOR inhibitor everolimus (20 nmol/L), PI3K inhibitor LY294002 (10 µmol/L) alone or combination for 24 hours in a normoxia or hypoxia condition, and were then used to detect the expressions of HIF-1α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by Western blot assays. The results were repeated independently at least 3 times. B, VEGF expression profile of MCF-7 cells in a hypoxia condition after tamoxifen, everolimus, LY294002 alone or combined treatment. After 24 hours of treatment with tamoxifen, everolimus, LY294002 alone or combination, medium free of serum were changed after 3 times washing with PBS, then supernatant were collected 24 hours later and were analyzed by ELISA. Results represent 3 independent experiments carried out in triplicate. *, statistically significant differences between control and treatment, P < 0.05. **, statistically significant differences between T + E or T + L group and T + E + L group, P < 0.01. C, VEGF expression profile of BT474 cells in a hypoxia condition after tamoxifen, everolimus, LY294002 alone or combined treatment. After 24 hours of treatment with tamoxifen, everolimus, LY294002 alone or combination, medium free of serum were changed after 3 times washing with PBS, then supernatant were collected 24 hours later, and were analyzed by ELISA. Results represent 3 independent experiments carried out in triplicate. *, statistically significant differences between control and treatment, P < 0.05. D, effect of PI3K pathway inhibitor on endothelial sprout formation in MCF-7 cells. Human dermal microvascular endothelial cells (HDMECs) were plated in growth factor-reduced Matrigel in the presence or absence of conditioned medium from MCF-7 cells after 24 hours of treatment with tamoxifen, everolimus, LY294002 alone or combination the same as C. The cultures were then fixed and stained. As a negative control, only HDMECs were plated. Original magnification ×20, where the black bars represent 100 µmol/L. Three independently experiments were done in triplicate. E, effect of PI3K pathway inhibitor on endothelial sprout formation in MCF-7 cells. Vessel sprout formation was quantified by direct microscopic counting. The data are presented as mean ± SD for triplicate determinations. *, statistically significant differences between control and treatment, P < 0.05. F, effect of PI3K pathway inhibitor on endothelial sprout formation in BT474 cells. HDMECs were plated in growth factor reduced Matrigel in the presence or absence of conditioned medium from BT474 cells after 24 hours of treatment with tamoxifen, everolimus, LY294002 alone or combination the same as C. The cultures were then fixed and stained. As a negative control, only HDMECs were plated. Original magnification ×20, where the black bars represent 100 µmol/L. Three independently experiments were done in triplicate. G, effect of PI3K pathway inhibitor on endothelial sprout formation in BT474 cells. Vessel sprout formation was quantified by direct microscopic counting. The data are presented as mean ± SD for triplicate determinations. *, statistically significant differences between T + E, T + L group, and T + E + L group, P < 0.05. **, statistically significant differences between control and treatment, P < 0.01. Ctrl, control; T, tamoxifen; E, everolimus; L, LY294002.

In summary, our results suggest that dual PI3K and mTOR inhibitors with concurrent endocrine therapy may offer a potential treatment strategy for endocrine therapy.
delay or reverse everolimus resistance in ER-positive breast cancer treatment, which warrants further clinical evaluation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 6. PI3K inhibitors enhance antitumor and antiangiogenesis activity of tamoxifen and everolimus in vivo. A, eight-week-old NOD/SCID mice were implanted subcutaneously with \(2 \times 10^5\) MCF-7 cells, and divided into 3 groups which received tamoxifen alone, combined with everolimus, or combined with everolimus and LY294002 when tumor volume was at least 100 mm\(^3\) at the same time. Tumor volume (mm\(^3\)) was evaluated at indicated times. B, volume of xenograft tumors derived from MCF-7 cells treated with tamoxifen alone, combined with everolimus, or combined with everolimus and LY294002. Tumor sizes were measured using a caliper at the indicated time points. Data are shown as mean ± SD. **", statistically significant differences between control and treatment, \(P<0.01\). C, effect of PI3K pathway inhibitors on PI3K/Akt/mTOR pathway and angiogenesis status in vivo. Subcutaneous tumors with treatment of tamoxifen alone, combined with everolimus, or combined with everolimus and LY294002 were harvested and processed for immunohistochemistry staining for p-Akt (Thr473), and human CD31 (1:100 anti-rabbit IgG). Original magnification, ×20, where the black bars represent 100 µm. TAM, tamoxifen; Eve, everolimus; LY, LY294002.