**Cancer Therapy: Preclinical**

**Targeting PI3K/mTOR Overcomes Resistance to HER2-Targeted Therapy Independent of Feedback Activation of AKT**


**Abstract**

**Purpose:** Altered PI3K/mTOR signaling is implicated in the pathogenesis of a number of breast cancers, including those resistant to hormonal and HER2-targeted therapies.

**Experimental Design:** The activity of four classes of PI3K/mTOR inhibitory molecules, including a pan-PI3K inhibitor (NVP-BKM120), a p110α isoform-specific PI3K inhibitor (NVP-BYL719), an mTORC1-specific inhibitor (NVP-RAD001), and a dual PI3K/mTORC1/2 inhibitor (NVP-BEZ235), was evaluated both in vitro and in vivo against a panel of 48 human breast cell lines.

**Results:** Each agent showed significant antiproliferative activity in vitro, particularly in luminal estrogen receptor–positive and/or HER2+ cell lines harboring PI3K mutations. In addition, monotherapy with each of the four inhibitors led to significant inhibition of in vivo growth in HER2+ breast cancer models. The PI3K/mTOR pathway inhibitors were also effective in overcoming both de novo and acquired trastuzumab resistance in vitro and in vivo. Furthermore, combined targeting of HER2 and PI3K/mTOR leads to increased apoptosis in vitro and induction of tumor regression in trastuzumab-resistant xenograft models. Finally, as previously shown, targeting mTORC1 alone with RAD001 leads to consistent feedback activation of AKT both in vitro and in vivo, whereas the dual mTOR1–2/PI3K inhibitor BEZ235 eliminates this feedback loop. However, despite these important signaling differences, both molecules are equally effective in inhibiting tumor cell proliferation both in vitro and in vivo.

**Conclusion:** These preclinical data support the findings of the BOLERO 3 trial that shows that targeting of the PI3K/mTOR pathway in combination with trastuzumab is beneficial in trastuzumab-resistant breast cancer. Clin Cancer Res; 1–14. ©2014 AACR.

**Introduction**

The HER2/neu oncogene is amplified and overexpressed in 25% to 30% of all breast cancers, and presence of this alteration is associated with significantly shortened disease-free and overall survival (1, 2). Targeted agents against HER2, such as the humanized mAb trastuzumab and the dual HER2–EGFR tyrosine kinase inhibitor lapatinib, result in significantly improved clinical outcomes in patients with HER2+ breast cancers (3–5). However, resistance to both agents exists. Approximately 50% of HER2-amplified patients exhibit de novo resistance to trastuzumab and a number of patients who initially respond to the drug will eventually develop acquired resistance (5–7). Lapatinib provides clinical benefit to some patients progressing on trastuzumab; however, less than 25% achieve an objective response and the majority of these eventually develop acquired lapatinib resistance (3). Consequently, these resistance phenomena and the mechanisms underlying them remain a clinically significant problem.

Numerous potential mechanisms for resistance to HER2-targeted therapies have been proposed. HER2 lacks a functional ligand-binding domain and must homodimerize or form heterodimers with ligand-activated receptor tyrosine kinases (RTK) such as EGFR, HER3, or HER4 to transduce a downstream signal to the PI3K/mTOR and/or Ras/MAPK pathways (8, 9). Trastuzumab does not inhibit ligand-dependent HER2 heterodimerization (10); therefore, signaling through HER2 dimerization partners via increased ligand activation or increased levels of cell surface receptors such as insulin-like growth factor 1 receptor (IGF-1R) could potentially reduce the activity of trastuzumab (11–13).
Alternatively, signaling through a non-RTK like src has been linked to resistance to HER2-targeted therapy (14). Another possible mechanism of resistance includes impairment of the capacity of trastuzumab to bind HER2 via cleavage of the extracellular domain or masking of the receptor by the membrane-associated glycoprotein, mucin-4 (15, 16). However, despite data proposing a multitude of different alterations as being responsible for resistance to HER2-targeted therapies, the most common alteration associated with this resistance is continued activation of the PI3K/mTOR signaling pathway (17–23).

Aberrant PI3K/mTOR signaling has been implicated in the pathogenesis of many cancers, including resistance to targeted therapies for both hormone receptor–positive and HER2þ breast cancers (17–20). Dysregulated PI3K/mTOR signaling can occur through loss of the protein phosphatase PTEN or by the presence of activating mutations in the PIK3CA gene encoding the p110α catalytic subunit of the PI3K enzyme (21, 22). Knockdown of PTEN confers trastuzumab resistance to HER2-amplified cells in culture and lower PTEN levels are associated with poor response to trastuzumab. Introduction of PIK3CA mutations confers resistance to HER2-targeted therapy in vitro (17, 19). Finally, combined measurement of PIK3CA mutations or PTEN loss significantly predicts for both de novo and acquired trastuzumab resistance in a large panel of breast cancer cell lines and patient tissues (17, 20).

Taken together, these data highlight the therapeutic potential of targeting PI3K/mTOR signaling in breast cancers and have led to the development of a number of targeted agents directed against specific signaling nodes in this pathway. However, the efficacy of these molecules may be limited by feedback activation of the pathway in response to mTOR inhibition (24, 25). Strategies to overcome this feedback inhibition include using dual-specific inhibitors that target both PI3K and mTORC1/2 complexes or pan/p110α–specific PI3K inhibitors that will inhibit both AKT and mTOR signaling (26–28). However, a major unanswered question has been, is this feedback activation predictive of a loss of clinical response (29).

In this study, we evaluated a panel of 48 human breast cell lines, representing the spectrum of breast cancer subtypes, for both in vitro and in vivo response to four novel PI3K/mTOR inhibitors; NVP-BKM120 (pan-PI3K), NVP-BYL719 (PI3K–p110α isoform-specific), NVP-RAD001 (mTORC1), and NVP-BEZ235 (dual PI3K/mTORC1/2). HER2-amplified cell lines were among the most sensitive to PI3K/mTOR inhibition and, thus, combined activity with trastuzumab was assessed in these cell lines. The effects of the different PI3K/mTOR inhibitors on intracellular signaling as well as the association between feedback activation and response to therapy were also evaluated.

Materials and Methods

Cell lines, cell culture, and reagents

Growth inhibitory activity of NVP-BKM120, NVP-BYL719, NVP-RAD001, and NVP-BEZ235 (provided by Novartis and, hereafter, referred to as BKM120, BYL719, RAD001, and BEZ235, respectively) was assessed in a panel of 48 human breast cell lines, including three immortalized and 45 malignant lines representing the spectrum of molecular subtypes of breast cancer (30). Cells were routinely assessed for mycoplasma contamination using a multiplex PCR method and mitochondrial DNA was sequenced using a 3730 DNA Analyzer (Applied Biosystems) to confirm identity (31, 32). Trastuzumab-resistant (BT474-TR) cells were established as previously described (20, 33).

In vitro proliferation assay

Cells were seeded in duplicate at 5,000 to 20,000 cells per well in 24-well plates, as described previously (30) and the following day 1 μmol/L of inhibitor and 2-fold serial dilutions over 9 to 12 concentrations was added to generate dose–response curves. Cells were counted on day 1 when drug was added, as well as day 6 using a Coulter-Z1 particle counter (Beckman Coulter Inc.). Suspension cultures were counted using a Coulter Vi-Cell counter (Beckman Coulter Inc.). Growth inhibition was calculated as a ratio of cell generation time in the presence of drug versus generation time in the absence of drug. Lethality was defined as any decrease in cell numbers in treated wells compared with counts on the initial day of treatment. IC50 values for each molecule were calculated by fitting curves to data points from each dose–response assay using the Proc NLIN function in SAS for Windows version 9.2 (SAS Institute, Inc.). An IC50 value of <1 μmol/L was used to define sensitivity and all experiments were performed in duplicate. For drug combination studies, cells were treated with either trastuzumab (10 μg/mL, with 2-fold dilutions of trastuzumab), one of the PI3K pathway inhibitors (10 μmol/L, with 10-fold dilutions over six concentrations) or the combination of both.
Western blot analysis

Protein lysates were obtained from cells growing in log-phase using a lysis buffer (Cell Signaling Technology) containing a mixture of protease inhibitors (Calbiochem) and 1 mmol/L phenylmethylsulfonylfluoride. Western blot analysis was performed as previously described (20). Total (p185) and phosphorylated (Tyr1221/1222) HER2 were detected using anti-HER2 monoclonal (Ab-3; Calbiochem) and the polyclonal anti-pHER2 (Cell Signaling Technology) antibodies, respectively. Total (C-17) and phosphorylated HER3 (Tyr1289) antibodies were purchased from Santa Cruz Biotechnology and Cell Signaling Technology, respectively. Total and phosphorylated AKT (Ser473 and Thr308), S6 (Ser225/236), 4EBP-1 (Thr47/46), and ERK (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technology. Anti-α-tubulin was purchased from Calbiochem. All blots were repeated in duplicate and relative levels of individual proteins were quantitated using the ECL Plus chemiluminescent reagent (Amersham Biosciences) and the Typhoon 9400 system (Amersham Biosciences).

In vitro apoptosis and cell-cycle analyses

Cells were seeded in 6-well plates at approximately 100,000 cells per well. Following 72 hours treatment, apoptosis analysis was performed by washing cells with PBS, trypsinization and centrifugation at 3,000 rpm for 5 minutes. Cells were resuspended in 300 μL of binding buffer and stained with 10 μL of Annexin V–FITC and 5 μL of propidium iodide at room temperature for 5 minutes (Medical & Biological Laboratories, Co.). Effects on cell cycle were assessed using 300 to 500 μL propidium iodide/Triton X-100 staining solution containing 2 mg/mL DNAse-free RNase A. Flow cytometry was performed using a CellQuanta (Beckman Coulter Inc.) flow machine and results analyzed using FlowJo software (Tree Star Inc.).

In vivo efficacy studies and biomarker analysis

Xenograft models of seven individual breast cancer cell lines were established in 6-week-old CD-1 athymic nude mice (Charles River Laboratories). The following conditions were optimized for the subcutaneous injection of each cell line: UACC812 1.7 × 10⁷ cells, KPL-1 2.2 × 10⁷ cells, ZR75-1 1.7 × 10⁷ cells, SUM190 1.5 × 10⁷ cells, MDA361 2.0 × 10⁷ cells, BT474 0.5 × 10⁷ cells with 50% Matrigel (BD Biosciences), and BT474-TR 0.5 × 10⁷ cells with 50% Matrigel. Development of the trastuzumab-resistant phenotype was used as a model to identify acquired HER2 amplification. In a panel of six breast cancer cell lines using a two-dimensional Western blot analysis, the following conditions were used to detect HER2 amplification: BT474, UACC812, KPL-1, ZR75-1, and MDA361. 17β-estradiol 60-day release pellets (Innovative Research of America) were implanted subcutaneously into the left flank 7 days before tumor inoculation. When tumors reached an average size of 150 to 200 mm³, mice (n = 8) were randomized into treatment groups. Tumor xenografts were measured with calipers three times per week, and tumor volume in mm³ was determined by multiplying height × width × length. Tissue acquisition studies were carried out in parallel to the xenografts experiments. Six additional mice from each treatment group were treated as described above for 7 days and sacrificed between 2 and 24 hours after final drug administration. Serum was isolated from whole-blood samples obtained through terminal bleed by cardiac puncture on each mouse. Tumors were excised, divided in half and either snap-frozen in liquid nitrogen, or prepared as formalin-fixed/paraffin-embedded (FFPE) tissues. Tumor and serum drug concentration determination and reverse phase protein arrays (RPPA) were performed as previously described (28, 34). All animal work was carried out under a protocol approved by the Institutional Animal Care and Use Committee and the University of California, Los Angeles Animal Research Committee.

Immunohistochemistry

FFPE tissue sections (4 μm) were deparaffinized in xylene, rehydrated in graded ethanol, and subjected to antigen retrieval (35). Immunohistochemistry was performed with the following antibodies: Ser473 AKT (Cell Signaling Technology), and Ki67 (DAKO), and detected using horseradish peroxidase–labeled secondary antibody (DAKO). Ser347/346 S6 was detected using the PS6 Kit from Eton Biosciences. Sections were counterstained with hematoxylin, dehydrated, mounted with Permount, and target antigens quantitated using standard immunohistochemical methods.

Statistical analysis

For in vitro studies, differences between groups were compared using a two-tailed Student t test. For in vivo studies, tumor growth in each of the treatment arms was compared with the vehicle control using repeated measures ANOVA (RMANOVA) followed by the Dunnett test for multiple comparisons. Statistical differences between mean tumor volumes at specific time points were performed using a two-tailed paired Student t test. Differences between groups were considered statistically significant at P < 0.05. All statistics were calculated using Microsoft Excel and StatView for windows (SAS Institute, Inc.).

Results

Effect of trastuzumab on PI3K/mTOR signaling in HER2-amplified breast cancer cell lines

The antiproliferative effects of trastuzumab were assessed in a panel of six breast cancer cell lines using a two-dimensional drug response assay (Fig. 1A). Typically, response to this mAb exhibited a flat dose–response curve over the 32-fold dose range. Responses in HER2-amplified cells ranged from 90% growth inhibition for BT474 cells to less than 5% for SUM190 cells. We have previously characterized a panel of 17 HER2-amplified breast cancer cell lines for response to trastuzumab and determined the cutoff for sensitivity to be >20% growth inhibition (20). On the basis of these criteria, the BT474 cells were classified as negative for trastuzumab, the MDA453, SUM225, and SUM190 cells were classified as de novo trastuzumab-resistant, and the BT474-TR cells (trastuzumab-conditioned BT-474 cells) were classified as acquired trastuzumab-resistant. The HER2-normal MCF7 cells were insensitive to trastuzumab (<10% growth inhibition).
In models of trastuzumab sensitivity, trastuzumab treatment induced a gradual decrease in PI3K/AKT/mTOR signaling over 72 hours (Fig. 1B). After 24 hours exposure, trastuzumab reduced the phosphorylation status of AKT at both phosphorylation sites necessary for catalytic activity (Ser473 and Thr308) and reduced levels of phosphorylated ribosomal protein S6 (Ser235/236). In contrast, pAKT and pS6 levels were unchanged after trastuzumab treatment in resistant MDA453, SUM225, SUM190, and BT474-TR cell lines (Fig. 1C), indicating that resistance is associated with a failure to inhibit PI3K/mTOR signaling.

### Activity of PI3K/mTOR inhibitors in breast cancer cell lines

The antiproliferative activity of four molecules that specifically target key nodes in the PI3K/mTOR signaling pathway were assessed in a panel of 48 breast cell lines, including 18 that were classified as HER2-amplified (Fig. 2B; Supplementary Table S1). For the purposes of this study, cell lines have been classified as PIK3CA mutant only if they harbor mutations in either exon 9 (E545K, E542K) or exon 20 (H1047R/L) that have been shown to be activating (21). Flat dose–response curves were observed for the allosteric mTORC1 inhibitor RAD001, making IC50 values less reliable for quantifying response across the panel (Fig. 2A). To address this, a combination of IC50 and average growth inhibition at 100 nmol/L was used to classify cells as either sensitive or resistant (S.A. Hurvitz and colleagues; unpublished data). According to these criteria, 13 of 18 HER2-amplified cell lines, six of 10 PI3K-mutant lines and five of the six lines carrying both mutations were sensitive to RAD001. Moreover, 10 of the 13 most sensitive cell lines to RAD001 are ER+. Remarkably, each of the 48 breast cell lines had an IC50 value
Figure 2. Targeting the PI3K/mTOR pathway in a panel of breast cancer cell lines. BKM120, BYL719, RAD001, and BEZ235 were prepared for in vitro studies as 10 mmol/L stock solutions (except RAD001; 20 mmol/L) in 100% dimethyl sulfoxide (DMSO). A, the mTORC1-specific inhibitor RAD001, #, IC₅₀ > 100 nmol/L. B, the dual PI3K–mTOR inhibitor, BEZ235. C, activity of the pan-PI3K inhibitor BKM120, #, IC₅₀ > 1 μmol/L. D, the p110α-specific inhibitor, BYL719, #, IC₅₀ > 10 μmol/L. A to D, black bars, HER2-amplified cell lines, *, cell lines with activating mutations of PIK3CA (exon 9 or 20). Data, mean ± SD.
Figure 3. Combination of HER2 and PI3K/mTOR targeting in trastuzumab-sensitive and trastuzumab-resistant breast cancer cell lines. A to C, cells were treated for 5 days with a range of concentrations of trastuzumab (Tz; 0.3125–10 μg/mL) plus RAD001 (R), BEZ235 (Z), BKM120 (K), or BYL719 (Y) (all 0.0001–10 μmol/L). Generational (Gen) inhibition of >100% considered to be induction of lethality, that is, the number of cells at day 5 was less than the number of cells at day 0. D, effect of targeting HER2 plus PI3K/mTORC1/2 (15 μg/mL trastuzumab + 500 nmol/L BEZ235) or HER2 plus mTORC1 (15 μg/mL trastuzumab + 500 nmol/L RAD001) on feedback activation of AKT in trastuzumab-sensitive (BT474) and trastuzumab-resistant cells (BT474-TR and MDA453).
100 nmol/L for the dual PI3K/mTOR inhibitor BEZ235 (Fig. 2B), making it difficult to stratify sensitivity to this molecule by particular breast cancer subtypes. Activity of the pan-PI3K inhibitor BKM120 showed a strong luminal ER\textsuperscript{+}/HER2\textsuperscript{+} signature, with the 15 most sensitive lines all luminal being ER\textsuperscript{+} or HER2\textsuperscript{+}. Also, eight of 10 cell lines with activating mutations in PIK3CA were sensitive to BKM120 (Fig. 2C). In contrast, only 10 lines were considered sensitive to the p110\textalpha-specific PI3K inhibitor BYL719; however, nine of these were HER2-amplified, four PIK3CA mutant, and three positive for both alterations (Fig. 2D).

Figure 4. Targeting PI3K/mTOR induces apoptosis and cell-cycle arrest in trastuzumab-sensitive and trastuzumab-resistant breast cancer cell lines. A, propidium iodide staining of trastuzumab-sensitive and trastuzumab-resistant cells treated for 72 hours with 15 μg/mL trastuzumab + 500 nmol/L BKM120, BYL719, RAD001, or BEZ235. Left, representative examples of trastuzumab-sensitive BT474 cells treated with trastuzumab compared with trastuzumab-resistant MDA453 cells treated with trastuzumab (both 15 μg/mL). B and C, Annexin V staining of cells treated as described previously. Histograms, the percentage of Annexin V\textsuperscript{−}positive (AN V\textsuperscript{−}) cells after each treatment. Data, mean ± SD. All experiments were repeated in at least duplicate. *, P < 0.05 versus vehicle control; #, P < 0.05 versus corresponding single-agent treatment, calculated using a paired two-tailed Student t test.
Figure 5. PI3K/mTOR inhibitors show in vivo efficacy in mouse xenograft models of multiple subtypes of human breast cancer. A, mice bearing tumor xenografts from the HER2-amplified breast cancer cell lines (UACC812, SUM190, and MDA361) were treated daily orally with BKM120 (35 mg/kg in 10% NMP/90% PEG300), BYL719 (50 mg/kg in 0.5% w/v methylcellulose/water), RAD001 (10 mg/kg in microemulsion; Novartis; content not disclosed), BEZ235 (35 mg/kg in 10% NMP/90% PEG300), or 10 mg/kg trastuzumab by intraperitoneal injection twice per week. (Continued on the following page.)
The effect of each of these molecules on cell signaling was assessed in two representative HER2-amplified cell lines; the PI3K wild-type SKBR3 and the PI3K-mutant MDA453 (Supplementary Fig. S1). Specifically targeting mTORC1 with RAD001 resulted in elimination of S6 phosphorylation followed by reactivation of AKT at Thr308 and Ser473 in both cell lines at concentrations as low as 0.1 nmol/L. In contrast, at concentrations of 500 nmol/L and above, where BEZ235 also targets both mTOR and PI3K (36), BEZ235 eliminates feedback activation of AKT (Supplementary Fig. S1A and S1B). BKM120 induced a concentration-dependent decrease in S6 phosphorylation, followed by a concomitant increase in phosphorylation of AKT at Thr308, suggesting that feedback activation can also occur through PI3K targeting (Supplementary Fig. S1C). Conversely, the p110α-specific PI3K inhibitor BYL719 induced a concentration-dependent elimination of AKT phosphorylation at both Thr308 and Ser473. Interestingly, there was no downstream effect on S6 phosphorylation in BYL719-resistant SKBR3 cells (IC50 1.807 μmol/L), whereas there was a concentration-dependent elimination of S6 signaling in BYL719-sensitive, PI3K-mutant MDA453 cells (IC50 0.547 μmol/L; Supplementary Fig. S1D). In contrast with what has previously been reported (37, 38), we did not observe compensatory activation of HER3 and/or ERK signaling in response to PI3K/mTOR pathway inhibition (Supplementary Fig. S1A–S1D).

Combined targeting of HER2 and PI3K/mTOR in trastuzumab resistance

Given the activity of the PI3K/mTOR inhibitors in the HER2-amplified models, the combination of trastuzumab plus each of the PI3K/mTOR inhibitors was evaluated in the three different trastuzumab response subtypes (Fig. 1). Because of the flat dose–response curves associated with the mAb (Fig. 1A and Fig. 3A–C), it was not possible to calculate combination indices (39). We were, however, able to observe in the trastuzumab-resistant cell lines that trastuzumab augmented the inhibition of proliferation induced by each of the four PI3K pathway inhibitors. Combination with RAD001 induced growth inhibition greater than 100%, indicating induction of cell death, which was not observed with either agent alone (Fig. 3A). The combination of RAD001 and trastuzumab also increased the anti-proliferative activity seen with either agent alone in MDA453 de novo trastuzumab-resistant cells (Fig. 3B). In BT474-TR acquired trastuzumab-resistant cells, RAD001 alone or in combination inhibited 100% of proliferation at concentrations as low as 0.1 nmol/L. Single-agent BEZ235 induced lethality in each of the models at concentrations above 10 nmol/L making it difficult to observe any combined activity with trastuzumab (Fig. 3A–C).

Treatment with BEZ235 and trastuzumab over a period of 72 hours eliminated phospho-AKT at Ser473, S6, and 4E-BP1 in all cell lines tested. Levels of phospho-AKT at Thr308 initially dropped in response to BEZ235, likely due to its direct activity on PI3K; however, levels returned to baseline within 24 hours (Fig. 3D). In contrast, the combination of RAD001 and trastuzumab over the same time-course induced significant feedback activation of AKT at both Thr308 and Ser473 phosphorylation sites (Fig. 3D). Similar results were observed in the trastuzumab-sensitive SKBR3 and de novo trastuzumab-resistant SUM190 and SUM225 models (data not shown). However, despite these contrasting effects on feedback activation, both molecules had similar efficacies in terms of inhibition of cell proliferation (Fig. 3A–C), indicating that the feedback activation of AKT may not limit the efficacy of these molecules.

Targeting PI3K/mTOR signaling induces apoptosis and cell-cycle arrest in trastuzumab-resistant cells

As expected, single-agent trastuzumab induced G0–G1 cell-cycle arrest in the trastuzumab-sensitive BT474 cells but not in the resistant MDA453 cells (Fig. 4A). Single-agent RAD001 and BEZ235 induced G0–G1 cell-cycle arrest in both trastuzumab-sensitive and -resistant cell lines. In contrast, BKM120 or BYL719 only induced significant G0–G1 arrest when combined with trastuzumab (Fig. 4A). BEZ235 was the most potent inducer of apoptosis, causing a significant increase in the apoptotic population by 24.7% in the BT474 cells and 16.3% in the MDA453 cells relative to untreated controls. BYL719 also induced a significant increase in apoptotic cells, whereas single-agent RAD001 and BKM120 did not induce significant apoptosis (Fig. 4B and C). Interestingly, despite the fact trastuzumab alone does not induce apoptosis in the cells, the combination of trastuzumab with BKM120, BYL719, or RAD001 resulted in a significant increase in apoptosis in both cell line models (Fig. 4B and C).

In vivo activity of PI3K/mTOR inhibitors in different PI3K mutation models of human breast cancer

The in vivo activity of these molecules was assessed in five HER2-amplified xenograft models. Significant antitumor
activity was observed with each of the inhibitors in the trastuzumab-sensitive UACC812, SUM190, and MDA361 xenograft models (Fig. 5A; Supplementary Table S2). Interestingly, the MDA361 and SUM190 cells, which have a reduced response to trastuzumab in 2D cell culture (20), were sensitive to trastuzumab in vivo (Fig. 5B; Supplementary Table S2). Similar responses to BKM120, BYL719, RAD001, and BEZ235 were observed in the PI3K-mutant SUM190H1047R and MDA361E545K xenografts as the PI3K wild-type UCACC812 xenografts.

Activity was also assessed in two in vivo models of trastuzumab resistance; BT474-TR and SUM190-TR. Resistance was confirmed in vivo for both models (Fig. 5B–D). Single-agent BKM120, BYL719, RAD001, and BEZ235 all significantly reduced tumor progression in the BT474-TR xenografts relative to vehicle- and trastuzumab-treated controls (Fig. 5B, bottom; Supplementary Table S2). Moreover, the addition of trastuzumab to each of the PI3K/mTOR inhibitors increased antitumor responses and induced tumor regression in these trastuzumab-resistant xenografts (Fig. 5C). A SUM190 xenograft model of acquired trastuzumab resistance was developed in vivo through long-term treatment of tumors that were initially responsive to trastuzumab. Xenografts treated with trastuzumab alone continued to progress over the 32 days of this signal finding study (173 mm$^3$ increase in mean tumor volume), whereas tumor regression was observed when BKM120 (122 mm$^3$ decrease in tumor volume), RAD001 (62 mm$^3$ decrease), and BEZ235 (8 mm$^3$ decrease) were added to the trastuzumab treatment (Fig. 5D). No significant toxicities were observed with the combination of trastuzumab and any of the PI3K/mTOR inhibitors in either trastuzumab-resistant xenograft models.

Our in vitro screen also revealed that these molecules had activity in ER$^+$ cell lines. On the basis of these findings, we expanded our in vivo studies to include to xenograft models of ER$^+$ breast cancer that also represent different molecular subtypes of PI3K/mTOR pathway activation status; PI3K mutant KPL-1 cells* and ZR75-1 PI3K wild-type/PTEN null cells. Significant anti-tumor activity was observed with each of the PI3K/mTOR pathway inhibitors in both the PI3K mutant KPL-1 and PTEN-null ZR75-1 xenograft models (Fig. 5E and F; Supplementary Table S2). Tumor regression was observed with BKM120, BYL719, and RAD001 in both xenograft models, whereas BEZ235 induced tumor regression in the KPL-1 tumors and not in the ZR75-1s.

*The KPL-1 cells were originally isolated in 1995 from a 50 year old Japanese woman with ER+ breast cancer (Kurebayashi et al., British Journal of Cancer (1995) 71, 845–853). These cells were considered to be PIK3CA wild-type until studies from the German Cell Line Repository (DSMZ, www.dsmz.de) revealed the KPL-1 cells had in fact been cross contaminated with the MCF7 cell line. SNP and STR analysis has shown that the KPL1 cells are identical to the MCF7 cell lines and carry the ES45K activating mutation in exon 9 of the PIK3CA gene (http://www.sanger.ac.uk and http://www.broadinstitute.org/ccle/home). We recently have confirmed these findings through in house sequencing studies.

**In vivo targeting of PI3K/mTOR signaling and pathway feedback activation**

To assess whether response to PI3K/mTOR inhibition in vivo related to inhibition of feedback activation of PI3K/mTOR signaling, we performed reverse phase protein analysis on xenograft tissues from studies performed in parallel to the in vivo efficacy experiments. Analyses of SUM190 tumors treated daily with each inhibitor for 7 days revealed that at 2.5 hours after final dose, AKT phosphorylation was significantly reduced at both the Ser473 and Thr308 in BKM120-, BYL719- and BEZ235-treated tumors. In contrast, levels of phospho-AKT were found to be above that of the vehicle control in RAD001-treated tumors (Fig. 6A, left). However, RAD001 did induce significant decreases in pS6 S235–236 levels in SUM190 xenografts, consistent with that induced by BKM120, BYL719, and BEZ235 (Fig. 6A, right). Immunohistochemical analyses of the tissues confirmed these findings; although pAKT$^{S473}$ levels are decreased in the BKM120-, BYL719- and BEZ235-treated tumors, no decrease was observed in the RAD001-treated tumors (Fig. 6C). RPPA analyses were repeated on samples collected 24 hours after final dose in the UACC812 xenografts. At this time point, pAKT$^{S473}$/Thr308 levels were significantly increased in response to each molecule, indicating that feedback activation also occurs in vivo with these molecules (Fig. 6B, right). However, even at 24 hours after final dose, pS6$^{S235–236}$ levels were still significantly decreased in RAD001-treated tumors, despite the fact that drug concentrations in both tumor and serum were at zero at this time point, indicating a sustained and potent knockdown of mTORC1 activity. These data were reproduced in KPL-1 xenograft samples collected 24 hours after final dose (Supplementary Fig. S2). Once again, it is important to note that despite reactivation of pAKT signaling in the in vivo models, significant antitumor responses were still observed in both the tissue acquisition and larger efficacy studies (Supplementary Fig. S3 and Fig. 5).

**Discussion**

PI3K/mTOR signaling is frequently dysregulated in human cancers and plays a central role in tumor cell growth, survival as well as resistance to anticancer therapies (23, 40). We and others have previously shown that activation of this pathway is one of the few factors that is consistently associated with resistance to trastuzumab therapy (17, 20), making the targeting of this pathway an attractive strategy for overcoming trastuzumab resistance. However, the potential benefits of this approach may be dependent on the particular PI3K signaling nodes being targeted and the resultant effects on downstream signaling and feedback activation. In this study, the activity of four different classes of PI3K-, mTOR-, and PI3K/mTOR-targeting agents was compared in a panel 48 breast cell lines, including models of de novo and acquired trastuzumab resistance. The pan-PI3K inhibitor NVP-BKM120, the p110$\alpha$ isofrom–specific PI3K inhibitor NVP-BYL719, and the dual PI3K-mTOR inhibitor NVP-BEZ235 are all in phase I/II/III clinical testing, whereas NVP-RAD001 or everolimus...
Figure 6. Biomarkers of in vivo response to inhibitors of PI3K/mTOR signaling. A and B, RPPA analysis of tumor samples taken after 7 days treatment with the molecules indicated. SUM190 tumors taken 2.5 hours after final dose, UACC812 tumors taken 24 hours after final dose. C and D, representative images of immunohistochemical staining for pAKT Ser473 and pS6 Ser235–236 in SUM190 tumor xenografts (2.5 hours after final dose).
(Afinitor; Novartis) has already been approved as mono-therapy for advanced renal cell carcinomas, neuroendocrine tumors, and recently for hormone receptor–positive advanced breast cancer when used in combination with an aromatase inhibitor [41, 42].

Each of the four inhibitors showed significant in vitro antiproliferative activity in the breast cancer cell line panel, with increased sensitivity observed in the luminal ER+ and HER2+ cell lines. The pan-P13K inhibitor BKM120 was most active in ER+ cell lines with each of the seven most sensitive lines classified as ER+. The p110α-specific P13K inhibitor, BYL719, showed more activity in HER2+/P13K cells with each of the most sensitive lines being either HER2-amplified, P13K-mutant, or both. RAD001 was also very active in HER2+/HER2+/PI3K-mutant lines. Because of the profound sensitivity of the panel to the dual PI3K/mTOR inhibitor, BEZ235 (IC50 consistently <100 nmol/L), it was difficult to stratify the lines into response subgroups; however, the HER2+/P13K-mutant lines were among the most sensitive.

On the basis of our observations that these molecules clearly have in vitro activity in the HER2+ cells lines and the fact that trastuzumab is ineffective in downregulating P13K/mTOR signaling in trastuzumab-resistant models, we compared the relative ability of these molecules with target P13K/mTOR signaling in two representative HER2+ cell lines; P13K wild-type SKBR3 cells and P13K-mutant MDA453H1047R cells. These studies showed that mTORC1 inhibition by RAD001 leads to complete inhibition of S6K phosphorylation at concentrations as low as 50 nmol/L; however, this was followed by feedback activation of pAKTTh308 and pAKT S473. RAD001 specifically targets the mTORC1 complex, deactivating ribosomal protein S6K signaling and removing its suppression of IRS1. This allows IRS1 to activate IGF-1R signaling and ultimately results in phosphorylation of AKT at Th308 (25, 43). Also, because RAD001 is mTORC1 specific, it does not inhibit AKT phosphorylation at the S473 site via mTORC2 (29, 44, 45). As a result, despite potent activity of RAD001 at mTORC1, it ultimately causes AKT to be phosphorylated at both sites. These data demonstrate that feedback activation of AKT occurs to a similar degree in both SKBR3 and MDA453 cells treated with RAD001, yet SKBR3 cells are resistant to this drug, whereas MDA453 cells are sensitive. The dual P13K/mTOR inhibitor BEZ235 is very active in both cell lines and treatment completely eliminates feedback activation of the pathway. Because BEZ235 targets P13K, reactivation of AKT at Th308 is blocked and inhibition of the mTORC2 complex blocks reactivation of AKT at S473. The activity of the pan-P13K inhibitor, BKM120, on P13K signaling results in downstream loss of mTOR activity, ultimately leading to feedback activation of AKT at Th308. These data demonstrate that directly targeting P13K also leads to feedback activation of the pathway through reactivation of AKT; however, despite this, both SKBR3 and MDA453 cells are sensitive to the antiproliferative effects of BKM120. Conversely, the p110α-specific P13K inhibitor, BYL719, has a more potent effect on P13K activity as indicated by persistent dephosphorylation of AKT at 24 hours after treatment in contrast with BKM120. Interestingly, dephosphorylation of S6 was only observed in the BYL719-sensitive MDA453H1047R and not in the BYL719-resistant SKBR3 cells. Together, these data indicate that single-agent treatment with the four classes of P13K/mTOR-targeting molecules have differing effects on the degree of feedback activation of AKT but this is not predictive of response. Similar observations were made in the analyses of xenograft tumor tissue from our in vivo studies. Thus, our data indicate that dephosphorylation of S6 tracks more consistently with a growth inhibitory response rather than AKT phosphorylation.

In vitro studies comparing effects of combining trastuzumab with BEZ235 or RAD001 on P13K/mTOR signaling in models of trastuzumab sensitivity and resistance provide further evidence that feedback activation of AKT is not predictive of response. Despite the fact that these two combinations have significantly different effects on feedback activation, they have very similar antiproliferative activity with almost complete growth inhibition at very low concentrations (10 nmol/L). The significant single-agent activity of each drug makes quantitating of any potential additive efficacy of combining either with trastuzumab difficult to determine by cell count assay. However, the value of combining these agents was shown when we looked at the effects of combinations via cell-cycle/apoptosis analysis. The combination of trastuzumab plus each of the P13K/mTOR pathway inhibitors either increased the degree of cell death induced by the single agent (BEZ235 and BYL719) or induced apoptosis where little or no cytotoxicity was observed with single agent (RAD001 and BKM120), highlighting the potential benefit of combined targeting of HER2 and P13K/mTOR signaling.

We further investigated the activity of these molecules in in vivo models of HER2+ and ER+ breast cancer representing different activation status of the P13K/mTOR pathway. In HER2+ breast cancer, impressive efficacy was observed for each molecule independent of P13K mutation status. In ER+/HER2+ breast cancer, significant antitumor activity was observed independent of PTEN mutation status. Furthermore, in two xenograft models of trastuzumab resistance, single-agent treatment with each of the P13K/mTOR pathway inhibitors induced significant tumor growth inhibition, whereas the combination of these agents with trastuzumab resulted in overt tumor regressions. Thus, treatment with each P13K/mTOR-targeting molecule gave antitumor activity across a panel of breast cancer xenografts irrespective of trastuzumab response or the presence of P13K or PTEN mutations. Future studies, using orthotopically engrafted tumors in the mammary fat-pad, will assess the impact these inhibitors may have on metastases, particularly to the brain, given that these molecules have the capacity to cross the blood–brain barrier.

RPPA analysis of xenograft tumor tissues confirmed our in vitro findings that treatment with each of the inhibitors induced significant reduction of S6 phosphorylation at two hours after dosing. This was accompanied by a loss of AKT phosphorylation in the BKM120-, BYL719-, and BEZ235-
treated mice but feedback activation of Akt in the RAD001-treated mice. RAD001 also differed from the other molecules by inducing a sustained knockdown of S6 phosphorylation, which could be attributed to the superior half-life of RAD001 relative to the other molecules. This sustained inhibition may be responsible for the potential antiangiogenic activity associated with mTORC1 inhibition (46, 47).

Taken together, the data provide further evidence that targeting the PI3K/mTOR pathway may be a logical strategy for overcoming resistance to HER2-targeted therapy. What is unexpected is the significant antitumor activity observed with RAD001, BKM120, and BYL719 that have efficacies comparable with the dual inhibitor, BEZ235. Moreover, these tumor growth inhibitory responses are achieved despite persistent reactivation of upstream AKT signaling as seen most prominently with RAD001 treatment. In fact, RAD001 induced superior tumor growth inhibition relative to the dual inhibitor BEZ235 in 6 of 7 (86%) of the xenograft models tested. Our preclinical findings are supported by data from phase I/II clinical trials that show RAD001 provides benefit in combination with trastuzumab in trastuzumab-refractory breast cancer (48, 49). Furthermore, data recently reported from the large phase III clinical trial (BOLERO 3) showed that targeting HER2 and mTOR in combination with vinorelbine significantly prolongs progression-free survival compared with trastuzumab plus vinorelbine in trastuzumab-pretreated HER2 metastatic breast cancer (50).

The impressive in vivo efficacies we observed with the two PI3K-targeting molecules BKM120 and BYL719, suggest that these agents may also be useful in targeting ErbB/HER2 breast cancer, and should also be tested in combination with trastuzumab in patients with trastuzumab-refractory disease.

Disclosure of Potential Conflicts of Interest
S. Hurvitz reports receiving a commercial research grant from Novartis. R. Linnartz is an employee of Novartis. R. Finn is a consultant/advisory board member for Novartis. S. Hirawat is an employee of and has ownership interest (including patents) in Novartis. No potential conflicts of interest were disclosed by the other authors.

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Targeting PI3K/mTOR Overcomes Resistance to HER2-Targeted Therapy Independent of Feedback Activation of AKT


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