Combined Use of Metformin and Everolimus Is Synergistic in the Treatment of Breast Cancer Cells

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Everolimus inhibits mammalian target of rapamycin (mTOR) and leads to decreased protein synthesis and decreased cancer cell proliferation in many experimental systems. Adenosine 5'-monophosphate-activated protein kinase (AMPK) activators such as metformin have similar actions in keeping with the TSC2/1 pathway linking activation of AMPK to inhibition of mTOR. Histopathological and biochemical studies of breast cancer show frequent dysregulation of the AMPK and the mTOR pathway. Therefore, we investigated the efficacy of the mTOR inhibitor everolimus and metformin in the treatment of breast cancer cells. This study evaluated the in vitro and in vivo effects of everolimus alone or in combination with metformin on breast cancer cells. MTT assay was used to quantify the inhibitory effect of the drugs on breast cancer cells in vitro. SCID mice injected with HCC1428 cells followed by different treatments were used to assess the in vivo efficacy of different agents. Data showed that the combination of everolimus and metformin exerted synergistic inhibitory effects on the growth of breast cancer cells both in culture and in a mouse xenograft model. Further, this combination abrogated S6 and 4EBP1 phosphorylation. Collectively, we suggest that the combination of everolimus and metformin may be an effective regimen for treatment of breast cancer, hence warranting further evaluation of the combination in the clinic.

Key words: Metformin; Everolimus; Breast cancer; Adenosine 5'-monophosphate-activated protein kinase (AMPK); Mammalian target of rapamycin (mTOR)

INTRODUCTION
Aberrant activation of the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway occurs in many types of cancers, including breast cancer, largely due to frequent mutations of K-Ras, PTEN, LKB1, and/or epidermal growth factor receptor (EGFR) (1–4). Therefore, the PI3K/Akt/mTOR signaling pathway has become an area of intensive research and has attracted extensive attention for drug discovery. Consequently, everolimus, an inhibitor of this signaling pathway, has been developed and is currently used or being evaluated for cancer therapy in the clinic.

Metformin is an oral biguanide agent widely used for the treatment of type 2 diabetes mellitus. Recently, several studies have indicated that metformin could lower the risk of developing several cancers, including those of the breast, pancreas, colon, and prostate (5–7). Furthermore, metformin can inhibit cancer cell proliferation and tumor growth in animal models (8). The mechanisms underlying the anticancer effects of metformin are varied, and among these the activation of AMP-activated protein kinase (AMPK) is pivotal (9). AMPK activators, such as metformin, have similar actions, in keeping with the TSC2/1 pathway linking activation of AMPK to inhibition of mTOR (10). Everolimus is an analog of rapamycin with similar function to rapamycin as an allosteric inhibitor of mTOR. In patients with advanced renal cell cancer previously treated with VEGF-targeted agents, everolimus improves progression-free survival (11). Recently, it has also been shown to significantly prolong progression-free survival of patients with progressive advanced pancreatic neuroendocrine tumors with a low rate of severe adverse events (12). In many other solid organ malignancies, everolimus and other rapamycin analogs exert modest anticancer effects (13,14), which, though promising, are not sufficient to warrant monotherapy with these agents.

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Everolimus causes Akt activation in human cancer cells, including breast cancer cells, and in tumor biopsies while inhibiting mTOR signaling (3). AMPK activation during mTOR inhibition by metformin is likely PI3K dependent (10). Thus, at the same time, activation of AMPK via metformin and inhibition of mTOR via everolimus might be an effective therapeutic strategy. In this study, we focused on determining whether the combination of everolimus and metformin exerts enhanced therapeutic efficacy against breast cancer cells and found that this combination was more effective than either agent alone in inhibiting the growth of breast cancer cells both in vitro and in vivo.

MATERIALS AND METHODS

Reagent, Cell Culture, and Antibodies

Metformin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile phosphate-buffered saline (PBS). Everolimus was a kind gift of Novartis Pharma Stein AG. Antibodies against p-S6, S6, p-4EBP-1, 4EBP-1, and β-actin were from Cell Signaling Technology (Danvers, MA, USA). Unless otherwise noted, all other chemicals were from Sigma-Aldrich. HCC1428, MDA-MB-468, and BT549 human breast cancer cell lines were used for this study. All of the cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. These cells lines were maintained at 37°C in a humidified atmosphere with 5% CO2.

MTT Assay

A 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay was used to assess cell proliferation. The cells were seeded, and 20 ml of the MTT solution (5 mg/ml) was then added to each well at the indicated time. The absorbance at 490 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Flow Cytometry Analysis of CD44 and CD24 Expression

Cells growing in 60-mm dishes were washed once with PBS and then harvested with 0.05% trypsin/0.025% EDTA. Cell suspensions were washed with PBS and resuspended in wash buffer (1% BSA in PBS). Cells (10⁶/100 μl) were incubated with the combinations of fluorochrome-conjugated monoclonal antibodies against human CD44-APC, CD24-PE, or IgG isotype controls for APC and PE in the dark for 30 min on ice. The labeled cells were washed with PBS and then analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA).

Mammosphere Culture

Cells were trypsinized and mechanically disrupted to obtain single-cell suspensions. Single-cell suspensions were then plated in ultralow attachment 96-well plates (Corning Costar Corp., Cambridge, MA, USA) at different densities of viable cells in a serum-free mammary epithelial growth medium supplemented with 1:50 B27 (Invitrogen), 20 ng/ml epithelial growth factor, 20 ng/ml basic fibroblast growth factor (BD Biosciences), and 10 μg/ml heparin (Sigma-Aldrich) for 7–10 days. Mammospheres were imaged and counted using phase-contrast microscopy.

Immunohistochemistry

Tumors from xenograft cells were fixed in 10% neutralized buffered formalin for 48 h and embedded in paraffin. Four-micrometer-thick consecutive sections were cut and processed for immunohistochemistry with antibodies. Briefly, tissue sections were deparaffinized with xylene, dehydrated with a graded series of alcohols, and then incubated in 3% (v/v) hydrogen peroxide for 10 min at room temperature. After three washes of 3 min each in PBS, tissue sections were microwaved for 20 min in 10 mM citrate buffer (pH 6.0). Then the sections were washed a further three times in PBS for 5 min each, and incubated with normal goat serum to reduce nonspecific binding. Tissue sections were then incubated with antibodies at 4°C overnight. Sections were washed three times in PBS, and biotinylated goat anti-mouse serum IgG was used as a secondary antibody. After washing three times in PBS, the sections were incubated in streptavidin–biotin conjugated with horseradish peroxidase, and the peroxidase reaction was developed with 3,30-diaminobenzidine tetrahydrochloride. The slides were examined under a light microscope, and representative images were taken from a minimum of five different slides of each group.

Western Blot Assay

Equal amounts of protein were separated using SDS polyacrylamide gels and were electrotransferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were immunoblotted overnight at 4°C with primary antibodies, followed by their respective secondary antibodies. β-Actin was used as the loading control.

In Vivo Tumorigenesis Assays

Six-week-old female BALB/c-nu mice were obtained from Shanghai Slac Laboratory Animal Co. Ltd. Female BALB/c-nu mice were maintained in a specific pathogen-free facility. All experimental protocols were reviewed by the Committee on the Ethics of Animal Experiments of Shandong University and were executed according to the Guidelines for Animal Experiments of Shandong University. HCC1428 cells (1.0×10⁶) were injected subcutaneously into the abdominal mammary fat pads of these mice after they had acclimated to their new...
environment. The mice had continuous free access to sterilized food and autoclaved water. When the tumor size was approximately 4 mm in diameter, the animals were randomly divided into four groups (five mice per group), and treatment was initiated with oral gavage of everolimus (2 mg/kg, once daily) alone, oral gavage of metformin alone (100 mg/kg, once daily), or a combination of both drugs. Tumor length ($L$) and width ($W$) were measured every 3 days, and tumor volume was calculated using the equation: $\text{volume} = \frac{W^2 \times L}{2}$. After 5 weeks, the mice were killed, and the tumor volume and weight were measured.

**Statistical Analysis**

Experimental data are shown as mean ± standard deviation (SD). The results from different treatment groups were compared using a two-tailed Student’s $t$-test. Differences were considered to be statistically significant at a value of $p < 0.05$. Statistical analysis was done with SPSS/Win11.0 software (SPSS, Inc., Chicago, IL, USA).

**RESULTS**

The Combination of Everolimus With Metformin Synergistically Inhibits the Growth of Breast Cancer Cells In Vitro

The in vitro effect of everolimus and metformin on cell growth was determined using a standard MTT assay. Results of dose–response studies are shown in Figure 1A and B. HCC1428, MDA-MB-468, and BT549 breast cancer cell growth was inhibited by both metformin and everolimus. Compared with the group treated with everolimus or metformin alone, the antiproliferative effect of the combination of everolimus with metformin on breast cancer cells was significantly greater ($p < 0.001$) (Fig. 1C).

The Combination of Everolimus and Metformin Synergistically Inhibits Breast Cancer Cell Stemness

Many studies have identified pivotal roles for cancer stem cells (CSCs) in breast cancer growth, invasion, metastasis, and resistance to chemotherapy. Thus, we evaluated whether combining treated everolimus with metformin could significantly inhibit breast CSCs. We performed the mammosphere formation assay to determine whether everolimus and metformin could synergistically inhibit the stemness of breast cancer cells. The results showed that control group HCC1428 cells produced more and larger spheres (Fig. 2A). The combination of everolimus and metformin (Fig. 2D) significantly

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**Figure 1.** Inhibition of HCC1428, MDA-MB-468, and BT549 breast cancer cell growth by metformin and everolimus. (A) Dose–response curves were obtained by MTT assays after 48-h exposure to everolimus alone (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM). (B) Dose–response curves were obtained by MTT assays after 48-h exposure to metformin alone (1 μM, 10 μM, 100 μM, 1 mM, 10 mM, 100 mM). (C) The inhibitory effects of combination treatment with metformin and everolimus on breast cancer cells. Each column represents the mean ± SD ($n = 5$). The statistical analysis was performed with two-way ANOVA using Tukey’s test for pairwise comparisons. **$p < 0.01$** compared with the everolimus or metformin alone treated group.
inhibited the growth of HCC1428 mammosphere, whereas everolimus (Fig. 2B) or metformin (Fig. 2C) alone at the tested doses only minimally or partially inhibited the growth of HCC1428 mammosphere. Compared with the everolimus or metformin alone groups, a significantly greater mammosphere inhibitory effect was observed with the combination treatment in the everolimus + metformin group (p<0.01) (Fig. 2E). A human breast cancer cell population characterized by a CD44+/CD24− surface marker profile has been reported to be highly enriched in CSCs. Flow cytometry showed that control group HCC1428 had a higher proportion of CD44+/CD24− cells in the population (Fig. 3A). The combination of everolimus and metformin (Fig. 3D) significantly inhibited the growth of proportion of CD44+/CD24− cells, whereas everolimus (Fig. 3B) or metformin (Fig. 3C) alone at the tested doses only minimally or partially inhibited the growth of HCC1428 CD44+/CD24− cells. Compared with the everolimus or metformin alone groups, a significantly greater CD44+/CD24− cell inhibitory effect was observed with the combination treatment in the everolimus + metformin group (p<0.01) (Fig. 3E).

**The Combination of Everolimus and Metformin Synergistically Enhanced Effects on Suppression of S6 and 4EBP1 Phosphorylation**

To understand the mechanisms by which the combination of everolimus and metformin exert enhanced anticancer activity, we analyzed the effects of the combination on the phosphorylation of S6 and 4EBP1, which are translational repressors whose action is attenuated when phosphorylated by mTOR. As presented in Figure 4, everolimus or metformin alone decreased the levels of both p-S6 and p-4EBP-1 in the HCC1428 cell lines. Compared with the everolimus or metformin alone, significantly decreased levels of both p-S6 and p-4EBP-1 were observed with the combination treatment in the everolimus + metformin cells.

**The Combination of Everolimus and Metformin Exerts Augmented Activity Against the Growth of Breast Cancer Cell Xenografts in Nude Mice**

Because of the promising growth-inhibitory effects of the everolimus and metformin combination in breast cancer cells in vitro, we also validated the efficacy of the combination against the growth of breast cancer tumors in mice. HCC1428 cells were injected subcutaneously...
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into the flank of SCID mice (n=5 per group). On establishment of palpable tumors, mice were randomly assigned to one of four groups and treated with placebo, everolimus, metformin, or a combination of both compounds, until tumor size in control mice reached termination criteria. The combination of everolimus and metformin significantly inhibited the growth of HCC1428 xenografts (p<0.01 compared with control group), whereas everolimus or metformin alone at the tested doses only minimally or partially inhibited the growth of HCC1428 xenografts as measured by both tumor sizes and weights. Moreover, the combination was significantly more potent than each single agent in inhibiting the growth of the xenografts (p<0.01 compared with everolimus or metformin alone treated group) (Fig. 5A–C). These in vivo data further demonstrate that the combination of everolimus and metformin displays augmented anticancer activity. During the treatment, we did not see a significant effect of the combination on body weight loss of the mice (data not shown), suggesting

Figure 3. Effect of the combination of everolimus and metformin on breast cancer cell stemness measured by flow cytometry. Flow cytometry shown from control group (A), everolimus alone (B), metformin alone (C), and combination of everolimus and metformin (D) treatment groups. (E) The CD44+/CD24− cells were valued in different treatment groups. **p<0.01 compared with the control group. ##p<0.01 compared with the everolimus or metformin alone treated group.

| Treatment | p-S6 | S6 | p-4EBP1 | 4EBP-1 | β-actin |
|-----------|-----|----|---------|--------|--------|
| Control   | +   | −  | −       | −      | −      |
| Everolimus (100 nM) | −   | +  | −       | +      | +      |
| Metformin (100μM)  | −   | −  | +       | +      | +      |

Figure 4. The combination of everolimus and metformin synergistically enhanced effects on suppression of S6 and 4EBP-1 phosphorylation in vitro. The expression of p-S6, S6, p-4EBP-1, and 4EBP-1 in different treatment groups was assayed by Western blot.
that the combination is well tolerated. By analyzing tumor tissues, we detected decreased levels of p-S6 and p-4EBP-1 in xenograft tumors treated with everolimus or metformin alone. Moreover, we found that either everolimus or metformin alone partially decreased p-S6 and p-4EBP-1 levels; however, their combination was much more potent than either single agent in reducing p-S6 and p-4EBP-1 levels in xenograft tumors (Fig. 6), demonstrating that the combination of everolimus and metformin also exerts an augmented effect on the suppression of S6 and 4EBP-1 phosphorylation in vivo.

**DISCUSSION**

Accumulated evidence from preclinical research suggests that metformin exerts its positive effect on the clinical course of neoplastic diseases and, in particular, on breast cancer, primarily through the stimulation of AMPK in association with the upstream liver kinase B1 (LKB1) (15). AMPK is a key cellular energy sensor, activation of which by metformin leads to suppression of energy-consuming processes, such as gluconeogenesis, protein, and fatty acid synthesis and, in type 2 diabetics, results in a metabolic normalization of hyperglycemia and of insulin resistance.
In carcinoma cells, the stimulation of AMPK, mediated by metformin, resulted in the inhibition of the mTOR/ribosomal S6 kinase pathway and thus in inhibition of pathological cell cycle progression, cell growth, and angiogenesis (17). Likewise, the stimulation of AMPK by metformin led to significant repression of cell proliferation in both estrogen receptor α (ERα)-negative and -positive human breast cancer cell lines (18,19).

Dysregulation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) activities occurs frequently in breast cancer (20). Inhibition of the mTOR activity by everolimus or its analogs results in the translational inhibition of the proteins required for cell cycle progression, survival, and resistance to apoptosis, thereby inhibiting the growth and the progression of breast tumors, both in vitro and in vivo (4). However, the activity of everolimus as single agent in solid tumors is limited. The limited clinical benefit of everolimus may be associated with the mTOR-dependent feedback loop that acts to inhibit PI3K/AKT activity. Everolimus, by inhibiting mTOR, not only inhibits protein translation, but also inhibits this feedback loop, leading to increased AKT activation (11). This in turn may limit some of the antiproliferative actions of this class of agents. Recently published data indicated that mTOR inhibition, resulting from metformin exposure through AMPK activation, reduced AKT activation, an action opposite to that of everolimus (11). These findings suggested that by integrating metformin into the treatment regime of everolimus in combination with chemotherapeutic agents, the antineoplastic efficacy might be further enhanced.

This study is the first to demonstrate that everolimus combination with metformin inhibited the growth of breast cancer cells both in vitro and in vivo. In previous studies, stem cells are considered to be resistant to antitumor agents. The antitumor drugs kill differentiated cells, which account for the majority of the cells within a tumor, but do not affect the small number of stem cells (21). In this study, we also found that compared with alone, the combination with everolimus and metformin significantly inhibited breast CSCs.

First, we studied the in vitro anticancer effects of everolimus and metformin on breast cancer cell lines and showed that everolimus or metformin suppresses the proliferation of different breast cancer cell lines, including HCC1428, MDA-MB-468, and BT549 cell lines, in a concentration-dependent manner (Fig. 1A, B). We then investigated for the combinatorial effect of everolimus and metformin on tumor cell proliferation in a panel of breast cancer cell lines (Fig. 1C). To our surprise, the cell proliferation inhibition was further intensified when everolimus was added in the treatment regime of metformin. Because of the promising growth-inhibitory effects of the everolimus
and metformin combination in breast cancer cells in vitro, we also validated the efficacy of the combination against the growth of breast cancer tumors in mice. The combination of everolimus and metformin significantly inhibited the growth of HCC1428 xenografts, whereas everolimus or metformin alone at the tested doses only minimally or partially inhibited the growth of HCC1428 xenografts as measured by both tumor sizes and weights. Moreover, the combination was significantly more potent than each single agent in inhibiting the growth of the xenografts ($p<0.01$ compared with everolimus or metformin group). These in vitro and in vivo data further demonstrate that the combination of everolimus and metformin displays augmented anticancer activity.

Previous studies have identified pivotal roles for CSCs in breast cancer growth, invasion, metastasis, and resistance to chemotherapy (22,23). Thus, we evaluated whether combining everolimus with metformin could significantly inhibit breast CSCs. We performed the mammosphere formation assay to determine whether everolimus and metformin could synergistically inhibit the stemness of breast cancer cells. The results showed that control group HCC1428 cells produced more and larger spheres. The combination of everolimus and metformin significantly inhibited the growth of HCC1428 mammosphere, whereas everolimus or metformin alone at the tested doses only minimally or partially inhibited the growth of HCC1428 mammosphere. In addition, compared with the everolimus or metformin alone groups, a significantly greater CD44+/CD24− cell inhibitory effect was observed with the combination treatment in the everolimus + metformin group.

To understand the mechanisms by which the combination of everolimus and metformin exerts enhanced anticancer activity, we analyzed the effects of the combination on the phosphorylation of S6 and 4EBP-1, which are translational repressors whose action is attenuated when phosphorylated by mTOR (14). Everolimus or metformin alone decreased the levels of both p-S6 and p-4EBP-1 in the HCC1428 cell lines. Compared with everolimus or metformin alone, significantly decreased levels of both p-S6 and p-4EBP-1 were observed with the combination treatment in the everolimus + metformin cells. Moreover, we found that either everolimus or metformin alone partially decreased p-S6 and p-4EBP-1 levels; however, their combination was much more potent than either single agent in reducing p-S6 and p-4EBP-1 levels in xenograft tumors, demonstrating that the combination of everolimus and metformin also exerts an augmented effect on suppression of S6 and 4EBP-1 phosphorylation in vivo. Our results suggest that this combination can provide a new strategy for breast cancer treatment. This effect may be sequence dependent, and determining the in vitro mechanism of action still requires further studies.

In summary, the in vivo and in vitro results in this study indicate that the combination of everolimus and metformin can somewhat enhance the inhibitory effect of each individual drug on breast cancer cells. This combination provides a new therapeutic option for breast cancer patients. However, the combined effect of these drugs still requires a large-scale clinical trial for further validation.

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