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

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List of Abbreviation: PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; PTEN, phosphatase and tensin homolog; ER, estrogen receptor; PR, progesterone receptor; TNBC, triple negative breast cancer; IHC, immunohistochemistry; RTV, relative tumor volume; TGI, tumor growth inhibition; I.P., intraperitoneal; TCGA, the Cancer Genome Atlas; aCGH, array Comparative Genomic Hybridization

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Abstract

Basal-like breast cancer is an aggressive disease, for which targeted therapies are lacking. Recent studies demonstrated 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 anti-tumor 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 demonstrated high levels of AKT phosphorylation and loss of phosphatase and tensin homolog (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 to 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.
Introduction

Phosphatidylinositol 3-kinase (PI3K)-AKT pathway plays a cardinal role in the transduction of extra-cellular and intra-cellular 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 (mammalian target of rapamycin). PTEN, on the other hand, negatively regulates PI3K by dephosphorylating PIP3. 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 (PR) 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.

We have decided to start with an mTOR inhibitor as our agent of choice since 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 anti-tumor 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 two 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 (10)), with IC50 being 5 nmol/L, 12 nmol/L, and 65 nmol/L toward purified human AKT1, AKT2, and AKT3, respectively. There is an over 100 fold selectivity for AKT over 250 protein kinases tested (11). MK-2206 has shown single agent anti-proliferative effect in vitro and in vivo, especially for tumor 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 anti-tumor 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 reference (13)) that is undergoing clinical development for cancer therapy (14, 15). It has demonstrated broad anti-tumor activity in preclinical models of a variety of cancer types (13, 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 capecitabine (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) (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 (12mg/ml) was prepared in 30% captisol solution (W/V) (CyDex, Lenexa, KS, Cat. No. CY-04A-05006) freshly made on the day of therapy. MK-8669 was prepared in a stock solution of 10mg/ml in DMA (N,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, Pittsburgh, PA, Cat. No. 10317080), 40% PEG-400 (Polyethylene glycol 400) (Sigma, Cat. No. 91893) in sterile water.

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

PTEN and GFP (control) shRNA Lentiviral packaging vectors (pLKO.1) were obtained from the Genome Institute at the Washington University School of Medicine in St Louis. The targeting sequences are: shPTEN, CGTGCAGATAATGACAAGGAA; shGFP, CTCTCGGCATGGACGAGCTGT). PTEN shRNA vector or the control vector was co-transfected into 293T cells with two packaging vectors (pHR'8.2ΔR and pCMV-VSV-G) by using FuGENE 6 transfection reagent (Roche, Indianapolis, IN, Cat. No. 11815091001) according to the manufacturer's instructions as previously described (23). Three days post-transfection, the culture supernatant containing shRNA lentiviruses was harvested, and filtered through a 0.45 µm filter to ensure removal of any non-adherent 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. 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 RPIM-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/well and treated with MK-2206 and/or MK-8669 at various concentrations in triplicates. Cell growth was assessed 6 days later by measuring Alamar Blue reduction (555λ_{Ex}/585λ_{Em}) using a fluorescent microplate reader. For Western blot analysis of treatment effect from MK-2206 and/or MK-8669, cells were plated at a density of 6 × 10^5 cells/well in 6-well plate and cultured for 1 day, followed by the addition of each agent alone or in combination at various concentrations. Cells were harvested 24 hours later and lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton, 1 mM PMSF and Protease Inhibitor Cocktail (Sigma) for 20 min on ice. Western blot procedures were performed using the standard techniques as described previously (23).

**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 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. 1 x $10^6$ tumor cells of each line and 5 x $10^5$ fibroblasts (2.5 x $10^5$ that were exposed to 4 Gy IR and 2.5 x $10^5$ 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, Billerica, MA, Cat. No. 08-115) to each side of the 4th mammary fat pad that has been cleared of any mouse mammary tissues in female NU/J homozygous mice (Jackson lab, Cat. No. 2019) to propagate xenografts for tumor growth inhibition and biomarker assays.

For tumor growth inhibition study, xenografts were allowed to grow to approximately 0.5cm 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; 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 since a higher or more frequent dosing was difficult to tolerate in the mice. MK-8669 was administered I.P. (intraperitoneal injection) 1 mg/kg on days 1-5, and 8-12. 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 two dimensions (length and width) using Traceable Digital Calipers. The following formula was used to calculate tumor volume: tumor volume (cm$^3$) = (length x width$^2$) x 0.5. The individual relative tumor volume (RTV) was defined as $V_x/V_I$, where $V_x$ is the volume at a given time and $V_I$ at the start of treatment. The percentage tumor growth inhibition (% TGI) was calculated using the equation 100-($T/C$ x 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; PO, days 1 and 8) (n=4); MK-8669 alone (n=3) (1 mg/kg, I.P. days 1, 2, 3, 4, 5, 8); and the combination of MK-2206 and MK-8669 (n=4). Tumors were harvested 4 hours post 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; PO, 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 post 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; PO, days 1 and 3) (n=2); MK-8669 alone (n=2) (0.5 mg/kg, I.P. days 1, 2, 3); and the combination of MK-2206 and MK-8669 (n=2). Tumors were harvested 4 hours post day 3 therapy. Each xenograft tumor was cut into 3 pieces with one piece in 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 and IHC were the same for PTEN (Cell Signaling Technology, Danver, MA, Cat. No. 9559), pAKT<sup>473</sup> (Cell Signaling Technology, Danvers, MA, Cat. No. 4060), pS6<sup>240/244</sup> (Cell Signaling Technology, Cat. No. 2215). The primary antibodies
for Western blot included those that are against p4EBP1^{Ser65} (Cat. No.9456), 4EBP1 (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, Fremont, CA, Cat. No. RM-9106-S0) and CD31 (BD Biosciences, Cat. No. 550274).

**Western Blot**

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

**Immunohistochemistry (IHC)**

IHC of interested markers were performed on five-micron 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) (23). The primary antibodies and dilutions are as follows: pAKT^{473} (1:200), pS6^{240/244} (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 min. at room temperature, fixed in ice cold acetone for 10 min, and hydrated in PBS. The endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in PBS for 15 min 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's hematoxylin for 1 min, 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 (Standard Error of Mean) unless otherwise indicated. IC50 was defined as the drug concentration that decreases the cell growth by 50%. The relative IC50 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 MK8669 on cell growth was analyzed using the software CalcuSyn (Biosoft, Ferguson, MO) 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 400X or 200X magnification, respectively. Approximately 1500 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 while post-hoc Tukey's comparison was used to obtain p-values comparing between two treatments. Tumor volume data were compared using two-way ANOVA (dose, treatment and their interaction) and treatments were pair-wise 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 two factors (+/-MK-2206 and +/- MK-8869) (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 et al. (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 over expression (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 since 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 AKTI 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 anti-tumor 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 gavaging 60 mg/kg on day 1 and 80 mg/kg on day 8), MK-8669 (IP 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 performed every 2-3 days starting day 1 therapy (Figs. 1c and 1d). In both models, single agent MK-8669 significantly inhibited tumor growth compared to 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% tumor growth inhibition in WU-BC5 compared to vehicle treatment (p<0.001). Combination therapy of MK-2206 and MK-8669 led to a mean tumor growth inhibition of 82% in WU-BC4 and 91% in WU-BC5 (p<0.001 compared to 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 (Supplemental Fig. 1).

**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 performed on WU-BC4 tumors harvested on day 8 and WU-BC5 tumors on day 11 from each treatment group. Compared to 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 2b). 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) (Supplemental Fig. 2). 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**

Since PI3K pathway inhibition has been shown to inhibit angiogenesis, we performed IHC analysis of CD31, a marker of endothelial cells, on tumors post treatment with either vehicle, MK-2206, MK-8669, alone or in combination. 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 two 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 to 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 co-administration 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) (Figs. 4b, c, d, and Table 1). The IC50 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 two agents on cell proliferation was observed in WU-BC3 (shPTEN), 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 two agents. The established xenografts of WU-BC3 (shPTEN) demonstrated loss of PTEN expression (Fig. 5a) and a higher baseline level of pAKT compared to WU-BC3 (shGFP) (Fig. 5b). 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 tumor growth inhibition (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). 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) (Supplemental Fig. 3). These data confirmed the in vitro finding 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 one of the five 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 10-15% of all 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). Additionally, PIK3CA mutation occurred in 7% and INPP4B mutation/loss occurred in 30% of basal-like breast cancer. Furthermore, the highest PI3K pathway activity demonstrated 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 demonstrated 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 demonstrated 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 two 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 et al recently reported their in vitro data of rapamycin in combination with A-443654, an AKT inhibitor, in human breast cancer cell lines, which demonstrated that the combination therapy inhibited cell proliferation by G2/M arrest and induced apoptosis (35). In another report, similar to our finding, the combination of MK-2206 and MK-8669 was also found to be synergistic in a genetically engineered mouse model of prostate cancer in a recent publication (36).

In addition to inhibiting PI3K pathway signaling in tumor cells, we demonstrated that mTOR inhibition with or without AKT inhibition was associated with a reduction in angiogenesis, although this effect on tumor growth inhibition is uncertain. This data is consistent with the
literature report the mTOR inhibition decreased expression of hypoxia inducible factor-1 (HIF-1), an important stimulator of vasculogenesis (37).

An advantage of our study is the use of early passage patient-derived xenograft models, which are more closely related to the human tumor counterpart. These xenograft models preserve cell morphology, architecture and molecular features and recapitulate the biology of their human counterpart (23, 38-41). Importantly, drug response of a tumor xenograft and its original human counterpart has been found to be highly correlated (38, 39). Patient-derived xenografts were first used in the 1970s and have shown encouraging results in predicting clinical drug response. A particularly important, large panel of xenografts, derived directly from biopsies, was established by Fiebig and colleagues at the University of Freiburg in Germany (42). In a comparison of cancer treatment response of direct patient-derived xenograft models from breast, lung, ovarian and testicular cancers and their human treatment, the xenografts correctly predicted response in 90% of patients (19 of 21 tumors) and resistance in 97% (57 of 59) (43). We have confirmed previously the similarities between the xenograft and the human tumor counterpart by gene expression for WU-BC4 and WU-BC5 (23). Compared to xenograft models established from existing cell lines these models have the potential to be more predictive in the testing of cancer therapeutic agents.

In summary, our preclinical study in patient-derived xenograft models of basal-like breast cancer indicated that a subgroup of basal-like breast cancer could benefit from 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 (ERK) or upstream receptor tyrosine kinases could contribute to tumor resistance to PI3K pathway inhibitors and combination therapy may be most effective (44-50). Preclinical studies that address these questions in the context of the heterogeneity of TNBC are needed.

Acknowledgement

We thank the patients who donated the tumor specimens for the generation of patient-derived xenograft models of TNBC.

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References


11. Yan L. MK-2206: A potent oral allosteric AKT inhibitor. AACR Meeting Abstracts. 2009; Abstract #DDT01-1


<table>
<thead>
<tr>
<th></th>
<th>IC50</th>
<th>Combination Index (MK-2206 plus MK-8669)</th>
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<tbody>
<tr>
<td></td>
<td>MK-2206</td>
<td>MK-8669</td>
</tr>
<tr>
<td>WU-BC3 (shGFP)</td>
<td>&gt;8000 nM</td>
<td>&gt;250 nM</td>
</tr>
<tr>
<td></td>
<td>CI (ED50) 0.87369</td>
<td>CI (ED75) 0.94280</td>
</tr>
<tr>
<td>WU-BC3 (shPTEN)</td>
<td>3478 nM</td>
<td>41 nM</td>
</tr>
<tr>
<td></td>
<td>CI (ED50) 0.43957</td>
<td>CI (ED75) 0.20633</td>
</tr>
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The Combination Index (CI) value was calculated for ED50, ED75, and ED90; 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 legend

Fig. 1 MK-8669, either alone or in combination with MK-2206, inhibited tumor growth in patient-derived xenograft models of basal-like breast cancer with increased PI3K pathway activity

a. Tumor lysates of WU-BC3, 4 and 5 were subjected to Western blot analysis of indicated markers.  b. IHC of PTEN.  c and d. Tumor growth curves for mice bearing WU-BC4 and 5 treated with vehicle, MK-2206, MK-8669 or the combination. ***p<0.001

Fig. 2 MK-2206 in combination with MK-8669 inhibited tumor cell proliferation and angiogenesis in WU-BC4 and WU-BC5

Mice harboring WU-BC4 and 5 were treated as indicated and tumors were analyzed for Ki67 (a and b) and CD31 (c and d) and by IHC. Representative images are shown in panels a and c, respectively, for Ki67 and CD31 (total magnification x400). Quantitations on percentage of tumor cells positive for staining ± 95% confidence intervals for Ki67 and number of CD31 positive blood vessels per 200x magnification are shown in panels b and d, respectively. Notable comparisons to Vehicle group are marked with *** if \( p < 0.001 \) and notable comparisons to the Combination group are marked with ## if \( p < 0.01 \) and ### if \( p < 0.001 \).

Fig. 3 MK-2206 inhibits MK-8669 induced AKT activation

Mice harboring WU-BC4 were treated as indicated and tumors were analyzed for pAKT\(^{473}\) and pS6 by IHC post treatment.
Fig. 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 and 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 panel b are averages of 3 independent experiments of triplates. *<0.05,**p<0.01 and ***p<0.001.

Fig. 5  MK-2206 and MK-8669 reduced tumor growth synergistically in the PTEN knockdown WU-BC3 xenograft model

a, IHC of PTEN indicated reduced PTEN expression in the WU-BC3 (shPTEN) xenograft model. b, Western blot analysis of indicated markers were performed on xenografts of WU-BC3 (shGFP) or WU-BC3 (shPTEN) harvested on day 3 post treated with either vehicle, MK-2206, MK-8669, or the combination. c and d, Tumor growth curves for mice bearing WU-BC3 (shGFP) and WU-BC3 (shPTEN) treated with vehicle, MK-2206, MK-8669 or the drug combination. *<0.05,**p<0.01 and ***p<0.001.
Fig. 1

a) Western blot analysis showing expression levels of pS6\textsuperscript{240/244}, S6, pAKT\textsuperscript{473}, AKT, and Actin in WU-BC3, WU-BC4, and WU-BC5.

b) Representative immunohistochemical images of WU-BC3, WU-BC4, and WU-BC5.

c) Graph depicting tumor volume cm\(^3\) over 40 days for WU-BC4 with the following treatments:
- Vehicle
- MK-2206
- MK-8669
- Combination

MK-2206 and MK-8669 are administered at specific time points indicated by the horizontal lines.

d) Similar graph for WU-BC5.

Vehicle, MK-2206, MK-8669, and Combination treatments are represented by different markers and lines.
Fig. 2

(a) Vehicle  MK-2206  MK-8669  Combination

(b) KI67 Positive Cells %

(c) Vehicle  MK-2206  MK-8669  Combination

(d) CD31 (+) Blood Vessels/field
Fig. 3

Vehicle       MK-2206    MK-8669    Combination

pS6240/244

PAK7

Vehicle       MK-2206    MK-8669    Combination
**Fig. 4**

(a) Western blot analysis showing the effects of MK2206 and MK8669 on PTEN, pAKT^{473}, AKT, pS6, S6, p4EBP1, 4EBP1, and Actin in WU-BC3 (shGFP) and WU-BC3 (shPTEN) cells.

(b) Graphical representation of relative cell growth percentages in WU-BC3 (shGFP) and WU-BC3 (shPTEN) cells treated with MK-2206, MK-8669, and MK-2206 + MK-8669 at various concentrations.
Fig. 5

(a) WU-BC3 (shGFP) vs WU-BC3 (shPTEN)

(b) Western blot analysis of WU-BC3 (shGFP) and WU-BC3 (shPTEN) cells treated with vehicle, MK-2206, MK-8669, or a combination.

(c) Tumor volume cm³ over 60 days for WU-BC3 shGFP treated with Vehicle, MK-2206, MK-8669, or a combination.

(d) Tumor volume cm³ over 60 days for WU-BC3 shPTEN treated with Vehicle, MK-2206, MK-8669, or a combination.
Molecular Cancer Therapeutics

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

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