Targeting PI3K/mTOR overcomes resistance to HER2-targeted therapy independent of feedback activation of AKT

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CONFLICTS OF INTEREST: Emmanuelle di Tomaso, Christian Schnell, Ronald Linnartz and Samit Hirawat are employees of Novartis Pharmaceuticals.
TRANSLATIONAL RELEVANCE:

Although trastuzumab provides clinical benefit for patients with HER2-amplified breast cancer, resistance to therapy remains a significant clinical problem. Alterations in the PI3K/mTOR signaling pathway, due to loss of PTEN or activating mutations in PI3K, have been shown to track consistently with resistance to HER2-targeted therapy. In this study, we show that targeting the PI3K/mTOR pathway with either the pan-PI3K inhibitor (NVP-BKM120), the p110α-isoform-specific PI3K inhibitor (NVP-BYL719), the mTORC1-specific inhibitor (NVP-RAD001) or the dual PI3K/mTORC1/2-inhibitor (NVP-BEZ235) can overcome resistance to HER2-targeted therapy either as single agents or in combination with trastuzumab. Furthermore, this activity is independent of feedback activation of the pathway induced by mTORC1 inhibition. These preclinical data support the findings of the BOLERO 3 clinical trial that shows targeting of the PI3K/mTOR pathway in combination with trastuzumab is beneficial in trastuzumab resistant breast cancer.

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) were evaluated both in vitro and in vivo against a panel of 48 human breast cell lines.
Results: Each agent showed significant anti-proliferative activity \textit{in vitro}, particularly in luminal ER-positive and/or HER2-positive cell lines harboring PI3K mutations. In addition, monotherapy with each of the four inhibitors led to significant inhibition of \textit{in vivo} growth in HER2-positive breast cancer models. The PI3K/mTOR pathway inhibitors were also effective in overcoming both \textit{de novo} and acquired trastuzumab resistance \textit{in vitro} and \textit{in vivo}. Furthermore, combined targeting of HER2 and PI3K/mTOR lead to increased apoptosis \textit{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 \textit{in vitro} and \textit{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 \textit{in vitro} and \textit{in vivo}.

Conclusion: These pre-clinical data support the findings of the BOLERO 3 trial that shows targeting the PI3K/mTOR pathway in combination with trastuzumab is beneficial in trastuzumab resistant breast cancer.

Introduction:

The HER2/neu oncogene is amplified and overexpressed in 25-30\% 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 monoclonal antibody trastuzumab and the dual HER2-EGFR tyrosine kinase inhibitor lapatinib, result in significantly improved clinical outcomes in patients with HER2-positive breast cancers (3-5). However, resistance to both agents exists. Approximately 50\% of HER2-amplified patients exhibit \textit{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 that 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 (RTKs) 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 heterodimerisation (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 (IGF-1R) could potentially reduce the activity of trastuzumab (11-13). Alternatively, signaling through a non-receptor tyrosine kinase 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 (ECD) 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 HER2-positive 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). Knock-down 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 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 amongst 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 (Basel, Switzerland) 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/well in 24-well plates, as described (30) and the following day 1µM of inhibitor and 2-fold serial dilutions over 9-12 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, Fullerton, CA). Suspension cultures were counted using a Coulter Vi-Cell counter (Beckman Coulter Inc, Fullerton, CA). 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 to 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., Cary, NC). An IC50 of <1µM/L was used to define sensitivity and all experiments were performed in duplicate. For drug combination studies, cells were treated with either trastuzumab (10µg/ml with 2-fold dilutions over 6 concentrations), one of the PI3K pathway inhibitors (10µM with 10-fold dilutions over 6 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 1mM/L phenylmethlysulphonylflouride (PMSF). Western blot analysis was performed as previously described.
Total (p185) and phosphorylated (Tyr 1221/1222) HER2 were detected using anti-HER2 monoclonal (Ab-3, Calbiochem) and the polyclonal anti-pHER2 (Cell Signaling Technologies) antibodies, respectively. Total (C-17) and phosphorylated HER3 (Tyr 1289) antibodies were purchased from Santa Cruz and Cell Signaling Technology respectively. Total and phosphorylated AKT (Ser 473 and Thr 308), S6 (Ser 225/236) 4EBP-1 (Thr 37/46) and ERK (Thr 202/Tyr 204) 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/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 300μL of binding buffer and stained with 10μL of Annexin V-FITC and 5μL of PI at room temperature for 5 minutes (Medical & Biological Laboratories, Co.). Effects on cell cycle were assessed using 300-500μl PI/Triton X-100 staining solution containing 2mg DNAse-free RNAseA. Flow cytometry was performed using a CellQuanta (Beckman Coulter) flow machine and results analyzed using Flow Jo software (Tree Star Inc., Asland, OR).

**In vivo efficacy studies and biomarker analysis:** Xenograft models of seven individual breast cancer cell lines were established in six-week-old CD-1 athymic nude mice (Charles River Laboratories). The following conditions were optimized for the sub-cutaneous injection of each cell line: UACC812 1.7x10^7 cells, KPL-1 2.2x10^7 cells, ZR75-1 1.7x10^7 cells, SUM190 1.5x10^7 cells, MDA361 2.0x10^7 cells, BT474 0.5x10^7 cells with 50% matrigel (BD Biosciences) and BT474-TR 0.5x10^7 cells with 50% matrigel. Development of the trastuzumab acquired resistant SUM190-TR model is described in the
Supplementary Materials and Methods. For mice receiving estrogen receptor (ER) positive cell lines (BT474, UACC812, KPL-1, ZR75-1, MDA361), 17-β-estradiol 60-day release pellets (Innovative Research of America) were implanted sub-cutaneously into the left flank seven days prior to tumor inoculation. When tumors reached an average size of 150-200mm³, mice (n = 8) were randomized into treatment groups. Tumor xenografts were measured with calipers 3 times/week, and tumor volume in mm³ was determined by multiplying heightXwidthXlength. 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 seven 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 IACUC and the UCLA Animal Research Committee.

Immunohistochemistry (IHC): FFPE tissue sections (4µm) 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 Technologies), and Ki67 (DAKO), and detected using horseradish peroxidase-labeled secondary antibody (DAKO). Ser²³⁵/²³⁶. S6 was detected using the PS6 kit from Eton Biosciences. Sections were counterstained with hematoxylin, dehydrated, mounted with Permount and target antigens quantified using standard IHC methods.

Statistical analysis: For in vitro studies, differences between groups were compared using a two-tailed paired Student’s t-test. For in vivo studies, tumor growth in each of the treatment arms was compared
to the vehicle control using Repeated Measures ANOVA (RMANOVA) followed by Dunnett test for multiple comparisons. Statistical differences between mean tumor volumes at specific time points was performed using a two-tailed paired Student’s 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).

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 monoclonal antibody 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 cut-off for sensitivity to be >20% growth inhibition of proliferation (20). Based on these criteria, the BT474 cells were classified as sensitive to trastuzumab, the MDA453, SUM225 and SUM190 cells were classified as *de novo* trastuzumab resistant and the BT474-TR (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 anti-proliferative 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.2 and 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 IC$_{50}$ values less reliable for quantifying response across the panel (Fig.2A). To address this, a combination of IC$_{50}$ and average growth inhibition at 100nM was used to classify cells as either sensitive or resistant (Hurvitz et al, manuscript in preparation). According to these criteria, 13 of 18 HER2-amplified cell lines, six of ten 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-positive. Remarkably, each of the 48 breast cell lines had an IC$_{50}$ <100nM/L for the dual PI3K/mTOR inhibitor BEZ235 (Fig.2B), making it difficult to stratify sensitivity to this molecule by particular breast cancer sub-types. Activity of the pan-PI3K inhibitor BKM120 showed a strong luminal ER-positive/HER2-positive signature, with the 15 most sensitive lines all luminal being ER+ or HER2+. Also 8 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 p110α-specific PI3K inhibitor BYL719, however, nine of these were HER2-amplified, four PIK3CA mutant and three positive for both alterations (Fig.2D).

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$_{H1047R}^{10}$ (Supplemental Figure
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 50nM/L. In contrast, at concentrations of 500nM/L and above, where BEZ235 also targets both mTOR and PI3K (36), BEZ235 eliminates feedback activation of AKT (Fig. S1A and B). 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 (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 1.807µM/L), whereas there was a concentration dependent elimination of S6 signaling in BYL719-sensitive, PI3K mutant MDA453 cells (IC₅₀ 0.547 µM/L) (Fig. S1D). In contrast to 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 (Fig. S1A-D).

**Combined targeting of HER2 and PI3K/mTOR in trastuzumab resistance.**

Given the activity of the PI3K/mTOR inhibitors in the HER2-amplified cells, the combination of trastuzumab plus each of the PI3K/mTOR inhibitors was evaluated in the three different trastuzumab response sub-types (Figure 1). Due to the flat dose response curves associated with the monoclonal antibody (Figure 1A and Figure 3A-C), it was not possible to calculate combination indices (39). We were however, able to observe in the trastuzumab sensitive cells 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 0.1nM/L. Single agent BEZ235 induced lethality in each of the models at concentrations above 10nM/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 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 & 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&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 anti-tumor 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, Table S2). Similar responses to BKM120, BYL719, RAD001 and BEZ235 were observed in the PI3K mutant SUM190<sup>H1047R</sup> and MDA361<sup>E545K</sup> xenografts as the PI3K wild-type UACC812 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, lower panel and Table S2). Moreover, the addition of trastuzumab to each of the PI3K/mTOR inhibitors increased anti-tumor 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 (173mm<sup>3</sup> increase in mean tumor volume), whereas tumor regression was observed when BKM120 (122mm<sup>3</sup> decrease in tumor volume), RAD001 (62mm<sup>3</sup> decrease) and BEZ235 (8mm<sup>3</sup> 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. Based on these findings, we expanded our *in vivo* studies to include xenograft models of ER+ breast cancer that also represent different molecular sub-types of PI3K/mTOR pathway activation status; PI3K/PTEN wild-type 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 wild-type KPL-1 and ‘PI3K activated’ ZR75-1 xenograft models (Fig. 5E & F, Table S2). Tumor regression was observed with BKM120, BYL719 and RAD001 in both xenografts models, whereas BEZ235 induced tumor regression in the KPL-1 tumors and not in the ZR75-1s.

**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 post 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 panel). However, RAD001 did induce significant decreases in pS6^{5235-236} levels in SUM190 xenografts, consistent with that induced by BKM120, BYL719 and BEZ235 (Fig. 6A, right panel). IHC analyses of the tissues confirmed these findings; although pAKT^{5473} 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 post final dose in the
UACC812 xenografts. At this time point, pAKT<sup>S473/T308</sup> levels were significantly increased in response to each molecule, indicating that feedback activation also occurs in vivo with these molecules (Fig. 6B, right panel). However, even at 24 hours post final dose, pS6<sup>S235-236</sup> 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 post final dose (Supplementary Figure S2). Once again, it is important to note that despite reactivation of pAKT signaling in the in vivo models, significant anti-tumor responses were still observed in both the tissue acquisition and larger efficacy studies (Supplementary Figure S3 & Figure 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 anti-cancer 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 p110α isoform-specific PI3K inhibitor NVP-BYL719 and the dual PI3K-mTOR inhibitor NVP-BEZ235 are all in phase I/II/III clinical testing while NVP-RAD001 or everolimus (Afinitor, Novartis) has already been approved as monotherapy 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 anti-proliferative activity in the breast cancer cell line panel, with increased sensitivity observed in the luminal ER+ and HER2+ cell lines. The pan-PI3K inhibitor BKM120 was most active in ER+ cell lines with each of the seven most sensitive lines classified as ER+. The p110α-specific PI3K inhibitor, BYL719, showed more activity in HER2+/PI3K cells with each of the most sensitive lines being either HER2-amplified, PI3K-mutant or both. RAD001 was also very active in HER2+/ER+/PI3K mutant lines. Due to the profound sensitivity of the panel to the dual PI3K/mTOR inhibitor, BEZ235 (IC50 consistently <100nM) it was difficult to stratify the lines into response subgroups, however the HER2+/PI3K mutant lines were among the most sensitive.

Based on our observations that these molecules clearly have in vitro activity in the HER2+ cells lines and the fact that trastuzumab is ineffective in down-regulating PI3K/mTOR signaling in trastuzumab-resistant models, we compared the relative ability of these molecules to target PI3K/mTOR signaling in two representative HER2+ cells lines; PI3K wild-type SKBR3 cells and PI3K mutant MDA453 H1047R cells. These studies showed that mTORC1 inhibition by RAD001 leads to complete inhibition of S6K phosphorylation at concentrations as low as 50nM however, this was followed by feedback activation of pAKT T308 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 T308 (25, 43). Also, since 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 SKRB3 and MDA453 cells.
treated with RAD001, yet SKBR3 cells are resistant to this drug while MDA453 cells are sensitive. The dual PI3K/mTOR inhibitor BEZ235 is very active in both cell lines and treatment completely eliminates feedback activation of the pathway. Since BEZ235 targets PI3K, reactivation of AKT at Thr308 is blocked and inhibition of the mTORC2 complex blocks reactivation of AKT at Ser473. The activity of the pan-PI3K inhibitor, BKM120, on PI3K signaling results in downstream loss of mTOR activity, ultimately leading to feedback activation of AKT at Thr308. These data demonstrate that directly targeting PI3K also leads to feedback activation of the pathway through reactivation of AKT, however despite this, both SKBR3 and MDA453 cells are sensitive to the anti-proliferative effects of BKM120. Conversely, the p110α specific PI3K inhibitor, BYL719, has a more potent effect on PI3K activity as indicated by persistent dephosphorylation of AKT at 24 hours post-treatment in contrast to BKM120. Interestingly, dephosphorylation of S6 was only observed in the BYL719 sensitive MDA453^H1047R^ and not in the BYL719 resistant SKBR3 cells. Together, these data indicate that single agent treatment with the four classes of PI3K/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 PI3K/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 anti-proliferative activity with almost complete growth inhibition at very low concentrations (10nM/L). The significant single-agent activity of each drug makes quantitating 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 PI3K/mTOR pathway inhibitors either increased the degree of cell death induced by the single agent (BEZ235 & BYL719) or induced apoptosis where little or no cytotoxicity was observed with single agent (RAD001 & BKM120), highlighting the potential benefit of combined targeting of HER2 and PI3K/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 PI3K/mTOR pathway. In HER2+ breast cancer, impressive efficacy was observed for each molecule independent of PI3K mutation status. In ER+/HER2- breast cancer, significant anti-tumor activity was observed independent of PTEN mutation status. Furthermore, in two xenograft models of trastuzumab resistance, single agent treatment with each of the PI3K/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 PI3K/mTOR targeting molecule gave anti-tumor activity across a panel of breast cancer xenografts irrespective of trastuzumab response or the presence of PI3K 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 post 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 anti-angiogenic 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 anti-tumor activity observed with RAD001, BKM120 and BYL719 that have efficacies comparable to 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 6/7 (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 to trastuzumab plus vinorelbine in trastuzumab-pretreated HER2-positive 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 ER+/HER2+ breast cancer, and should also be tested in combination with trastuzumab in patients with trastuzumab-refractory disease.
FIGURE LEGENDS:

Figure 1. Trastuzumab effectively shuts down AKT/mTOR pathway signaling in trastuzumab sensitive cells but not in resistant cells. A, Generational inhibition (inhibition of cell doubling time) in a panel of breast cancer cell lines in response to 5 days trastuzumab treatment (10 – 0.3125 µg/ml), 20% cut-off for sensitivity. Response for BT474 cells plotted on the left y-axis so that values below 20% can be seen more clearly (right y-axis). B, representative blots showing AKT/mTOR signaling over time in response to 15 µg/ml trastuzumab in BT474 cells (trastuzumab sensitive). C, representative blots showing AKT/mTOR signaling following trastuzumab treatment in trastuzumab sensitive cells (BT474), resistant cells (MDA453, SUM225, SUM190 & BT474-TR) and HER2 normal cells (MCF7), all lysates taken following 72 hours exposure to 15 µg/ml trastuzumab. Data represent mean +/- SD. Proliferation assays are prepresented as mean +/- SD of three replicate assays. Western blots were repeated in at least duplicate.

Figure 2. Targeting the PI3K/mTOR pathway in a panel of breast cancer cell lines. BKM120, BYL719, RAD001 and BEZ235 were and prepared for in vitro studies as 10mM/L stock solutions (except RAD001; 20 mM/L) in 100% dimethyl sulfoxide (DMSO). A, mTORC1 specific inhibitor RAD001, #: IC50 > 100 nMol/L. B, Dual PI3K-mTOR inhibitor, BEZ235. C, Activity of the pan-PI3K inhibitor BKM120, #: IC50 > 1 µMol/L. D, P110α-specific inhibitor, BYL719, #: IC50 > 10 µMol/L. A-D, Black bars indicate HER2 amplified cell lines, * indicates cell lines with activating mutations of PIK3CA (exon 9 or 20). Data represent mean +/- SD.

Figure 3. Combination of HER2 and PI3K/mTOR targeting in trastuzumab-sensitive and trastuzumab-resistant breast cancer cell lines. A-C, Cells were treated for 5 days with a range of concentrations of trastuzumab (Tz) (0.3125 to 10 µg/ml) plus RAD001 (R), BEZ235 (Z), BKM120 (K) or BYL719 (all 0.0001 to
10 µM). Generational (Gen) inhibition of >100% considered to be induction of lethality, i.e., 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).

Figure 4. Targeting PI3K/mTOR induces apoptosis and cell cycle arrest in trastuzumab-sensitive and trastuzumab-resistant breast cancer cell lines. A, Propidum Iodide staining of trastuzumab-sensitive and trastuzumab-resistant cells treated for 72 hours with 15 µg/ml trastuzumab +/- 500 nM BKM120, BYL719, RAD001 or BEZ235. Left panels show representative examples of trastuzumab sensitive BT474 cells treated with trastuzumab compared to trastuzumab resistant MDA453 cells treated with trastuzumab (both 15 µg/ml). B-C, Annexin V staining of cells treated as described. Histograms represent % Annexin V positive (AN V+) cells after each treatment. Data represent 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’s t-test.

Figure 5. PI3K/mTOR inhibitors show in vivo efficacy in mouse xenograft models of multiple sub-types of human breast cancer. A, Mice bearing tumor xenografts from the HER2 amplified breast cancer cell lines (UACC812, SUM190 and MDA361) were treated daily oral 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 IP injection twice per week. Data presented as mean tumor volume per group +/- SEM. *, P < 0.001, indicates statistically significant tumor growth in each treatment arm compared with the vehicle control. B, In vivo characterization of a BT474 trastuzumab resistant model (BT474-TR).
Mice bearing BT474 or BT474-TR xenografts (8 per group) were treated with PBS or 10 mg/kg trastuzumab by IP injection twice per week for seven weeks (top panel). "P < 0.05 indicates a statistically significant reduction in trastuzumab response in the BT474-TR relative to the BT474 xenografts at day 35 of study. BT474-TR xenografts (8 per arm) were also treated with trastuzumab alone or trastuzumab plus BKM120, BYL719, RAD001 by daily oral gavage for seven weeks (bottom panel). *, P < 0.001 for each treatment arm within the bracket relative to the vehicle control. C, Quantification of the mean changes in tumor volume of xenografts after seven weeks treatment, negative values indicate tumor regression, "P < 0.05. D, SUM190 xenografts were conditioned in vivo to acquire resistance to trastuzumab. Tumors progressing on trastuzumab were divided into four treatment groups of four mice. 1. Trastuzumab: continued on biweekly 10 mg/kg trastuzumab, 2. BKM120: continued trastuzumab plus 35 mg/kg BKM120, 3. RAD001: continued on trastuzumab plus 10 mg/kg RAD001, 4. BEZ235: continued on trastuzumab plus 35 mg/kg BEZ235, *, P < 0.001 relative to vehicle control. E & F, activity of PI3K/mTOR pathway inhibitors in ER+ breast cancer cell line xenografts, *, P < 0.001. *, P-values calculated by RMANOVA followed by Dunnett test for multiple comparisons. "P-values according to Student t-test.

Figure 6: Biomarkers of in vivo response to inhibitors of PI3K/mTOR signaling. A-B, Reverse Phase Protein Analysis (RPPA) of tumor samples taken after 7 days treatment with the molecules indicated. SUM190 tumors taken 2.5 hours post final dose, UACC812 tumors taken 24 hours post final dose. C-D, Representative images of Immunohistochemical (IHC) staining for pAKT Ser473 and pS6 Ser235-236 in SUM190 tumor xenografts (2.5 hours post final dose).
References


Fig 1.

A

% Generational Inhibition (BT-474)

\[ \text{\( \mu g/ml \ Tz \)} \]

B

15 \( \mu g/ml \) Trastuzumab

PS473-AKT

\( \text{P}^{\text{th}}\text{308-AKT} \)

AKT

P-S6

S6

Tubulin

C

Trastuzumab Sensitive

Trastuzumab Resistant

HER2 Normal

BT474

MDA453

SUM225

SUM190

BT474-TR

MCF7

HER2

PS473-AKT

\( \text{P}^{\text{th}}\text{308-AKT} \)

AKT

\( \text{P}^{\text{235/236}}\text{S6} \)

S6

C  Tz

C  Tz

C  Tz

C  Tz

C  Tz

C  Tz

C  Tz
Fig. 2

A. RAD001 - IC₅₀

B. BEZ235 - IC₅₀

C. BKM120 - IC₅₀

D. BYL719 - IC₅₀
Figure 3

A  BT474, Trastuzumab Sensitive

B  MDA453, De Novo Trastuzumab Resistant

C  BT474-TR, Acquired Trastuzumab Resistant

D  pS473_AKT

pS235/236_S6

S6

p4EBP

4EBP

Tubulin

BT-474

Fig.3
Fig. 4

A  

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<td>MDA453 + Trastuzumab</td>
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B  

BT-474: Trastuzumab Sensitive

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MDA453: Trastuzumab Resistant

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Fig. 5

**A** UACC812, HER2+/ER+, wt PTEN/PI3K
- Control
- RAD001
- BEZ235

**B** SUM190, HER2+/ER+, PI3K (H1047R)
- Control
- RAD001
- BEZ235
- BKM120
- BYL719
- Trastuzumab

**C** MDA361, HER2+/ER+, PI3K (E545K)
- Control
- RAD001
- BEZ235
- BKM120
- BYL719
- Trastuzumab

**D** Change in tumor volume post treatment (day 35 – day 1)
- BT474-TR Control
- BKM120 + Tz
- RAD001 + Tz
- BYL719 + Tz
- BEZ235 + Tz
- Trastuzumab

**E** KPL-1, ER+/HER2-, wt PTEN/PI3K
- Control
- RAD001
- BEZ235
- BKM120
- BYL719

**F** ZR75-1, ER+/HER2-, PTEN mt, wt PI3K
- Control
- RAD001
- BEZ235
- BKM120
- BYL719
Targeting PI3K/mTOR overcomes resistance to HER2-targeted therapy independent of feedback activation of AKT


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