Dual Blockade of HER2 in HER2-Overexpressing Tumor Cells Does Not Completely Eliminate HER3 Function

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Abstract

Purpose: Dual blockade of HER2 with trastuzumab and lapatinib or with pertuzumab is a superior treatment approach compared with single-agent HER2 inhibitors. However, many HER2-overexpressing breast cancers still escape from this combinatorial approach. Inhibition of HER2 and downstream phosphoinositide 3-kinase (PI3K)/AKT causes a transcriptional and posttranslational upregulation of HER3 which, in turn, counteracts the antitumor action of the HER2-directed therapies. We hypothesized that suppression of HER3 would synergize with dual blockade of HER2 in breast cancer cells sensitive and refractory to HER2 antagonists.

Experimental Design: Inhibition of HER2/HER3 in HER2⁺ breast cancer cell lines was evaluated by Western blotting. We analyzed drug-induced apoptosis and two- and three-dimensional growth in vitro. Growth inhibition of PI3K was examined in vivo in xenografts treated with combinations of trastuzumab, lapatinib, and the HER3-neutralizing monoclonal antibody U3-1287.

Results: Treatment with U3-1287 blocked the upregulation of total and phosphorylated HER3 that followed treatment with lapatinib and trastuzumab and, in turn, enhanced the antitumor action of the combination against trastuzumab-sensitive and -resistant cells. Mice bearing HER2⁺ xenografts treated with lapatinib, trastuzumab, and U3-1287 exhibited fewer recurrences and better survival than mice treated with lapatinib and trastuzumab.

Conclusions: Dual blockade of HER2 with trastuzumab and lapatinib does not eliminate the compensatory upregulation of HER3. Therapeutic inhibitors of HER3 should be considered as part of multidrug combinations aimed at completely and rapidly disabling the HER2 network in HER2-overexpressing breast cancers.

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Introduction

Gene amplification and/or overexpression of the receptor tyrosine kinase (RTK) HER2 occur in about 20% of human breast cancers and are associated with poor patient prognosis (1, 2). The antibodies trastuzumab and pertuzumab and the tyrosine kinase inhibitor (TKI) lapatinib are approved by the U.S. Food and Drug Administration for the treatment of HER2-overexpressing breast cancer. Trastuzumab is a humanized monoclonal antibody that binds domain IV in the extracellular region of HER2. Mechanisms of action of the antibody include blockade of ligand-independent HER2–HER3 dimerization (3) and induction of antibody-dependent cellular cytotoxicity and innate immunity to HER2 (4, 5). Trastuzumab in combination with chemotherapy significantly improves the survival of patients with early HER2⁺ breast cancer (6–8). The small-molecule lapatinib is an ATP-mimetic that reversibly binds the ATP pocket in the HER2 tyrosine kinase domain, thus inhibiting its catalytic activity (9). It is active as first-line monotherapy in patients with HER2⁺ metastatic breast cancer and in combination with chemotherapy improves progression-free survival compared with chemotherapy alone (10, 11). Pertuzumab is a humanized antibody that binds the heterodimerization loop in subdomain II of HER2 and, as such, prevents HER2 from dimerizing with ligand-bound HER3 (12). In 2 recent seminal studies, the combination of trastuzumab and pertuzumab was superior to trastuzumab in patients with operable and metastatic HER2⁺ breast cancer (13, 14). Several preclinical and clinical reports have already suggested that dual blockade of HER2 with a combination of HER2 antagonists with complementary mechanisms of action, such as trastuzumab and lapatinib, is a more robust approach to inhibit the HER2 signaling network and, in turn, induce an antitumor effect (15–17).

In patients with HER2⁺ metastatic breast cancer, resistance to trastuzumab and/or lapatinib, either as single agents or in combination with chemotherapy, commonly...
Translational Relevance

There are mounting data that dual blockade of HER2 with inhibitors such as trastuzumab with lapatinib or with pertuzumab is a superior treatment approach. Although these anti-HER2 therapies have improved outcome for patients with HER2+ breast cancer, patients frequently develop acquired drug resistance. Recent reports suggest that inhibition of HER2/PI3K/AKT causes upregulation in HER3 which may attenuate the antitumor action of these inhibitors. We show herein that the neutralizing HER3 antibody, U3-1287, in combination with dual blockade of HER2 further suppresses the growth of HER2+ human breast cancer cells and xenografts. Furthermore, treatment with lapatinib, trastuzumab, and U3-1287 in HER2+ human breast cancer xenografts in vivo results in a statistical increase in survival compared with treatment with lapatinib and trastuzumab. These findings support the use of combinations of HER2 and HER3 inhibitors for the treatment of patients with HER2+ breast cancer.

occurs within months of starting therapy. Only a fraction of patients with HER2+ metastatic breast cancer respond to single-agent trastuzumab (18, 19), suggesting de novo mechanisms of resistance in advanced cancers. These mechanisms include signaling from other HER (ErbB) receptors (20, 21), compensatory signaling from RTKs outside of the HER family (22, 23), aberrant phosphoinositide 3-kinase (PI3K) signaling as a result of mutations in this pathway (24, 25) and the presence of truncated forms of HER2 (26), among few others. Mechanisms of resistance to lapatinib also point to increased (PI3K) signaling, derepression/activation of compensatory survival pathways (27, 28) and defects in proapoptotic molecules such as BIM (29).

HER2 (ErbB2) is a member of the ErbB family of transmembrane RTKs, which also includes the EGF receptor (EGFR, ErbB1), HER3 (ErbB3), and HER4 (ErbB4). Binding of ligands to the extracellular domain of EGFR, HER3, and HER4 induces the formation of kinase active homo- and heterodimers to which activated HER2 is recruited as a preferred partner (30). HER3, which lacks potent intrinsic kinase activity, is able to strongly activate the PI3K/Akt via its 6 docking sites for the p85 regulatory subunit of PI3K, whereas HER2 is unable to directly bind to and activate PI3K/Akt. Loss of HER3 inhibits viability of HER2-overexpressing breast cancer cells (31, 32), and HER2-overexpressing cells are particularly sensitive to apoptosis induced by PI3K inhibitors (33), thus suggesting that the HER3/PI3K axis is essential for survival of HER2-dependent cells.

We and others have shown that inhibition at multiple levels of the PI3K pathway results in FoxO-dependent feedback reactivation of several RTKs which, in turn, limit the sustained inhibition of PI3K and attenuates the action of PI3K pathway antagonists (34–36). In a clinical trial where patients with HER2+ breast cancer were treated with lapatinib, we showed there was upregulation of HER3 protein and maintenance of active AKT in tumor core biopsies obtained at 2 weeks of treatment (34, 37). These studies suggest that treatment approaches aimed at disabling the reactivation of HER3 should improve the antitumor effect of HER2/PI3K-directed therapies.

In this study, we examined whether the neutralizing HER3 monoclonal antibody U3-1287, currently in clinical development, would prevent the upregulation of active HER3 after dual blockade of HER2 with lapatinib and trastuzumab in HER2-overexpressing cells sensitive and refractory to HER2 inhibitors. U3-1287 has been shown to inhibit ligand-induced p-HER3 and cause growth inhibition of pancreatic, non–small cell lung cancer (NSCLC), and colorectal xenograft tumors (38, 39). It has recently completed safety and dose-finding studies in patients with advanced cancer (40). Herein, we show that U3-1287 downregulates HER3 from the cell surface and blocks the upregulation of HER3 that follows the inhibition of HER2. Moreover, U3-1287 in combination with the HER2 inhibitors enhanced apoptosis in vitro, partially restored sensitivity to trastuzumab in trastuzumab-resistant xenografts, and improved the survival of mice bearing BT474 tumors compared with lapatinib and trastuzumab. These studies suggest that (i) dual blockade of HER2 does not eliminate HER3 function completely and (ii) HER3 inhibitors sensitize HER2-overexpressing breast cancers to dual blockade of HER2.

Materials and Methods

Cells and reagents

BT474, SKBR3, and MDA453 cells were obtained from American Type Culture Collection. HR6 cells were derived from a trastuzumab-resistant BT474 xenograft in our laboratory and have been described previously (20). Cells were grown as described (20, 34). The following drugs were used: lapatinib (GW-572016, LC Laboratories); trastuzumab and pertuzumab (Vanderbilt University Hospital Pharmacy, Nashville, TN); and U3-1287 (kindly provided by Johannes Bange at U3 Pharma).

Immunoblot analysis

Cells were prepared as described (34). Lysates were separated by 7% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Bio-Rad). Primary antibodies included Y1197 and Y1289 p-HER3, S473 and T308 p-Akt, total Akt, T202/Y204 p-Erk, total Erk, p-GSK3α/β, p-S6 (Cell Signaling); HER3 (Santa Cruz Biotechnology); and β-actin (Sigma). Immunoreactive bands were detected by enhanced chemiluminescence after incubation with horseradish peroxidase–conjugated secondary antibodies (Promega).

Cell surface biotinylation

Cells were treated with a receptor saturating concentration of U3-1287 (20 μg/mL) alone or in combination with the HER2 inhibitors at 37°C for 24 hours; cells were treated and lysed as described (41). Equal amounts of protein extracts (500 μg) were precipitated using immobilized...
Neutavidin gel (Pierce) followed by SDS-PAGE and HER3 immunoblot analysis.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling
To measure apoptosis, adherent cells were serum-starved for 24 hours and then treated with 1.0 μmol/L lapatinib, 20 μg/mL trastuzumab, and or/and 20 μg/mL U3-1287. After 48 hours, both detached and adherent cells were pooled and subjected to terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis using the APO-Bromodeoxyuridine Kit (Phoenix Flow Systems) following the manufacturer’s protocol.

Monolayer and three-dimensional growth assays
Cells were seeded in 6-well plates (5 × 10^4/well) in 10% FBS-containing medium followed by treatment with inhibitors. Media and inhibitors were replenished every 2 to 3 days until 60% to 80% confluence was achieved in untreated cells. Cells were then fixed and stained with 20% methanol/79.5% water/0.5% crystal violet for 3 minutes, washed with water, and dried. Crystal violet staining intensity was quantified by scanning plates using an Odyssey Infrared Imaging System (LI-COR Biosciences), followed by analysis using manufacturer’s software. For growth in 3-dimension, cells were seeded on growth factor–reduced Matrigel (BD Biosciences) in 48-well plates following published protocols (42). Inhibitors were added to the medium at the time of cell seeding; 12 to 16 days later, the plates were scanned, and colonies measuring ≥25 μm were counted using GelCount software (Oxford Optronix). Colonies were photographed using an Olympus DP10 camera mounted in an inverted microscope. In Fig. 3B, the mean colony size was determined using the imaging software ImageJ (NIH).

Xenograft studies
A 21-day 17β-estradiol pellet (Innovative Research of America) was inserted subcutaneously in the dorsum of 4- to 6-week-old female athymic mice (Harlan Sprague Dawley Inc.) 1 day before cell injection. Approximately, 5 × 10^6 BT474 or HR6 cells were injected s.c. into the mouse right flank. Once tumors reached a volume ≥200 mm^3, mice were randomized to different treatments including U3-1287, trastuzumab, and/or lapatinib using doses as described (34). Tumor xenografts were measured as described (34). After 5 days of treatment, some of the animals were anesthetized with 1.5% isoflurane–air mixture and sacrificed by cervical dislocation. All mouse experiments used in this study were approved by the Institutional Animal Care Committee of Vanderbilt University.

Immunohistochemistry
Tumor fragments were harvested and immediately fixed in 10% buffered neutral formalin for 24 hours at room temperature and then dehydrated and paraffin-embedded. Five-micrometer sections were used for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) using an antibody against S473 p-AKT (Cell Signaling). The intensity of cytoplasmic p-AKT staining were graded by an expert breast pathologist (M.G. Kuba) using a score of 0 to 300 as described (34). Scoring was blinded to treatment arms.

Results

U3-1287 downregulates HER3 and blocks HER3 phosphorylation following inhibition of HER2
We initially examined the temporal effect of the HER3-neutralizing antibody U3-1287 (38, 39) on HER3 protein levels in the HER2-overexpressing human breast cancer cell lines BT474, SKBR3, and MDA453. In all 3 cell lines, there was a modest decrease in HER3 protein levels starting at 1 hour and lasting through 48 hours of treatment (Fig. 1A). Treatment with U3-1287 induced a modest dose-dependent downregulation of HER3, more evident in SKBR3 cells than in BT474 cells (Supplementary Fig. S1). We and others have reported that inhibition of the HER2 kinase and downstream PI3K/AKT with lapatinib results in increased FoxO-dependent HER3 transcription followed by recovery of HER3 phosphorylation (34). Thus, the potent inhibition of PI3K/Akt with the combination of lapatinib and trastuzumab should still result in an increase in FoxO-dependent HER3 transcription. Trastuzumab has been shown to dampen the recovery of HER3 phosphorylation (and hence recovery of PI3K/Akt) by inhibiting ligand-independent HER2–HER3 dimers (3). However, as HER3 can be phosphorylated by other kinases including EGFR, MET (43), Src (44), and FGFR2 (45), it is possible that p-HER3 may still recover, thus engaging PI3K and decreasing the action of the anti-HER2 combination.

Therefore, we examined the effect of U3-1287 on total, phosphorylated, and cell surface HER3 and p-AKT. There was moderate to marked upregulation of cell surface HER3 in all 3 cell lines treated with both HER2 inhibitors (Fig. 1B). Trastuzumab is overall a weak inhibitor of PI3K/Akt. As this inhibition is required to derepress FoxO-mediated transcription of HER3 mRNA (34), we did not observe a consistent increase in HER3 levels in cells treated with trastuzumab alone. The addition of U3-1287 to lapatinib and trastuzumab blocked this increase and almost completely downregulated cell surface HER3 levels. p-HER3 as measured with site-specific antibodies was lower in cells treated with the triple combination. Treatment with lapatinib inhibited both S473 and T308 p-Akt levels. In SKBR3 and MDA453 cells, the addition of U3-1287 to lapatinib and trastuzumab enhanced the inhibition of S473 p-Akt. Furthermore, in BT474 and SKBR3 cells, phosphorylation of the PIP3-dependent PDK-1 site in AKT, as measured by immunoblot with a T308 p-AKT antibody, was lower in cells treated with all 3 inhibitors than in the other treatments (Fig. 1C).

Neutralizing HER3 antibody sensitizes to combination of HER2 inhibitors
We next examined the apoptotic effect of lapatinib, trastuzumab, and U3-1287 using the TUNEL assay. Lapatinib and the combination of lapatinib and trastuzumab...
induced BT474 and SKBR3 cell apoptosis whereas trastuzumab or U3-1287 did not (Fig. 2A). Addition of the HER3 antibody to lapatinib- and trastuzumab-treated cells resulted in a clear increase in apoptosis in all of the cell lines. We next conducted a 2D focus-forming assay as readout for cell growth. The combination of lapatinib and trastuzumab was variably effective against all cell lines. BT474 cells are most sensitive to lapatinib and trastuzumab, and the addition of U3-1287 resulted in a statistical, albeit small decrease in growth compared with lapatinib and trastuzumab. However, SKBR3 and MDA453 cells, which are less sensitive to lapatinib and trastuzumab, showed a more obvious decrease in cell growth upon the addition of U3-1287 (Fig. 2B). In 3-dimensional growth assays in Matrigel, the addition of U3-1287 to lapatinib and trastuzumab resulted in a statistical decrease in total BT474 and MDA453 colony formation compared with that in the presence of both HER2 inhibitors (Fig. 2C). These data suggest that the HER3 antibody sensitizes breast cancer cells to dual HER2 blockade.

**Trastuzumab-resistant cells remain sensitive to HER2-HER3 blockade**

We next expanded our studies to trastuzumab-refractory breast cancer cells. HR6 cells are derived from a BT474 xenograft that was rendered resistant to trastuzumab in vivo (20). In these cells, the addition of U3-1287 to lapatinib and trastuzumab reduced levels of p-HER3, total HER3, and S473 p-AKT (Fig. 3A). Moreover, the triple combination resulted in a statistical reduction of colony formation in 3D Matrigel (Fig. 3B), 2D growth (Fig. 3C), and an increase in apoptosis compared with that induced by the combination of both HER2 antagonists (Fig. 3D).

We next tested whether the addition of U3-1287 would sensitize HR6 xenografts to trastuzumab and lapatinib. Mice bearing established HR6 xenografts were randomized to therapy with vehicle (control), trastuzumab, U3-1287, lapatinib, trastuzumab and U3-1287, lapatinib and trastuzumab, or the combination of all 3 drugs. Trastuzumab as a single agent had no antitumor activity (Fig. 3E), whereas single-agent lapatinib, U3-1287, or the combination of trastuzumab and U3-1287 showed statistical reduction compared with vehicle-treated mice. Tumors treated with the combination of trastuzumab and U3-1287 exhibited a statistical reduction in tumor volume compared with the trastuzumab arm. The addition of U3-1287 to lapatinib/trastuzumab did not reduce tumor volume further compared with lapatinib/trastuzumab. HR6 cells overexpress EGFR and ligands for EGFR including EGF, TGFα, and HB-EGF (20). Thus, being an EGFR-TKI, lapatinib would have a significant antitumor effect, but U3-1287 would be unable to significantly add to the combination, as it cannot completely block ligand-induced EGFR–HER3 dimers, nor prevent EGFR signaling...
However, in each case, the addition of the HER3 antibody was variably effective against MDA453, HR6, and SKBR3 cells. The combination of pertuzumab and trastuzumab was monolayer focus-forming assay as readout for cell growth. MDA453 and HR6 cells (Fig. 4A). We next conducted as well as enhanced inhibition of T308 p-Akt in both result in further inhibition of Y1197 and Y1289 p-HER3 addition of U3-1287 to trastuzumab and pertuzumab. This combination has recently been approved for patients with metastatic HER2 breast cancer (14, 46). Indeed, the addition of U3-1287 to trastuzumab and pertuzumab enhances inhibition of HER3/PI3K signaling and tumor growth.

Addition of U3-1287 to the combination of trastuzumab and pertuzumab enhances inhibition of HER3/PI3K signaling and tumor growth

We next considered a second approach for dual blockade of HER2: the combination of trastuzumab and pertuzumab. This combination has recently been approved for patients with metastatic HER2 breast cancer (14, 46). Indeed, the addition of U3-1287 to trastuzumab and pertuzumab resulted in further inhibition of Y1197 and Y1289 p-HER3 as well as enhanced inhibition of T308 p-Akt in both MDA453 and HR6 cells (Fig. 4A). We next conducted monolayer focus-forming assay as readout for cell growth. The combination of pertuzumab and trastuzumab was variably effective against MDA453, HR6, and SKBR3 cells. However, in each case, the addition of the HER3 antibody resulted in a statistical decrease in cell growth compared with both HER2 inhibitors (Fig. 4B).

Dual blockade of HER2 in combination with HER3 antibody reduces tumor growth

To expand our findings to an in vivo setting, we determined the activity of lapatinib, trastuzumab, U3-1287, or combinations of these against trastuzumab-sensitive BT474 xenografts. We had previously reported that U3-1287 (previously called AMG-888) does not inhibit BT474 xenograft growth and single agent lapatinib has a modest antitumor effect (34). Mice bearing BT474 xenografts measuring ≥350 mm³ were treated with trastuzumab, trastuzumab + U3-1287, lapatinib + U3-1287, lapatinib + trastuzumab or lapatinib + trastuzumab + U3-1287. All treatments significantly inhibited BT474 xenograft growth, particularly the combinations of lapatinib + trastuzumab, U3-1287 + trastuzumab, and the triple combination (Fig. 5A). Mice treated with lapatinib + trastuzumab or lapatinib +...
trastuzumab + U3-1287 had almost no palpable tumor (mean tumor volume = 60 and 41 mm³, respectively) remaining after 3 weeks of treatment.

To document pharmacodynamic biomarkers of PI3K pathway inactivation, some xenografts (n = 4–6) were harvested after 5 days of treatment. All treatment groups had a reduction in S473 p-Akt as assessed by IHC (Fig. 5B). Mice treated with trastuzumab alone exhibited the weakest reduction in S473 p-AKT levels, consistent with the weak anti-signaling effect of trastuzumab (47). All the remaining treatment groups exhibited a statistical reduction in S473 p-Akt compared with vehicle-treated (control) tumors.

**Dual HER2 blockade in combination with HER3 antibody improves survival**

Finally, we sought to determine whether the combined inhibition of HER2 and HER3 will improve long-term survival over dual HER2 blockade with trastuzumab and lapatinib. To test this, we treated mice bearing large BT474 xenografts with (i) lapatinib and trastuzumab or (ii) lapatinib, trastuzumab, and U3-1287. Treatment was initiated when tumors were ≥350 mm³; after 3 weeks of treatment, tumors were undetectable in both groups (data not shown). Treatment was stopped at this time and tumor regrowth was monitored. Eighteen weeks after treatment was discontinued, 6 of 8 (75%) mice treated with lapatinib and trastuzumab, whereas only 3 of 11 (27%) mice treated with lapatinib, trastuzumab, and U3-1287 had to be euthanized because of tumors measuring ≥2000 mm³. This translated to a significant increase in survival in mice treated with the triple therapy compared with mice treated without the HER3 antibody (Fig. 6).

**Discussion**

There is clear evidence to suggest that the HER3 co-receptor is essential for HER2-mediated transformation...
Fig. 4. Addition of U3-1287 to the combination of trastuzumab and pertuzumab enhances inhibition of HER3/PI3K signaling and tumor growth. A, cells were treated with U3-1287 (20 μg/mL), pertuzumab (20 μg/mL), or the indicated combinations for 24 hours. Whole-cell lysates were prepared and separated in a 7% SDS gel followed by transfer to nitrocellulose and immunoblot analysis with the indicated antibodies. B, cells were seeded in triplicate and treated with vehicle, U3-1287 (20 μg/mL), pertuzumab (20 μg/mL), or the indicated combinations. Media and inhibitors were replenished every 3 to 4 days. The monolayers were stained with crystal violet when the untreated cells became confluent after 14 to 21 days. Quantification of integrated intensity (% control) is shown (*, P < 0.05, t test).

and tumor progression as well as for the acquired resistance to HER2 inhibitors in HER2-overexpressing breast cancers. For example, loss of HER3 prevents HER2-mediated transformation of mammary epithelium (48). We show herein that an HER3-neutralizing antibody blocks the upregulation of total and activated HER3 that follows inhibition of PI3K/AKT upon treatment with lapatinib and trastuzumab. In turn, the HER3 antibody enhanced the antitumor effect of the anti-HER2 drug combination. Finally, mice treated with lapatinib, trastuzumab, and U3-1287 exhibited an increase in overall survival compared with mice treated with lapatinib and trastuzumab. These data suggest that HER3 antagonists should be an integral part of treatment strategies aimed at completely disabling the output of the HER2 network to PI3K in HER2-overexpressing cancers.

Inhibition of HER2/PI3K/AKT has been shown to induce an upregulation of several RTKs including HER3, insulin-like growth factor-1 receptor (IGF-1R), and insulin receptor among others (34–36). In turn, this compensatory feedback dampens the antitumor effect of HER2/PI3K/AKT antagonists. Treatment of HER2+ cells with lapatinib and trastuzumab resulted in an increase in both cell surface and total HER3 while not completely eliminating HER3 phosphorylation (Fig. 1B and C). However, addition of the HER3 antibody U3-1287 decreased cell surface and total HER3 as well as phosphorylated HER3 below basal levels. In BT474 and SKBR3 cells, phosphorylation of the PIP3-dependent PDK-1 site in AKT, as measured by immunoblot with a T308 p-AKT antibody, was lower in cells treated with all 3 inhibitors than in the other treatments. This suggests that a more complete and sustained inhibition of HER3 is necessary to block PI3K function and the subsequent production of PIP3, which maintains PDK-1 and AKT at the plasma membrane. Pulsatile and less frequent higher doses of lapatinib have been proposed as a means of sustained inhibition of HER3 in HER2+ tumors (49). This schedule is currently under investigation. However, higher concentrations than the one we are using (1 μmol/L) against cells in culture are not achieved in patients with the current daily schedule and may introduce off-target effects.

The combination of trastuzumab and U3-1287 was particularly effective against BT474 xenografts, significantly more so than the combination of lapatinib and U3-1287. Of note, the antitumor action of this combination was associated with moderate inhibition of tumor p-AKT levels (Fig. 5A), not as potent as that seen in lapatinib-treated tumors. Trastuzumab is thought to work mainly via blockade of ligand-independent HER2-HER3 dimerization (3) and induction of antibody-dependent cellular cytotoxicity and innate immunity to HER2 (4, 5). Therefore, this result suggests that the immune effector mechanisms of trastuzumab are central to its antitumor action.

It is also possible that U3-1287 and trastuzumab block ligand-induced (U3-1287) and ligand-independent HER2–HER3 dimers (trastuzumab). This speculation is consistent with modest inhibition of p-AKT, a downstream effect of HER2–HER3 dimerization and activation of PI3K. This result is reminiscent of the proposed mechanism of synergy between trastuzumab and pertuzumab (16). Thus, we investigated the addition of the HER3 inhibitor to dual blockade of HER2 with pertuzumab and trastuzumab. The combination of U3-1287, trastuzumab, and pertuzumab induced a greater inhibition of PI3K signaling and tumor growth in vitro compared with trastuzumab and pertuzumab (Fig. 4). This suggests that dual blockade...
of ligand-induced and ligand-independent HER2–HER3 dimers (by pertuzumab and trastuzumab, respectively) is not completely effective at removing HER3 function in HER2-dependent tumors.

In summary, dual blockade of HER2 does not eliminate HER3 function completely. Targeted inhibition of HER3 improved the response to HER2 antagonists in several HER2-dependent models of breast cancer. On the basis of these data, we conclude that therapeutic inhibitors of HER3 should be considered as part of multidrug combinations aimed at completely and rapidly disabling the HER2 network in HER2-overexpressing breast cancers. Furthermore, the combination with of HER3 antibodies with trastuzumab represents another potential strategy for dual blockade of HER2.

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

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