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Biomarkers of Response to Akt Inhibitor MK-2206 in Breast Cancer

Takafumi Sangai¹, Argun Akcakanat¹, Huiqin Chen², Emily Tarco^{1,2}, Yun Wu³, Kim-Anh Do⁴, Todd W. Miller⁶, Carlos L. Arteaga^{6,7}, Gordon B. Mills⁵, Ana Maria Gonzalez-Angulo^{2,5}, and Funda Meric-Bernstam¹

¹Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas

²Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas

³Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁴Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁵Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁶Department of Cancer Biology, Medicine, Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University, Nashville, Tennessee

⁷Department of Medicine, Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University, Nashville, Tennessee

Abstract

Purpose—We tested the hypothesis that allosteric Akt inhibitor MK-2206 inhibits tumor growth, and that *PTEN/PIK3CA* mutations confer MK-2206 sensitivity.

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Corresponding Author: Funda Meric-Bernstam, Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, 1400 Pressler Street, Unit 1484, Houston, TX 77030-4009. Phone: 713-745-4453; Fax: 713-745-4926; fmeric@mdanderson.org.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Sangai, A.M. Gonzalez-Angulo, F. Meric-Bernstam

Development of methodology: T. Sangai, K.-A. Do, G.B. Mills, A.M. Gonzalez-Angulo, F. Meric-Bernstam

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Sangai, A. Akcakanat, T.W. Miller, C.L. Arteaga, F. Meric-Bernstam

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Sangai, H. Chen, Y. Wu, K.-A. Do, G.B. Mills, A.M. Gonzalez-Angulo, F. Meric-Bernstam

Writing, review, and/or revision of the manuscript: T. Sangai, A. Akcakanat, Y. Wu, K.-A. Do, T.W. Miller, G.B. Mills, A.M. Gonzalez-Angulo, F. Meric-Bernstam

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Sangai, E. Tarco, F. Meric-Bernstam

Study supervision: G.B. Mills

Experimental Design—MK-2206 effects on cell signaling were assessed *in vitro* and *in vivo*. Its antitumor efficacy was assessed *in vitro* in a panel of cancer cell lines with differing PIK3CA and PTEN status. Its *in vivo* efficacy was tested as a single agent and in combination with paclitaxel.

Results—MK-2206 inhibited Akt signaling and cell-cycle progression, and increased apoptosis in a dose-dependent manner in breast cancer cell lines. Cell lines with *PTEN* or *PIK3CA* mutations were significantly more sensitive to MK-2206; however, several lines with *PTEN*/*PIK3CA* mutations were MK-2206 resistant. siRNA knockdown of PTEN in breast cancer cells increased Akt phosphorylation concordant with increased MK-2206 sensitivity. Stable transfection of PIK3CA E545K or H1047R mutant plasmids into normal-like MCF10A breast cells enhanced MK-2206 sensitivity. Cell lines that were less sensitive to MK-2206 had lower ratios of Akt1/Akt2 and had less growth inhibition with Akt siRNA knockdown. In PTEN-mutant ZR75-1 breast cancer xenografts, MK-2206 treatment inhibited Akt signaling, cell proliferation, and tumor growth. *In vitro*, MK-2206 showed a synergistic interaction with paclitaxel in MK-2206-sensitive cell lines, and this combination had significantly greater antitumor efficacy than either agent alone *in vivo*.

Conclusions—MK-2206 has antitumor activity alone and in combination with chemotherapy. This activity may be greater in tumors with PTEN loss or *PIK3CA* mutation, providing a strategy for patient enrichment in clinical trials.

Introduction

PI3K/Akt/mTOR signaling plays key roles in cell growth, protein translation, autophagy, metabolism, and cell survival (1). Activation of Akt signaling contributes to the pathogenesis of cancer. *PTEN* is mutated in many tumor types, and PTEN expression is decreased in many cancers, including sporadic breast cancer. Mutations in the *PIK3CA* gene, which encodes the catalytic subunit of PI3K, have been reported in many cancer types, and occur in more than 20% of breast cancers (2).

Although controversial, breast cancers with an increased level of Akt phosphorylation/activation or a gene expression signature of *PTEN* loss have been proposed to have a poor outcome (3). Although *PIK3CA* mutations have not been uniformly associated with activation of Akt signaling in patient tumors (2, 4), several *PIK3CA* mutations have been shown to have a gain-of function, leading to an increase in Akt phosphorylation in preclinical models (5, 6). The existing preclinical data suggest that most tumors expressing a low level of PTEN and many with a mutant PIK3CA rely on Akt for oncogenic signaling. Loss of PTEN activity and activation of phosphatidylinositol 3-kinase (PI3K) signaling are associated with resistance to endocrine therapy (7, 8), and resistance to trastuzumab (9–11). Thus, Akt is a very promising target for breast cancer therapy.

MK-2206 (Merck Oncology) is a novel selective allosteric inhibitor of Akt. Phase II clinical trials of MK-2206 have begun for the treatment of a variety of tumor types, including breast cancer. Thus, there is a pressing need to better understand the antitumor efficacy of this novel compound, both when used alone and in combination regimens, and to define markers that predict benefit from this agent. We sought to determine the antitumor efficacy of MK-2206 in breast cancer cell lines with varying genetic backgrounds. Our data show that MK-2206 inhibited Akt signaling and cell-cycle progression, and increased apoptosis in a dose-dependent manner. MK-2206 sensitivity was significantly greater in cell lines with PTEN loss or *PIK3CA* mutation. In MK-2206-sensitive cells, MK-2206 was synergistic with paclitaxel. MK-2206 also had a dose-dependent growth-inhibitory effect *in vivo*, and enhanced the antitumor activity of paclitaxel.

Materials and Methods

Cell lines and cultures

Cell lines were obtained from American Tissue Culture Collection: BT474, MCF7, HCC70, HCC1954, HT29, MCF10A, MCF12A, MDA-MB-231, MDA-MB-435, MDA-MB-453, MDA-MB-468, NCI/H727, SKBR3, U87MG, and ZR75-1. NCI/ADR-RES cells were obtained from the National Cancer Institute. MCF7 cells stably transfected with PTEN small hairpin RNA (shRNA) or mismatch control shRNA were cultured with 1 µg/mL of puromycin (7). Before starting experiment, these cells were cultured in phenol red-free medium supplemented with 2% charcoal-stripped FBS for 24 hours. PIK3CA p110 wild-type, E545K mutant, and H1047R mutant plasmids were stably transfected into MCF10A cells and cultured with 4 µg/mL of blasticidin. All cell lines were passaged less than 6 months after resuscitation and authenticated by vendors.

Reagents

MK-2206 was provided by Merck and Co., Inc. and used in all *in vitro* studies and most *in vivo* studies. *In vivo* studies in BT474 and MCF7 were conducted with MK-2206 produced in-house. For *in vivo* experiments, 30% Captisol (CYDEX Pharmaceuticals) was used as a vehicle. Rapamycin was purchased from LC Laboratories, Inc.

Cell growth assay

Antiproliferative activity was tested by sulforhodamine B (SRB) assay (12). The median inhibitory concentration (IC₅₀) and combination index (CI) were determined from dose-response curves for 4 days treatment (13).

Cell-cycle analysis and annexin V binding assay

For cell cycle assay, cells that were attached to the petridish and floating cells were collected. Samples were analyzed by flow cytometry and ModFit LT software (Verity Software House). Apoptosis was identified by using the annexin V apoptosis kit (Roche) according to the manufacturer's protocol, and cells were analyzed by flow cytometry and FlowJo (Tree Star; 13).

siRNA

siRNA duplexes (Sigma) were used to silence PTEN (14). Negative control siRNA was purchased from Life Technologies Co. Akt1-, Akt2-, and Akt3-specific siRNA pool, non-targeting pool siRNA, and DharmaFECT transfection reagent were purchased from Thermo Fisher Scientific, Inc.

Reverse-phase protein arrays

Reverse-phase protein array (RPPA) analysis was conducted as described previously (15–17). Cell lines were treated with MK-2206 (50 nmol/L, 150 nmol/L, 500 nmol/L, and 5 µmol/L) or 0.1% dimethyl sulfoxide (DMSO) for 24 hours. Each condition was carried out in 3 biologic replicates.

Multiplex phosphoprotein assays

Xenograft samples were lysed in RPPA lysis buffer. Final protein concentration was corrected to 1 µg/µL. Akt, pAkt S473, pGSK3 S9 were measured by MSD (Meso Scale Discovery) assay following the vendor's instructions.

Western blotting

Immunoblotting was conducted as described previously (18) with the following antibodies: Akt, Akt1, Akt2, Akt3, pAktT308, pAktS473, GSK3, pGSK3 S9, pPRAS40T246, pFOXO1/FOXO3a T24/T32, pBad S112, pBad S136, mTOR, pmTOR S2448, S6K, pS6K T389, pS6 S235/236, 4E-BP1, p4E-BP1 T37/46, p4E-BP1 S65, p4E-BP1 T70, PTEN (Cell Signaling Technology, Inc.), PHLPP1, PHLPP2 (Bethyl Laboratories, Inc.), actin (Sigma-Aldrich), INPP4B (Santa Cruz Biotechnology), pmTOR S2481 (Millipore), and vinculin (Abcam).

Immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Immunohistochemical analysis was conducted using pAkt S473 (Cell Signaling) and Ki67 antibodies (Dako) on the same samples as MSD assay and Western blotting from the first *in vivo* study ($n = 4$).

For detection of apoptosis, tissue sections of formalin-fixed, paraffin-embedded xenografts were stained using Peroxidase *In Situ* Apoptosis Detection Kit (Millipore) following the manufacturer's instructions.

In vivo studies

All animal experiments were approved by the MD Anderson Animal Care and Use Committee. ZR75-1 (1×10^7), MCF7 (5×10^6), and BT474 (5×10^6) cells were inoculated in the mammary fat pads of female nu/nu mice (Department of Experimental Oncology, MD Anderson). MCF7 and BT474 cell suspensions were mixed with Matrigel (BD Biosciences). All mice were implanted with 17 β -estradiol pellets (Innovative Research of America) subcutaneously. More than one mice death in the study arm was considered toxicity.

In the single-agent MK-2206 treatment experiment, the mice bearing ZR75-1 xenografts were randomized into 3 groups (vehicle, MK-2206 240 mg/kg, or 480 mg/kg, $n = 5-6$). The mice bearing MCF7 and BT474 xenografts were randomized into 2 groups (MCF7: vehicle and MK-2206 360 mg/kg, $n = 7$; and BT474: vehicle and MK-2206 360 mg/kg, $n = 6$).

To determine the *in vivo* efficacy of MK-2206 in combination with paclitaxel, mice were randomized into 4 groups (vehicle, 15 mg/kg paclitaxel, 360 mg/kg MK-2206, or both, $n = 7$). In the second combination therapy experiment, the mice were randomized into 5 groups (vehicle, 5 mg/kg paclitaxel, 240 mg/kg MK-2206, both simultaneously, or paclitaxel followed by MK-2206 24 hours later, $n = 6-8$).

All of the treatments were given weekly in these 3 experiments. Tumor volumes were calculated as previously described (13). Mice were euthanized 24 hours after the last treatment, and half of each tumor was snap-frozen and the other half was fixed in formalin and embedded in paraffin.

To determine whether apoptosis is induced *in vivo* by MK-2206 and paclitaxel, the mice bearing ZR75-1 tumor were randomized into 5 groups [vehicle, 15 mg/kg paclitaxel, 360 mg/kg MK-2206, paclitaxel (15 mg/kg) and MK-2206 (360 mg/kg), and 480 mg/kg MK-2206, $n = 3-4$]. All of the treatments were given once only in this experiment. Mice were euthanized 48 hours after the treatment and tumors were fixed in formalin and embedded in paraffin.

Statistical analysis

For *in vitro* studies, comparison between 2, and multiple, groups were carried out by the Student *t* test and 1-way ANOVA followed by Tukey multiple comparison test, respectively.

An isotonic regression model was used to identify a subset of proteins that had a monotone relationship between protein expression and dosage [false discovery rate (FDR) <0.3; refs. 19 and 20].

Association between PIK3CA/PTEN mutation status and MK-2206 sensitivity was tested with Fisher exact test. For the *in vivo* study, pairwise *t* tests were adjusted by the FDR method. The Tukey and FDR methods were used to adjust for multiplicities. All *in vitro* experiments were conducted at least 3 times. Data were presented as means $\pm$ SE.

Results

MK-2206 inhibits Akt signaling

MK-2206 is a novel allosteric Akt inhibitor with selective activity against Akt1 and Akt2 (21). To determine the effect of MK-2206 on cell signaling, we assessed the effect of MK-2206 on the functional proteomic profiles of 7 breast cancer cell lines of different subtypes and genetic backgrounds. RPPA showed a dose-dependent decrease in the expression of 10 markers, including pAkt T308 and pAkt S473 (FDR < 0.3, Supplementary Table S1), and Akt downstream signaling (Fig. 1A).

To further study the effects of MK-2206 treatment dose and duration, we treated ZR75-1 breast cancer cells, a cell line with a hemizygous deletion of *PTEN* and a missense mutation in the remaining allele (22), with either rapamycin (100 nmol/L), an allosteric mTOR inhibitor, or increasing doses of MK-2206 for 24 hours (Fig. 1B). An MK-2206 concentration of 50 nmol/L, a concentration that is clinically achievable in plasma (23), led to decreases in pAkt T308 and pAkt S473 levels and inhibited Akt signaling. Inhibition of Akt activity was confirmed by dose-dependent decreases in phosphorylation of Akt downstream targets GSK3 β , PRAS40, FOXO1/FOXO3a, and Bad. None of these non-mTOR-mediated signaling events was inhibited by rapamycin. Phosphorylation of mTOR target S6K and its target S6 were inhibited by MK-2206, although not as robustly as by rapamycin. In contrast, high doses MK-2206 (500 nmol/L and 5,000 nmol/L) inhibited 4E-BP1 phosphorylation more than rapamycin.

To determine the time course of MK-2206 effects, ZR75-1 cells were treated with MK-2206 150 nmol/L and collected after 1, 2, 6, 24, and 48 hours (Fig. 1C). MK-2206 inhibited Akt phosphorylation and downstream signaling within 1 hour, and this inhibition continued for at least 48 hours in terms of pAkt levels. Intriguingly, mTOR targets S6K/S6 and 4E-BP1 returned to baseline phosphorylation at delayed time points despite continued inhibition of pAkt, potentially representing other signaling pathways integrating with mTOR signaling.

MK-2206 inhibits cell cycle and induces apoptosis

The effect of MK-2206 on cell-cycle progression was analyzed by flow cytometry (Fig. 1D). Breast cancer cell lines were treated with vehicle, rapamycin, or MK-2206 for 4 days, and percentages of cells in G₁, S, and G₂-M phases of the cell cycle were determined. Both MK-2206 and rapamycin significantly inhibited cell-cycle progression from G₁ to S-phase in ZR75-1 and MCF7 cells, but not in MDA-MB-231 cells, which were resistant to both agents. In MDA-MB-468, rapamycin significantly increased the percentage of cells in G₁ phase, but MK-2206 did not.

To determine whether MK-2206 induces apoptosis, 3 of the most MK-2206-sensitive breast cancer cell lines were treated with vehicle or increasing concentrations of MK-2206 for 3 days, and the percentages of annexin V-positive cells were determined (Fig. 1E). High doses MK-2206 (500 nmol/L and 5,000 nmol/L) significantly induced apoptotic cell death in all 3 cell lines.

Sensitivity to MK-2206 is associated with PTEN and PIK3CA status

We tested the MK-2206 sensitivity of 16 cell lines; the panel was enriched for breast cancer cell lines, and consisted of cell lines with varying genotypes and a range of sensitivity to allosteric mTOR inhibitor rapamycin (24). MK-2206 sensitivity was assessed by SRB assay (Fig. 2A). Five of the cell lines were sensitive to MK-2206 (defined as greater than 50% growth inhibition at concentrations less than 500 nmol/L); all 5 had either a *PIK3CA* or *PTEN* mutation. Overall, 5 of 9 cell lines that had *PIK3CA* and/or *PTEN* mutation were MK-2206 sensitive, whereas none of the 7 *PTEN*/*PIK3CA* wild-type cell lines were MK-2206 sensitive ($P = 0.0337$, Fig. 2B). One of the 5 MK-2206-sensitive cell lines had a *RAS* or *RAF* mutation, although 4 of the 11 resistant cell lines had a *RAS*/*RAF* mutation ($P = 1.0000$). Notably ZR75-1 has a *HRAS* E162K mutation as well as a *PTEN* mutation whereas among the resistant cell lines there was 1 *KRAS* mutant, 2 *BRAF* mutants, and 1 with both mutations (25, 26).

To confirm this finding in a wider variety of cancer cell lines, we assessed the association between *PTEN* or *PIK3CA* mutation status and MK-2206 sensitivity in 444 cancer cell lines using the Catalogue of Somatic Mutations in Cancer (COSMIC) database (27). Cell lines had been treated with MK-2206 for 72 hours and their IC_{50} was recorded; we used the mean IC_{50} value of wild-type *PTEN*/*PIK3CA* as a cutoff for sensitivity as per their definition. Both *PIK3CA* and *PTEN* mutations were associated with increased MK-2206 sensitivity ($P = 0.0043$ and $P = 0.0062$, respectively; Fig. 2C). Mean IC_{50} value of *PIK3CA* and *PTEN* mutant cell lines was also lower than wild type (Supplementary Fig. S1). In contrast, cell lines with *RAS*/*RAF* mutations had higher MK-2206 IC_{50} (Supplementary Fig. S1).

As *PTEN* loss and *PIK3CA* mutations are relatively common in breast cancer (2, 28), we assessed the functional impact of these aberrations on *in vitro* MK-2206 sensitivity. To determine whether *PTEN* loss confers MK-2206 sensitivity, we assessed MK-2206 sensitivity in isogenic cell lines with differing expression levels of *PTEN*. We first studied MDA-MB-231, a triple-negative breast cancer cell line with normal *PTEN* levels and relative MK-2206 resistance. *PTEN* siRNA knockdown increased expression of pAkt S473 more than 3-fold compared with control siRNA, and this Akt activation caused an increase of pBad S136, a downstream target of Akt (29, 30). Increases in both pAkt and pBad were reversed by MK-2206 treatment (Fig. 3A). After *PTEN* knockdown with siRNA, growth of MDA-MB-231 cells was inhibited to a significantly greater extent by MK-2206 treatment than by control siRNA in 3 independent experiments. Similar results were observed in the HER2-positive breast cancer cell line SKBR3 (Fig. 3B).

Next, MCF7 cells stably transfected with mismatch control shRNA or *PTEN* shRNA were cultured in estrogen-depleted condition. Akt phosphorylation at both the T308 and S473 residues was increased in *PTEN* knockdown cells compared with mismatch control shRNA cells, and this was reversed by treatment with MK-2206 500 nmol/L. The IC_{50} of MK-2206 was significantly lower in MCF7 *PTEN* shRNA knockdown cells than in controls (Fig. 3C).

We next tested the effect of *PIK3CA* mutations on MK-2206 sensitivity. *PIK3CA* wild-type, E545K mutant, and H1047R mutant plasmids were stably transfected into normal-like breast epithelial cell line MCF10A. In the *PIK3CA* mutant-transfected cells, Akt phosphorylation at both T308 and S473 residues was increased compared with that in *PIK3CA* wild-type–

transfected cells, and this was reversed by treatment with MK-2206 500 nmol/L (Fig. 3D). The IC₅₀ of MCF10A cell lines transfected with PIK3CA E545K or PIK3CA H1047R was significantly lower than that of PIK3CA wild-type cells (Fig. 3D).

Cell lines sensitive to MK-2206 are also sensitive to Akt siRNA knockdown

Not all cell lines with PIK3CA or PTEN aberrations were sensitive to MK-2206, thus we also assessed expression of Akt isoforms, Akt phosphorylation, and the expression of Akt phosphatases in MK-2206-sensitive and -resistant cell lines (Fig. 4A). Baseline Akt phosphorylation did not show a strong relationship to MK-2206 sensitivity. However, both cell lines without pAkt expression were MK-2206 resistant. PTEN expression was decreased or lost in 4 of 5 cell lines with *PTEN* mutations (31), suggesting that loss of PTEN expression can be used to select for tumors with PTEN mutations. Although PH domain leucine-rich repeat-containing protein phosphatases (PHLPP) and inositol polyphosphate 4-phosphatase (INPP4B) have both been previously reported to regulate pAkt (32–34), PHLPP expression did not show an association with pAkt S473 expression or MK-2206 sensitivity (Fig. 4A). Contrary to our expectation, loss of INPP4B expression was observed in 4 of 9 MK-2206-resistant cell lines but in none of the MK-2206-sensitive cell lines; 2 of these cell lines also did not express pAkt.

MK-2206 inhibits all 3 Akt isoforms, Akt1, Akt2, and Akt3, but is 5- to 10-fold less potent against Akt3 (IC₅₀: Akt1 5 nmol/L, Akt2 12 nmol/L, Akt3 65 nmol/L; 21). Furthermore, Akt isoforms have been proposed to have distinct and separate roles in tumorigenesis (35). Thus, we assessed the expression of different Akt isoforms in MK-2206-sensitive and more resistant cell lines (Fig. 4A). The ratio of Akt1 to Akt2 was significantly higher in MK-2206-sensitive cells (Fig. 4A, right panel). Some MK-2206-resistant cells expressed Akt3; however, this was insufficient to explain relative sensitivity to MK-2206. We then sought to determine whether the Akt isoform ratio was predictive of MK-2206 sensitivity in a larger panel of cell lines. For 296 cell lines, we obtained MK-2206 IC₅₀ data from the COSMIC and transcriptional profiling data from the Cancer Cell Line Encyclopedia (26). Akt1/Akt2 mRNA expression did not show correlation with MK-2206 sensitivity.

We tested the Akt dependence of the growth of MK-2206-sensitive and -resistant cell lines by Akt siRNA and SRB assay. Expression of Akt1, 2, and 3 isoforms was knocked down with isoform-specific siRNA as shown by Western blotting (Fig. 4B). Combined treatment with siRNA to Akt1 and Akt2 led to statistically significant inhibition of growth not only in MK-2206-sensitive cell line ZR75-1 (PTEN loss), but also in MK-2206-resistant cell lines MDA-MB-468 (PTEN loss), HCC1954 (*PIK3CA* mutation), and MDA-MB-231 (wild-type PTEN and *PIK3CA*; Fig. 4B). There was greater than 50% suppression of cell growth in MK-2206-sensitive ZR75-1 cells but not in MK-2206-resistant cell lines. Knockdown of all 3 Akt isoforms in Akt3-expressing MDA-MB-231 still did not achieve greater than 50% suppression of growth inhibition.

MK-2206 inhibits Akt signaling and tumor growth *in vivo*

To determine the effect of MK-2206 *in vivo*, nu/nu mice bearing ZR75-1 xenografts were treated orally with MK-2206 240 mg/kg or 480 mg/kg once per week. Tumors were harvested 24 hours after the 4th treatment. Four tumors were randomly selected from each group and analyzed by multiplex proteomics (MSD) or Western blotting. MSD showed statistically significant inhibition of pAkt and its target pGSK3b (Fig. 5A). Western blotting showed a decrease in pAkt; with greater inhibition with the higher dose. There was an even greater dose-dependence of inhibition of downstream signaling targets such as pPRAS40, pBad, p4E-BP1, and pS6K (Fig. 5B); pAkt expression in the high- and low-dose MK-2206-treated lysates may have been beyond the linear range of Western blotting and MSD assays.

Immunohistochemical analysis showed inhibition of pAkt and a statistically significant decrease in proliferation marker Ki-67, again with greater effects with the higher MK-2206 dose (mean percentages of positive cells: control 85.0%, MK-2206 240mg/kg 72.5%, and MK-2206 480mg/kg 52.5%; Fig. 5C).

Both 240 mg/kg and 480 mg/kg MK-2206 weekly oral treatments inhibited tumor growth compared with vehicle control ($P < 0.0001$ for both comparisons; Fig. 5D). Tumors were significantly smaller in mice treated with MK-2206 480 mg/kg than in those treated with 240 mg/kg ($P = 0.0243$), suggesting that MK-2206 has a dose-dependent growth-inhibitory effect *in vivo*.

To further investigate the antitumor effect of MK-2206 *in vivo*, nu/nu mice bearing MCF7 and BT474 xenografts were treated orally with MK-2206. Mice bearing MCF7 xenografts were treated with an initial dose of 480 mg/kg MK-2206; the dose was reduced to 360 mg/kg weekly due to general fatigue. Mice bearing BT474 xenografts were treated with 360 mg/kg MK-2206 weekly. MK-2206 treatment was associated with tumor growth inhibition but not tumor regression in MCF7 ($P < 0.0001$; Supplementary Fig. S2A) and BT474 xenografts ($P < 0.0001$; Supplementary Fig. S2B). However, significant toxicity was observed in these 2 experiments in the study arms, with deaths in 3 of 7 mice in the MCF7 experiment (2 after second dose and 1 after third dose), and with deaths in 2 of 6 mice in the BT474 experiment, with none in the control groups. Unfortunately, autopsies were not obtained; thus cause of death is unknown. Notably, these studies were carried out at the same time, with the same mouse lot and with MK-2206 obtained from an alternate source (compound generated in-house). As there were no deaths in previous experiments conducted with MK-2206 obtained from Merck at 360 mg/kg or 480 mg/kg, it is possible that the formulations differed in amount, solubility, and ultimate drug concentrations achieved.

MK-2206 is synergistic with paclitaxel *in vitro* and enhances paclitaxel's antitumor efficacy *in vivo*

Next, we wanted to determine whether MK-2206 enhances the effect of paclitaxel, an antimicrotubule chemotherapeutic agent commonly used for breast cancer treatment. Apoptosis induced by MK-2206 and paclitaxel, alone or in combination, was assessed by flow cytometry with annexin V labeling in ZR75-1 cells. After cells were treated for 72 hours, the population of annexin V-positive cells was higher in paclitaxel + MK-2206-treated cells compared with cells treated with either agent alone (Fig. 6A). Apoptosis induced by treatment was calculated by subtracting the percentage of annexin V-positive cells in vehicle-treated cells from the annexin V-positive population in the treatment groups (Fig. 6B). The combination of paclitaxel 100 ng/mL + MK-2206 50 nmol/L induced significantly more annexin V-positive cells than MK-2206 50 nmol/L alone and paclitaxel alone.

We then tested whether there was an additive or synergistic treatment interaction between MK-2206 and paclitaxel in 5 breast cancer cell lines, 3 that were MK-2206 sensitive (ZR75-1, HCC70, MDA-MB-453) and 2 that were MK-2206 resistant (HCC1954, MDA-MB-468). The cells were treated with a range of doses of MK-2206 and paclitaxel simultaneously for 96 hours. The effects on cell growth were assessed by SRB assay and CI values were calculated. MK-2206 and paclitaxel combination was synergistic ($CI < 1.0$) in all 3 MK-2206-sensitive cell lines, but not in the 2 MK-2206-resistant cell lines tested (Fig. 6C). The synergy was greatest in ZR75-1 cells.

We next determined the *in vivo* effect of MK-2206 alone and in combination with paclitaxel. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay showed apoptosis induction in ZR75-1 xenografts harvested 48 hours after

MK-2206 treatment and a statistically significant increase in apoptosis when MK-2206 is administered in combination with paclitaxel [mean positive cells at 10 fields ($\times 400$ magnification): vehicle 17.3, 15 mg/kg paclitaxel 66.0, 360 mg/kg MK-2206 68.7, 480 mg/kg MK-2206 84.5, and 15 mg/kg paclitaxel and 360 mg/kg MK-2206 123.0; Fig. 6D].

For assessment of antitumor efficacy, nu/nu mice with ZR75-1 xenografts were treated weekly with vehicle, paclitaxel 15 mg/kg only, MK-2206 360 mg/kg only, or the combination of paclitaxel and MK-2206 (Fig. 6E). Pairwise *t* tests, which were adjusted by the FDR method, are shown. All 3 treatments significantly inhibited growth compared with controls ($P < 0.0001$ for all 3 comparisons). The combination of MK-2206 and paclitaxel inhibited growth significantly more than paclitaxel alone ($P = 0.0196$) or MK-2206 alone ($P = 0.0125$).

In our first *in vivo* experiment, 2 mice died after the third treatment (on day 17) in the combination arm. Therefore, we determined whether lower doses of MK-2206 and paclitaxel would also enhance the efficacy of paclitaxel *in vivo*. In addition, we explored the role of therapy sequence in this second experiment. In previous work, we found that rapamycin is synergistic with paclitaxel *in vitro*, and the antitumor efficacy is greater when rapamycin is given 24 hours after paclitaxel (13). It has been reported recently that MK-2206 is additive/synergistic *in vitro* with antimicrotubule agent docetaxel when MK-2206 and docetaxel are given simultaneously, but there is synergy when MK-2206 is given 24 hours after docetaxel (21). In our experiment, therefore, ZR75-1 xenografts were treated with a combination of paclitaxel and 240 mg/kg MK-2206 both synchronously and sequentially, with MK-2206 given 24 hours after paclitaxel (Fig. 6F). These treatments were well tolerated, with no symptoms or weight loss in the treatment groups. All 4 treatments (paclitaxel alone, MK-2206 alone, and synchronous or sequential paclitaxel+MK-2206) inhibited growth compared with vehicle ($P < 0.0001$). Synchronous treatment with paclitaxel and MK-2206 inhibited growth significantly compared with paclitaxel alone ($P = 0.0033$) or MK-2206 alone ($P = 0.0037$). Sequential treatment with paclitaxel followed by MK-2206 also inhibited growth significantly compared with paclitaxel alone ($P = 0.0159$) or MK-2206 alone ($P = 0.0159$). Tumor growth inhibition did not differ significantly in mice treated with paclitaxel and MK-2206 synchronously versus sequentially.

Discussion

Activated Akt signaling is a significant contributor to the pathogenesis of cancer. PTEN is a negative regulator of PI3K/Akt signaling whose expression is decreased in many tumor types, and PIK3CA is frequently mutated in many human cancers. MK-2206 is a selective allosteric inhibitor of Akt; we sought to determine the antitumor efficacy of MK-2206 in cell lines of varying genetic backgrounds. We show here that MK-2206 inhibits Akt signaling and cell-cycle progression, and increases apoptosis in a dose-dependent manner. MK-2206 sensitivity was significantly greater in cell lines with *PTEN* or *PIK3CA* mutation; however, not all lines with aberrations were sensitive. MK-2206 also had a growth-inhibitory effect *in vivo*, and enhanced the antitumor activity of paclitaxel.

There are several ongoing clinical trials of MK-2206 in multiple tumor types. Thus, there is a pressing need to identify predictive markers for selection of patients most likely to benefit. Although in our panel, *RAS/RAF* mutations were not associated with resistance to MK-2206. They were associated with increased resistance in the COSMIC cell line set; this finding is worthy of further exploration. In our study, cell lines with *PTEN* or *PIK3CA* mutations were more likely to be sensitive to MK-2206. Furthermore, loss of PTEN or transfection with mutant PIK3CA conferred greater MK-2206 sensitivity. Similarly, transfection of PIK3CA mutants was recently shown to enhance MK-2206 sensitivity of

thyroid cancer cell line SW176 (36). These findings provide support for use of *PTEN* mutation/loss or *PIK3CA* mutations as potential predictive biomarkers of response, and their use for patient enrichment in ongoing clinical trials [e.g., NCT01277757, Phase II Trial of Akt Inhibitor MK-2206 in Patients With Advanced Breast Cancer Who Have Tumors With a *PIK3CA* Mutation and/or *PTEN* Loss (37)].

Previously, we showed by immunohistochemical analysis that *PTEN* expression was lost in 30% of primary breast tumors and 25% of breast cancer metastases (28). *PIK3CA* mutations were detected in 40% of primary breast tumors and 42% of metastases. Thus, these aberrations are common enough to make their use for patient selection feasible. However, there was 26% discordance in *PTEN* expression status and 18% discordance in *PIK3CA* mutation status between primary and metastatic tumors. This high degree of discordance in *PTEN* level and *PIK3CA* mutations between primary tumors and metastases may have implications for patient selection in Akt-targeted therapy trials. Although primary tumor *PIK3CA* and *PTEN* status can be used to enrich for patients likely to have *PIK3CA* and *PTEN* alterations in their metastases, biopsy of the metastases may help confirm the metastatic tumor biomarker status and to determine whether patients with alterations that are preserved in both the primary and the metastases are more responsive to Akt-targeted therapy. It is notable that in our study not all cell lines with *PTEN* and/or *PIK3CA* mutation were sensitive to MK-2206. Thus, not all tumors with *PTEN/PIK3CA* mutation rely on Akt for oncogenic signaling.

Allosteric mTOR inhibitors have already been shown to have antitumor efficacy in renal carcinoma, neuroendocrine tumors, and breast cancer. It is interesting that some cell lines that were MK-2206 resistant in this study (e.g., MDA-MB-468) are sensitive to allosteric mTOR inhibitors (38). Which tumor types would preferentially benefit from Akt inhibitors rather than mTOR inhibitors is unclear. Furthermore, there are several Akt inhibitors in clinical development; antitumor efficacy may also differ among these drugs. A combination of biomarkers is likely needed to determine the best therapeutic approach in patients with aberrations in PI3K/Akt/mTOR signaling.

Only a few Akt targets have been shown to have isoform specificity to date: p21 CIP, SKP2, and palladin are Akt1 targets, and MDM2 and AS160 are Akt2 targets (39–44). Although Akt regulates cancer cell survival, signaling through Akt1 has been shown to block both cell invasion and migration. Actin-bundling protein palladin inhibits the breast cancer cell invasive phenotype, at least partly through regulation of phosphorylation of palladin by Akt1, whereas Akt2 regulates expression of palladin (35, 41). In our cell lines, MK-2206-sensitive cell lines had higher Akt1/2 ratios, however a high Akt1/2 mRNA ratio was not associated with MK-2206 sensitivity in a large cell line panel. The role of individual Akt isoforms in MK-2206 sensitivity needs to be further studied, and if there is an association, its downstream mediators need to be identified.

MK-2206 had a dose-dependent effect on cell signaling and tumor growth. Indeed, although Akt phosphorylation was inhibited with clinically relevant doses, dose escalation had a greater effect on downstream effectors such as 4E-BP1. Apoptosis was only seen with high doses of MK-2206. In xenograft models, furthermore, treatment with low-dose MK-2206, both as a single agent and in combination therapy, inhibited tumor growth. However, higher doses of MK-2206 led to tumor regression. Recently it has been shown that, although cancer cells treated with high doses of Akt inhibitors underwent apoptosis, those treated with moderate doses that only partially inhibited Akt signaling divided asymmetrically to produce an increased population of slowly proliferating G₀-like cells, representing a potential mode of therapy resistance (45). This dose dependence should be taken into consideration when dose reductions are considered in clinical trials for toxic effects such as skin rash. pAkt was

shown to decline in tumor biopsies and in hair follicles in the phase I MK-2206 trial (23). Correlative studies are ongoing in clinical trials to determine whether extent of Akt dephosphorylation and more complete downstream target inhibition correlates with clinical benefit.

MK-2206 was shown recently to be synergistic with several therapeutic agents (21, 36, 46–49). Synergy in combinatorial regimens has been attributed to enhanced apoptotic cell death and autophagy (21, 46–49). Moreover, blunting autophagic response to MK-2206 with elongation factor 2 kinase inhibitors has been shown to enhance apoptotic response to MK-2206 (50). Here, we showed synergy between MK-2206 and paclitaxel *in vitro*, and greater antitumor efficacy in combination of MK-2206 and paclitaxel than either agent alone *in vivo*. This finding is of particular clinical relevance as paclitaxel is used in the treatment of breast cancer in the adjuvant as well as metastatic setting. Our study shows synergy between MK-2206 and paclitaxel in MK-2206-sensitive but not in MK-2206-resistant breast cancer cell lines. This suggests that even when an Akt inhibitor is used in combination therapy, optimizing patient selection may enhance clinical benefit. Further study is needed to determine the mechanism of this synergy and to identify molecular predictors that can assist in prioritizing therapeutic regimens.

In summary, MK-2206 has antitumor activity alone and in combination with chemotherapy, and this activity may be greater in tumors with *PTEN* or *PIK3CA* mutation, supporting the concept of biomarker testing for patient enrichment in clinical trials. However, not all tumors with these aberrations are MK-2206 sensitive, emphasizing the need for additional predictive and pharmacodynamic markers of response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Activated Akt signaling is a significant contributor to the pathogenesis of breast cancer. *PTEN*, a negative regulator of PI3K/Akt signaling, is mutated or decreased, and *PIK3CA* is frequently mutated in multiple cancer lineages. In this study, we showed that cell lines with *PTEN* or *PIK3CA* mutations are more sensitive to MK-2206, a selective allosteric inhibitor of Akt. PTEN knockdown or introducing *PIK3CA* mutations in isogenic cell lines increased MK-2206 sensitivity. MK-2206 is synergistic with paclitaxel *in vitro* and enhances paclitaxel-mediated growth inhibition *in vivo*. These data show antitumor efficacy of MK-2206 *in vitro* and *in vivo*, and provide support for testing PTEN loss and *PIK3CA* mutations as potential predictors of response to MK-2206.

MK-2206 inhibits Akt signaling, causes cell-cycle arrest and apoptosis. A, 7 cancer cell lines were treated with vehicle or increasing doses of MK-2206 for 24 hours. Phosphoprotein levels were assessed by RPPA. Each dot indicates triplicated samples and the solid line connects the means. B, ZR75-1 cells were treated with vehicle, rapamycin 100 nmol/L, or increasing concentrations of MK-2206 for 24 hours. Western blotting was conducted to assess Akt signaling. C, ZR75-1 cells were treated with MK-2206 150 nmol/L and collected after indicated hours. Akt signaling was assessed by Western blotting. D, breast cancer cell lines were treated with vehicle, rapamycin (10 or 100 nmol/L), or MK-2206 (50 or 500 nmol/L) in triplicate for 96 hours, and percentages of cells in G₁

(navy), S (blue), and G₂–M (light blue) phases of the cell cycle were determined by flow cytometry. The percentages of cells in G₁ phase in each treatment group were compared (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant vs. control). E, MK-2206–sensitive breast cancer cell lines were treated with vehicle or MK-2206 (50, 500, or 5,000 nmol/L) for 72 hours in triplicate. The percentages of annexin V–positive cells were determined with flow cytometry and were compared (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, vs. control).

Figure 2.

PTEN and PIK3CA status is associated with MK-2206 sensitivity. A, 16 cell lines with varying PIK3CA, PTEN, and RAS/RAF status were treated with increasing doses of MK-2206, and IC_{50} was determined by SRB assay. B, 5 of the 16 cell lines were MK-2206 sensitive ($IC_{50} < 500$ nmol/L). MK-2206 sensitivity in cell lines with *PTEN* or *PIK3CA* mutation was compared to sensitivity of *PTEN* and *PIK3CA* wild-type cells. C, 233 of the 444 cell lines in the COSMIC were MK-2206 sensitive ($IC_{50} < \text{mean } IC_{50}$ value of *PTEN*/*PIK3CA* wild type). MK-2206 sensitivity in cells with *PTEN* or *PIK3CA* mutation was compared with sensitivity of *PTEN* and *PIK3CA* wild-type cells. WT, wild-type; MT, mutant.

Figure 3.

PTEN loss and PIK3CA mutation confer MK-2206 sensitivity. A, MDA-MB-231 cells were treated with control or PTEN siRNA for 24 hours and were subsequently treated with vehicle or MK-2206 500 nmol/L for 24 hours. pAkt S473 and pBad S136 levels were assessed by Western blotting. The relative expression of pAkt S473 was calculated (** $P < 0.01$; *** $P < 0.001$ vs. indicated in the graph). B, MDA-MB-231 and SKBR3 cells were treated with control or PTEN siRNA for 24 hours and were subsequently treated with MK-2206 for 96 hours. Cell growth in response to MK-2206 treatment was assessed by SRB assay and was compared at each MK-2206 concentration (*, $P = 0.0410$; ** $P = 0.0038$; *** $P < 0.0001$). C, MCF7 cells transfected with mismatch control or PTEN shRNA were cultured in phenol red free medium supplemented with 2% charcoal-stripped FBS for 24 hours and subsequently treated with vehicle, rapamycin 100 nmol/L, or MK-2206 (50 or 500 nmol/L) for 24 hours. pAkt T308 and S473 levels were assessed by Western blotting. The graph represents MK-2206 IC₅₀ under estrogen-depleted conditions (*, $P = 0.0074$). D, MCF10A cells stably transfected with PIK3CA wild-type, E545K mutant, or H1047R mutant were treated with vehicle, rapamycin 100 nmol/L, or MK-2206 (50 or 500 nmol/L) for 24 hours. pAkt T308 and S473 were assessed by Western blotting. The graph represents MK-2206 IC₅₀ for each transfected cell line (*, $P < 0.05$; ** $P < 0.01$ vs. control).

Figure 4.

MK-2206 sensitivity is associated with Akt-dependent growth. A, baseline expression of Akt isoforms, pAkt, PTEN, INPP4B, and PHLPPs was assessed by Western blotting. Akt1/Akt2 ratios in MK-2206-sensitive and -resistant cell lines were compared (*, $P = 0.0163$). B, 4 breast cancer cell lines were treated with control siRNA or siRNA to different isoforms for 96 hours. Western blotting was conducted to assess expression of Akt and its isoforms. Cell growth was assessed by SRB assay (*, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control).

Figure 5.

MK-2206 inhibits Akt signaling and tumor growth *in vivo*. Nu/nu mice bearing ZR75-1 xenografts were treated with vehicle MK-2206 240 mg/kg, or MK-2206 480 mg/kg. Four randomly selected tumors from each group were harvested 24 hours after the 4th treatment. A, pAkt S473, pGSK3 S9, and total Akt expression were assessed by MSD assay. Relative expression as the ratio of pAkt S473 or pGSK3 S9 to total Akt was calculated (*, $P < 0.05$; ***, $P < 0.001$ vs. control). B, the same lysates were assessed by Western blotting for Akt signaling. C, expression of pAkt S473 and Ki-67 was assessed immunohistochemically. Scale bar, 0.02 mm. Percentage of Ki-67 cells in each treatment group were compared (**, $P < 0.01$; vs. control). D, the graph represents mean tumor volume of ZR75-1.

Figure 6.

MK-2206 is synergistic with paclitaxel. A, annexin V-positive cell populations induced by therapy were determined by comparing with cells treated with 0.1% DMSO as a baseline. Apoptosis induced by MK-2206 or paclitaxel or a combination of the 2 were compared (*, $P < 0.05$; ***, $P < 0.001$; ns, not significant, vs. indicated in the graph). B, ZR75-1 cells were treated with MK-2206 and/or paclitaxel for 72 hours, and apoptotic cells were determined with flow cytometry. The populations of annexin V-positive cells are shown in the top right corner of each panel. C, 5 breast cancer cell lines were treated with MK-2206 and paclitaxel simultaneously for 96 hours. The effect on cell growth was assessed by SRB assay, and CI values were calculated. The graph represents the CI of MK-2206 and paclitaxel at ED50, ED75, and ED90. CI < 1.0 represents synergy. D, the apoptosis induction *in vivo* was assessed by TUNEL. TUNEL-positive cells in 10 fields ($\times 400$ magnification) were compared in each treatment group. The graph represents mean number of apoptotic cells stained with TUNEL (***, $P < 0.001$; vs. control, and ¶¶¶, $P < 0.001$; vs. combination). E, mice bearing ZR75-1 xenografts were treated with vehicle, paclitaxel 15 mg/kg, MK-2206 360 mg/kg, or a combination of paclitaxel and MK-2206 at the same doses. F, mice bearing ZR75-1 xenografts were treated with vehicle, paclitaxel 5 mg/kg,

MK-2206 240 mg/kg, paclitaxel and MK-2206 synchronously (paclitaxel+MK-2206), or paclitaxel and MK-2206 sequentially (paclitaxel MK-2206).