Combined Targeting of mTOR and AKT Is an Effective Strategy for Basal-like Breast Cancer in Patient-Derived Xenograft Models

Siguang Xu1, Shunqiang Li1,3, Zhanfang Guo1, Jingqin Luo2,3, Matthew J. Ellis1,3, and Cynthia X. Ma1,3

Abstract

Basal-like breast cancer is an aggressive disease for which targeted therapies are lacking. Recent studies showed that basal-like breast cancer is frequently associated with an increased activity of the phosphatidylinositol 3-kinase (PI3K) pathway, which is critical for cell growth, survival, and angiogenesis. To investigate the therapeutic potential of PI3K pathway inhibition in the treatment of basal-like breast cancer, we evaluated the antitumor effect of the mTOR inhibitor MK-8669 and AKT inhibitor MK-2206 in WU-BC4 and WU-BC5, two patient-derived xenograft models of basal-like breast cancer. Both models showed high levels of AKT phosphorylation and loss of PTEN expression. We observed a synergistic effect of MK-8669 and MK-2206 on tumor growth and cell proliferation in vivo. In addition, MK-8669 and MK-2206 inhibited angiogenesis as determined by CD31 immunohistochemistry. Biomarker studies indicated that treatment with MK-2206 inhibited AKT activation induced by MK-8669. To evaluate the effect of loss of PTEN on tumor cell sensitivity to PI3K pathway inhibition, we knocked down PTEN in WU-BC3, a basal-like breast cancer cell line with intact PTEN. Compared with control (GFP) knockdown, PTEN knockdown led to a more dramatic reduction in cell proliferation and tumor growth inhibition in response to MK-8669 and MK-2206 both in vitro and in vivo. Furthermore, a synergistic effect of these two agents on tumor volume was observed in WU-BC3 with PTEN knockdown. Our results provide a preclinical rationale for future clinical investigation of this combination in basal-like breast cancer with loss of PTEN. Mol Cancer Ther; 12(8); 1665–75. ©2013 AACR.

Introduction

Phosphatidylinositol 3-kinase (PI3K)–AKT pathway plays a cardinal role in the transduction of extracellular and intracellular signals that regulate cell growth, proliferation, survival, migration, and angiogenesis (1). Activation of PI3K recruits AKT, a serine/threonine kinase, to the plasma membrane, allowing its activation, which in turn phosphorylates various intracellular proteins, including the serine/threonine kinase mTOR. PTEN, on the other hand, negatively regulates PI3K by dephosphorylatingPIP3. Activation of PI3K pathway is common in cancer, and inhibitors against various components of this pathway are at various stages of preclinical and clinical development for cancer treatment (1).

Basal-like breast cancer is the most common subtype of triple-negative [negative for estrogen receptor (ER), progesterone receptor, and Her-2/neu gene amplification] breast cancer (TNBC) that is associated with an aggressive clinical behavior and for which effective targeted therapies are lacking. Interestingly activation of PI3K/AKT pathway has been associated with basal-like or TNBC. A significantly higher level of AKT phosphorylation by immunohistochemistry (IHC) has been observed in TNBC patient specimens compared with non-TNBC (2). In a study of 292 patients with invasive breast cancer, PI3K pathway activation, assessed by IHC analysis of phosphorylated AKT (pAKT), was significantly associated with basal-like phenotype and breast cancer-specific mortality (3). Similarly the level of pAKT was found to be higher by using reverse-phase protein array analysis in basal-like breast cancers (4). In the recent report of The Cancer Genome Atlas (TCGA), the highest PI3K pathway activity was associated with basal-like breast cancer by both gene expression and phosphoproteomic analysis (5). The increased level of pAKT correlated with lower PTEN protein expression and DNA copy number (4, 5), which is frequently observed in basal-like breast cancer (3, 4, 6). It has been reported that inhibitors against PI3K and mTOR reduced the proliferation of basal-like breast...
cancer cells in cell culture studies (4), supporting PI3K pathway as a therapeutic target in this subtype of breast cancer.

mTOR is the major downstream target of the PI3K/AKT pathway, and inhibitors against mTOR are among the first that entered in the clinic. However, a low response rate (9%) of mTOR inhibitors in the treatment of breast cancer was observed in clinical trials of unselected patient population (7). One potential mechanism of resistance is the AKT activation induced by mTOR inhibitors through a negative feedback loop (8, 9). Therefore, we hypothesized that inhibition of AKT could potentiate the antitumor effect of mTOR inhibitors, and the combined targeting of mTOR and AKT could be an effective approach in treating basal-like breast cancer. In this study, we tested MK-2206, which is an AKT inhibitor, and ridaforolimus MK-8669, an mTOR inhibitor, either alone or in combination in 2 patient-derived xenograft models of basal-like breast cancer.

MK-2206 is an orally active highly potent and selective allosteric pan-AKT inhibitor (structure published in ref. 10) with IC50 being 5 nmol/L, 12 nmol/L, and 65 nmol/L toward purified human AKT1, AKT2, and AKT3, respectively. There is more than 100-fold selectivity for AKT over 250 protein kinases tested (11). MK-2206 has shown single-agent antiproliferative effect in vitro and in vivo, especially for tumors that carry PI3K pathway abnormalities. In addition, additive and synergistic effect has been observed when MK-2206 was combined with various chemotherapy agents and small-molecular inhibitors including erlotinib and lapatinib (10, 11). MK-2206 is the first allosteric AKT inhibitor to enter clinical trials and has been well tolerated (12). The safety and well-tolerated toxicity profile of MK-2206 makes it a feasible partner to combine with other agents to enhance its antitumor effect in clinical trials. In the treatment of breast cancer, MK-2206 is being developed as either a single agent (NCT01240928, NCT01319539, NCT01277757), or in combination with hormonal therapy (NCT01344031), lapatinib (NCT01245205, NCT01281163) or paclitaxel (NCT01263145). Results of these trials are pending (http://clinicaltrial.gov).

MK-8669 (ridaforolimus, deforolimus, AP23573) is a novel selective non-prodrug analogue of rapamycin (structure published in ref. 13) that is undergoing clinical development for cancer therapy (14, 15). It has shown broad antitumor activity in preclinical models of a variety of cancer types (15, 16). MK-8669 has been well tolerated with clinical efficacies observed either as a single agent (17–19) or in combination with paclitaxel (20) or capcitabine (21). A phase I study of MK-2206 in combination with MK-8669 is being conducted in patients with advanced cancer to determine the maximum-tolerated dose and the safety and tolerability of the combination (NCT01295632, ref. 22). Therefore, promising data from preclinical testing of MK-2206 and MK-8669 in basal-like breast cancer could be readily translated into future phase II trials.

Materials and Methods

Chemicals

MK-2206 and MK-8669 were kindly provided by Merck & Co., Inc. MK-2206 (12 mg/mL) was prepared in 30% captsol solution (W/V; CyDex, Cat. No. CY-04A-05006) freshly made on the day of therapy. MK-8669 was prepared in a stock solution of 10 mg/mL inN,N-Dimethylacetamide (Sigma, Cat. No. 270555), then diluted fresh on the day of therapy at 1:50 in vehicle diluents buffer: 10% Tween 80 (Fisher Scientific, Cat. No. 10317080), 40% polyethylene glycol 400 (Sigma, Cat. No.91893) in sterile water.

Generation of WU-BC3 (shPTEN) and WU-BC3 (shGFP)

PTEN and GFP (control) short hairpin RNA (shRNA) Lentiviral packaging vectors (pLKO.1) were obtained from the Genome Institute at the Washington University School of Medicine (St Louis, MO). The targeting sequences are: shPTEN, CGTGCAGATAATGACAAGCTGT; shGFP, CTCTCGGCAATGGACGAGCTGT. PTEN shRNA vector or the control vector was cotransfected into 293T cells with 2 packaging vectors (pHR’8.2 and pCMV-VSV-G) by using FuGENE 6 transfection reagent (Roche, Cat. No. 11815091001) according to the manufacturer’s instructions as previously described (23). Three days posttransfection, the culture supernatant containing shRNA lentiviruses was harvested and filtered through a 0.45 µm filter to ensure removal of any nonadherent cells. WU-BC3 cells were infected with the filtered supernatant in the presence of polybrene (4 µg/mL). Infected cells were selected with puromycin (1.7 µg/mL) for a week to generate stable cell line. Knockdown efficacy of PTEN was assayed by Western blot analysis. No authentication of these cell lines was done by the authors.

In vitro experiments assessing effects of MK-2206 and/or MK-8669 on WU-BC3 (shPTEN) and WU-BC3 (shGFP) cell lines

Cells were cultured in RPMI-1640 medium with 10% FBS at 37°C in an atmosphere of 5% CO2 and 95% air as described previously. For cell proliferation assay, cells were plated in 96-well plates at a density of 750 cells per well and cultured for 1 day, followed by the addition of each agent alone or in combination at various concentrations. Cells were assayed by Western blot analysis. No authentication of these cell lines was done by the authors.
In vivo experiments assessing effects of MK-2206 and/or MK-8669 on WU-BC4, WU-BC5, WU-BC3 (shPTEN), and WU-BC3 (shGFP) xenografts

WU-BC4 and WU-BC5 were established previously (23) and passaged in the “humanized” mammary fat pad of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Passage 4-5 xenografts of WU-BC4 and WU-BC5 were used for the therapy experiments described in this study. Cultured cells of WU-BC3 (shGFP) and WU-BC3 (shPTEN) were used to create respective xenografts for experimental therapy. A total of 1 × 10⁶ tumors of each line and 5 × 10⁵ fibroblasts (2.5 × 10⁵ that were exposed to 4 Gy IR and 2.5 × 10⁵ untreated cells) were mixed and added to an equal volume of a 1:1 mixture of Matrigel (BD Biosciences, Cat. No. 354234) and Collagen I (Millipore, Cat. No. 08-115) to each side of the fourth mammary fat pad that has been cleared of any mouse mammary tissues in female NU/J homozygous mice (Jackson Laboratory, Cat. No. 2019) to propagate xenografts for tumor growth inhibition (TGI) and biomarker assays.

For TGI study, xenografts were allowed to grow to approximately 0.5 cm in the maximum diameter. Mice were then divided into 4 treatment groups (n = 8 mice in each group): Vehicle (30% captisol or vehicle diluents), MK-2206 alone, MK-8669 alone, and the combination of MK-2206 and MK-8669. MK-2206 was administered by oral gavaging 60 mg/kg on day 1 and 80 mg/kg on day 8. This dosing schedule for MK-2206 was chosen because a higher or more frequent dosing was difficult to tolerate in the mice. MK-8669 was administered intraperitoneally (i.p.) 1 mg/kg on days 1 to 5 and 8 to 12. This dosing schedule was previously shown to be effective in sensitive tumors (13). Treatment was repeated for 2 more weeks in WU-BC5. Tumor volume was measured in 2 dimensions (length and width) using Traceable Digital Calipers. The tumor volume (cm³) was calculated as V = (length × width)²/2 × 0.5. The individual relative tumor volume (RTV) was defined as Vt/V0 where Vt is the volume at a given time and V0 at the start of treatment. The percentage tumour growth inhibition (% TGI) was calculated using the equation 100 − (T/C × 100) where T is the mean RTV of the treated tumor and C is the mean RTV in the vehicle-treated group at the time of sacrifice.

For Western blot and IHC biomarker studies on WU-BC4, mice bearing WU-BC4 were treated with either Vehicle (n = 4), MK-2206 alone (120 mg/kg; per os, days 1 and 8; n = 4), MK-8669 alone (n = 3; 1 mg/kg i.p. days 1, 2, 3, 4, 5, and 8), and the combination of MK-2206 and MK-8669 (n = 4). Tumors were harvested 4 hours after day 8 therapy. For biomarker studies in WU-BC5, mice bearing WU-BC5 were treated with either Vehicle (n = 4), MK-2206 alone (120 mg/kg; per os, days 1 and 8; n = 3), MK-8669 alone (1 mg/kg i.p., days 1–5, 8–11; n = 3), or the combination of MK-2206 and MK-8669 (n = 3). Tumors were harvested 4 hours after day 11 therapy. For WU-BC3 (shGFP) and WU-BC3 (shPTEN), mice were treated with either Vehicle (n = 2), MK-2206 alone (100 mg/kg; per os, days 1 and 3; n = 2), MK-8669 alone (n = 2; 0.5 mg/kg i.p. days 1, 2, and 3), and the combination of MK-2206 and MK-8669 (n = 2). Tumors were harvested 4 hours after day 3 therapy. Each xenograft tumor was cut into 3 pieces with one piece in optimum cutting temperature (OCT; Fisher Scientific, Cat. No. 14-373-65) for frozen section, one piece flash frozen for tumor lysate, and the third piece fixed in 10% neutral buffered formalin and embedded in paraffin blocks.

All animal studies were carried out using the appropriate NIH animal care, and the animal study protocol was approved by the Animal Studies Committee of Washington University.

Antibodies

The primary antibodies for both Western blot analysis and IHC were the same for PTEN (Cell Signaling Technology, Cat. No. 9559), pAKT473 (Cell Signaling Technology, Cat. No. 4060), and pS6240/244 (Cell Signaling Technology, Cat. No. 2215). The primary antibodies for Western blot analysis included those that are against p4EBP1Ser65 (Cat. No. 9456), EBP1 (Cat. No. 9452), AKT (Cat. No. 4685), and S6 (Cat. No. 2217) from Cell Signaling Technology, and Actin (BD Biosciences, Cat. No. 612656). The primary antibodies for IHC included those that are against Ki-67 (Lab Vision, Cat. No. RM-9106-50) and CD31 (BD Biosciences, Cat. No. 550274).

Western blot analysis

Western blot analysis was conducted according to previously published procedures (23).

Immunohistochemistry

IHC of interested markers were conducted on 5 μm tissue sections from paraffin-embedded tumor as described previously using the EnVision + Single Reagents HRP-Rabbit (Dako, Cat. No. K4003) and REAL substrate buffer (REAL DAB + chromogen, Dako, Cat. No. K3468; ref. 23). The primary antibodies and dilutions are as follows: pAKT473 (1:200), pS6240/244 (1:200), Ki-67 (1:200), PTEN (1:100). Appropriate positive and negative controls were included.

CD31 immunostaining

Xenograft tumors were removed and frozen in OCT on dry ice and sectioned into 5 μm slides for immunolabeling with anti-CD31 antibody (1:20). Slides were dried for 30 minutes at room temperature, fixed in ice cold acetone for 10 minutes, and hydrated in PBS. The endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in PBS for 15 minutes and in protein block (Dako, Cat. No. X0909) for 15 minutes. Slides were incubated with the primary antibody overnight at 4°C in 1% PBS-BSA. The primary antibody was visualized by using anti-rat Ig HRP Detection Kit (BD Biosciences, Cat. No. 551013). Subsequently, the slides were counterstained with Meyer hematoxylin for 1 minute, dehydrated...
in ascending ethanol series, cleared with xylene, and coverslipped using a permanent mounting medium. Appropriate positive and negative controls were tested.

Statistical analysis
Quantitative data are presented as mean ± SEM unless otherwise indicated. IC_{50} was defined as the drug concentration that decreases the cell growth by 50%. The relative IC_{50} values for MK2206 or/and MK8669 on day 6 in WU-BC3 (shPTEN) or control WU-BC3 (shGFP) cell line were calculated using Origin version 7.5 (OriginLab) software. The combination index of MK2206 and MK8699 on cell growth was analyzed using the software CalcuSyn (Biosoft) as described (24). For IHC data, tumor cells staining positive for Ki-67 or CD31 were counted in 4 randomly selected fields per tumor at ×400 or ×200 magnification, respectively. Approximately 1,500 cells were counted per tumor for Ki67 and the percentage of positively stained tumor cells was calculated. One-way ANOVA was applied to compare IHC measurements among treatments, whereas posthoc Tukey comparison was used to obtain P values comparing between 2 treatments. Tumor volume data were compared using two-way ANOVA (dose, treatment and their interaction) and treatments were pairwise contrasted at each time point of tumor measurement. The synergistic effect of the drug combination on tumor growth (at the end-of-study in natural log scale), Ki67 and CD31 for WU-BC4 and 5 was confirmed by examining the significance of the interaction effect in the two-way ANOVA model with the 2 factors (±MK-2206 and ± MK-8869; ref. 25). All statistical analyses were implemented in R 2.14.1 and GraphPad Prism. All tests were two-sided, and significance was claimed at the 5% level unless otherwise noted.

Results
MK-2206 in combination with MK-8669 inhibited tumor growth of patient-derived xenograft models of basal-like breast cancer with increased PI3K pathway activation
We had previously established WU-BC3, WU-BC4, and WU-BC5 TNBC xenograft models by direct implantation of patient tumor specimens to the humanized mammary fat pad of immune deficient NOD/SCID mice using a revised methodology described by Kuperwasser and colleagues (23, 26). All 3 models were confirmed to be of human origin based on human-specific cytokeratin staining and share similar gene expression pattern with their respective human tumor counterpart (23). WU-BC4 and WU-BC5 were classified as basal like by PAM50 (23, 27, 28). In vitro cultured WU-BC3 cell line, which was derived from WU-BC3 xenograft, was also basal like, although the in vivo xenograft tumor of WU-BC3 was more similarly clustered to the HER2-E subtype but without HER2 overexpression (23). Western blot analysis of pAKT and pS6 indicated an activated PI3K pathway signaling in WU-BC4 and WU-BC5 but not WU-BC3 (Fig. 1A). This is consistent with the loss of PTEN protein expression in tumor cells of WU-BC4 and WU-BC5 but not WU-BC3 (Fig. 1B). Furthermore, PTEN gene deletion was detected in WU-BC5 but not in WU-BC3 by comparative genomic hybridization (aCGH; data not shown). Loss of PTEN expression in WU-BC4 is likely epigenetic because PTEN is normal by both aCGH and genomic sequencing (data not shown). No somatic mutations were detected in other PI3K pathway genes including PIK3CA and AKT1 in these 3 models. These data suggest that the increased PI3K pathway activity in WU-BC4 and WU-BC5 is likely due to loss of PTEN expression.

To investigate the antitumor effect of MK-2206 in combination with MK-8669, WU-BC4, and WU-BC5 were tested as they both have increased PI3K pathway activity. Mice bearing WU-BC4 and WU-BC5 were separated in 4 treatment groups including vehicle, MK-2206 (oral gavage 60 mg/kg on day 1 and 80 mg/kg on day 8), MK-8669 (i.p. 1 mg/kg daily on days 1–5 and days 8–12), or the combination of MK-2206 and MK-8669 (the same dosing and scheduling as single agents; n = 8 in each treatment group). The same treatment was repeated for another 2 weeks in WU-BC5. Mice were followed for 42 days and caliber tumor measurements were conducted every 2 to 3 days starting day 1 therapy (Fig. 1C and D). In both models, single-agent MK-8669 significantly inhibited tumor growth compared with vehicle treatment (P < 0.001). The mean %TGI was 54% in WU-BC4 and 74% in WU-BC5. MK-2206 alone at the dose administered had little effect on WU-BC4, but achieved a mean of 43% TGI in WU-BC5 compared with vehicle treatment (P < 0.001). Combination therapy of MK-2206 and MK-8669 led to a mean TGI of 82% in WU-BC4 and 91% in WU-BC5 (P < 0.001 compared with each agent alone). The combination therapy was synergistic in both models (P = 2.21e-06 in WU-BC4; P = 0.0116 in WU-BC5) by examining the interaction effect in the two-way ANOVA model (Supplementary Fig. S1).

MK-2206 in combination with MK-8669 inhibited tumor cell proliferation in patient-derived xenograft models of basal-like breast cancer
To investigate the effect of MK-2206 and MK-8669 on tumor cell proliferation, IHC of Ki67, a marker of cell proliferation, was conducted on WU-BC4 tumors harvested on day 8 and WU-BC5 tumors on day 11 from each treatment group. Compared with vehicle, treatment with MK-2206, MK-8669, and the combination reduced Ki67 by 45% (P < 0.01), 81% (P < 0.001), and 85% (P < 0.001), respectively, in WU-BC4, and by 71% (P < 0.001), 64% (P < 0.001), and 88% (P < 0.001), respectively, in WU-BC5 (Fig. 2A and B). A synergistic effect on Ki67 was observed for combination therapy in both models by examining the interaction effect in the two-way ANOVA model (P = 0.0051 in WU-BC4 and P = 2.89e-08 in WU-BC5; Supplementary Fig. S2). The degree of apoptosis induction as assessed by IHC of cleaved caspase 3 was small, less than 3%, consistent with a cytostatic rather than cytotoxic effect of this treatment in these models.
MK-8669, either alone or in combination with MK-2206, reduced tumor angiogenesis

Because PI3K pathway inhibition has been shown to inhibit angiogenesis, we conducted IHC analysis of CD31, a marker of endothelial cells, on tumors after treatment with either vehicle, MK-2206, MK-8669, alone or in combination (Fig. 2C and Fig. 2D). Treatment with MK-8669 alone led to 47% reduction of CD31 staining in WU-BC4 (P < 0.05) and 71% reduction in WU-BC5 (P < 0.001). MK-2206 had minimal effect on CD31 staining in WU-BC4, but led to 37% reduction in WU-BC5 (P < 0.001). Combination therapy reduced CD31 staining by 54% in WU-BC4 (P < 0.001) and 71% in WU-BC5 (P < 0.001). There was no synergism observed between the 2 agents.

MK-2206 suppressed MK-8669–induced AKT activation

To investigate target inhibition by MK-2206 and MK-8669, WU-BC4 treated with either vehicle, MK-2206, MK-8669, or the combination of MK-2206 and MK-8669, were analyzed for the levels of pAKT and pS6 (Fig. 3). MK-2206 reduced the level of pAKT and, to a lesser degree, the level of pS6. MK-8669 inhibited S6 phosphorylation but increased that of AKT. The addition of MK-2206 abolished the pAKT expression, which provides a potential mechanism for the additive or synergistic effect observed for these 2 agents.

PTEN knockdown sensitizes the growth inhibitory effect of mTOR and AKT inhibition

PTEN loss is the most common cause of PI3K pathway activation in basal-like breast cancer. To evaluate the effect of PTEN status on tumor cell sensitivity to PI3K pathway inhibition, we generated stable knockdown of PTEN in the cell line derived from the WU-BC3 patient-derived xenograft model. As shown in Fig. 4, PTEN knockdown in WU-BC3 (shPTEN) led to an increase in the level of pAKT and pS6, indicating activated PI3K pathway signaling compared with control knockdown in WU-BC3 (shGFP). In cell culture experiments, treatment with MK-2206 led to a dose-dependent reduction in the levels of pAKT and, to a lesser degree, the levels of pS6 and p4EBP1 (Fig. 4A). Treatment with MK-8669 led to a concentration-dependent reduction in the level of pS6, but also an increase in pAKT at lower dose levels, consistent with the feedback upregulation of AKT activity following mTOR inhibition. The increase in pAKT level was effectively abolished by coadministration of increasing concentrations of MK-2206 (Fig. 4A). We then compared the effect of MK-8669 and MK-2206 on cell proliferation in WU-BC3 (shPTEN) and WU-BC3.
(shGFP) (Fig. 4B and Table 1). The IC\textsubscript{50} for both agents alone was lower in WU-BC3 (shPTEN) than that in WU-BC3 (shGFP), indicating a greater sensitivity to both agents with the loss of PTEN. A strong synergistic effect of these 2 agents on cell proliferation was observed in WU-BC3 (shPTEN) but not in WU-BC3 (shGFP) (Table 1). These in vitro cell culture studies indicated that mTOR and AKT inhibitors are more effective in tumor cells with loss of PTEN and the combination therapy may be most effective.

To further investigate the effect of loss of PTEN on tumor response to mTOR and AKT inhibitors, we established xenograft models of WU-BC3 (shPTEN) and WU-BC3 (shGFP) in the humanized mammary fat pad of NU/J homozygous mice followed by treatment with either vehicle, MK-8669, MK-2206, or the combination of the 2 agents. The established xenografts of WU-BC3 (shPTEN) showed loss of PTEN expression (Fig. 5A) and a higher baseline level of pAKT compared with WU-BC3 (shGFP) (Table 1).
Treatment with MK-8669 induced a higher pAKT level, which was abolished by the addition of MK-2206 in both models (Fig. 5B). In WU-BC3 (shGFP), only combination therapy induced significant TGI (21%; \( P < 0.001 \); Fig. 5C). In contrast, MK-8669 and the combination induced significant tumor growth inhibition, 21% (\( P < 0.001 \)) and 61% (\( P < 0.001 \)), respectively, in WU-BC3 (shPTEN) (Fig. 5D). The synergistic effect of combination therapy on tumor volume was confirmed by examining the significance of the interaction effect in the two-way ANOVA model in WU-BC3 (shPTEN) (\( P = 0.031 \); Supplementary Fig. S3). These data confirmed the in vitro finding

### Table 1. IC\(_{50}\) and combination index of MK-2206 and MK-8669 in WU-BC3 (shGFP or shPTEN)

<table>
<thead>
<tr>
<th></th>
<th>IC(_{50}) (MK-2206)</th>
<th>IC(_{50}) (MK-8669)</th>
<th>Combination index (MK-2206 plus MK-8669)</th>
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<tbody>
<tr>
<td>MK-2206</td>
<td>&gt;8000 nmol/L</td>
<td>&gt;250 nmol/L</td>
<td>CI (ED(<em>{50})) 0.87369 CI (ED(</em>{75})) 0.94280 CI (ED(_{90})) 1.02747 Avg CI 0.94798</td>
</tr>
<tr>
<td>MK-8669</td>
<td>3978 nmol/L</td>
<td>41 nmol/L</td>
<td>CI (ED(<em>{50})) 0.43957 CI (ED(</em>{75})) 0.2063 CI (ED(_{90})) 0.09949 Avg CI 0.24846</td>
</tr>
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**NOTE:** The combination index (CI) value was calculated for ED\(_{50}\), ED\(_{75}\), and ED\(_{90}\); the average CI value for each combination is also shown. A CI of <0.3 indicates a strong synergistic effect, 0.3 < CI < 0.7 a synergistic effect, 0.7 < CI < 0.9 moderate synergy, 0.9 < CI < 1 additive effect, and CI > 1 antagonistic effect.

Figure 4. MK-2206 and MK-8669 inhibit cell proliferation more effectively in PTEN knockdown WU-BC3 cells in vitro. WU-BC3 cell line was stably transfected with shRNA against PTEN or with control shGFP and treated with increasing concentrations of either MK-2206, MK-8669, or the combination followed by Western blot analysis of indicated proteins after 24 hours (A) and cell proliferation assay by AlamarBlue after 6 days of treatment (B). Results in B are averages of 3 independent experiments of triplicates. *, \( P < 0.05 \); **, \( P < 0.01 \); and ***, \( P < 0.001 \).
that loss of PTEN sensitized tumor cells to mTOR and AKT inhibitors, and combination therapy may be most effective.

Discussion
Basal-like breast cancer is 1 of the 5 intrinsic breast cancer subtypes that include luminal A, luminal B, HER2 enriched, basal like, and claudin-low, by gene expression profiling, and is associated with the worst clinical outcome in regards to early relapse and death (27, 29, 30). Although it accounts for about 10% to 15% of all the breast cancer cases, basal-like breast cancer is the most common molecular subtype in TNBC and it is more prevalent in African-American and young women (30–32). The aggressive nature of basal-like breast cancer and the lack of response to established targeted agents against ER and HER2 make it a particular clinical challenge. There is a pressing need to identify effective therapies.

Activation of the PI3K pathway has been increasingly recognized as a potential therapeutic target in TNBC. In the recent TCGA report, PTEN mutation/loss was identified in 35% of basal-like breast cancers (5). In addition, PIK3CA mutation occurred in 7% and INPP4B mutation/loss occurred in 30% of basal-like breast cancer. Furthermore, the highest PI3K pathway activity shown by phosphoproteomic and gene expression analysis was observed in basal-like breast cancer subtype (5). The importance of PI3K pathway activation in the pathogenesis of basal-like breast cancer was further supported by the development of basal-like breast cancer due to loss of PTEN in animal models (33, 34). Intriguingly, in the phase I study of the PI3K inhibitor BKM120 (Novartis) in 35 patients with resistant metastatic solid tumors, the only partial response was observed in a patient with TNBC, indicating the validity of PI3K pathway as a therapeutic target for this disease.

We showed in this study that mTOR and AKT inhibition is effective in reducing tumor growth in patient-derived basal-like breast cancer xenograft models with high levels of PI3K pathway signaling activity. In addition, we showed that PTEN knockdown sensitized tumor cells to treatment. These results provided a preclinical rational for clinical testing of PI3K pathway inhibition in basal-like breast cancer. If the efficacy is confirmed in clinical trials, this therapeutic approach has the potential to impact a large number of basal-like breast cancers because of the high prevalence of PTEN deficiency in this patient population.

The synergistic or additive antitumor effect between mTOR and AKT inhibitors observed in our study indicates that combination strategy of the 2 classes of agents are likely to be most effective in basal-like breast cancer. Our data confirmed the previous observation of AKT activation by mTOR inhibitors in cancer cells (8, 9). Zheng and colleagues recently reported their in vitro data of
The combined approach of targeting both mTOR and AKT, and loss of PTEN could be a potential predictor of response to this approach. Future studies are needed to identify additional predictors and resistant mechanisms to this treatment approach. Preclinical models of different tumor types, including TNBC, suggested that PI3K pathway inhibition may lead to activation of extracellular signal-regulated kinase, or upstream receptor tyrosine kinases could contribute to tumor resistance to PI3K pathway inhibitors, and the combination therapy may be more effective (43–49). Preclinical studies that address these questions in the context of the heterogeneity of TNBC are needed.

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

Authors’ Contributions
Conception and design: S. Xu, S. Li, M.J. Ellis, C.X. Ma
Development of methodology: S. Xu, S. Li, M.J. Ma
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Xu, S. Li, Z. Guo, M.J. Ellis, C.X. Ma
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Xu, S. Li, Z. Guo, J. Luo, M.J. Ellis, C.X. Ma
Writing, review, and/or revision of the manuscript: S. Xu, S. Li, Z. Guo, J. Luo, M.J. Ellis, C.X. Ma
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Xu, S. Li, C.X. Ma
Study supervision: S. Xu, M.J. Ellis, C.X. Ma

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