

Ligand-Independent HER2/HER3/PI3K Complex Is Disrupted by Trastuzumab and Is Effectively Inhibited by the PI3K Inhibitor GDC-0941

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SUMMARY

Herceptin (trastuzumab) is the backbone of HER2-directed breast cancer therapy and benefits patients in both the adjuvant and metastatic settings. Here, we describe a mechanism of action for trastuzumab whereby antibody treatment disrupts ligand-independent HER2/HER3 interactions in HER2-amplified cells. The kinetics of dissociation parallels HER3 dephosphorylation and uncoupling from PI3K activity, leading to downregulation of proximal and distal AKT signaling, and correlates with the antiproliferative effects of trastuzumab. A selective and potent PI3K inhibitor, GDC-0941, is highly efficacious both in combination with trastuzumab and in the treatment of trastuzumab-resistant cells and tumors.

INTRODUCTION

Herceptin (trastuzumab) was initially approved for the treatment of women with metastatic breast cancer overexpressing HER2/ErB2. The randomized clinical trial that led to this approval was conducted in combination with standard cytotoxic chemotherapy (Slamon et al., 2001). In addition, two single-agent, single-arm studies were also conducted in metastatic breast cancer patients, and these studies demonstrated that trastuzumab alone had a finite and measurable response rate (Cobleigh et al., 1999; Vogel et al., 2002). Long-term therapeutic benefit is also recognized in the adjuvant setting. For early-stage, HER2-positive breast cancer patients, trastuzumab therapy after chemotherapy offers significant benefit (Smith et al., 2007).

Trastuzumab is thought to manifest its mechanism of action in multiple ways. As a humanized IgG₁, it binds to Fcγ receptor III (RIII) and is a potent mediator of antibody-dependent, cell-mediated cytotoxicity (ADCC). Evidence for the importance of Fc-FcγRIII involvement in the mechanism of action of trastuzumab is provided by Clynes et al. (2000), who assessed trastuzumab response in tumor models deficient in FcγRIII function. In addition to engaging immune effector cells, numerous investigations suggest that trastuzumab also inhibits tumor cell signaling

or shows partial agonist activities (Sarup et al., 1991). The biochemical nature of these properties is confusing and controversial. Initially, it was thought that engagement of HER2 by trastuzumab led to an increase in HER2 endocytosis or degradation rate (Hudziak et al., 1989). Careful analysis of HER2 endocytosis led to the conclusion that the monoclonal antibodies do not affect HER2 internalization or endocytic rates (Austin et al., 2004; Hommelgaard et al., 2004). Additionally, trastuzumab is not effective in blocking dimerization of HER2 with ligand-activated EGFR or HER3 (Agus et al., 2002). Nevertheless, trastuzumab treatment does result in G₁-S cell cycle growth arrest that does not lead to apoptosis (Lewis et al., 1996). Several reports suggest that the cytostatic effect of trastuzumab correlates with a downregulation of AKT activity, which ultimately leads to an increase in the CDK2 inhibitor p27 (Lane et al., 2000; Lee et al., 2001; Yakes et al., 2002). Furthermore, trastuzumab is reported to have either no effect on HER2 activation status or to cause a rapid increase or decrease of HER2 phosphorylation (Lane et al., 2000; Nagata et al., 2004; Sarup et al., 1991).

A number of studies suggest that the transformation potential of HER2 is augmented by coexpression with HER3 (Cohen et al., 1996; Zhang et al., 1996). Interfering with or ablating HER3 expression or interaction with HER2 resulted in antiproliferative

SIGNIFICANCE

This study characterizes a mechanism of action for trastuzumab whereby trastuzumab disrupts the constitutively signaling ligand-independent HER2/HER3/PI3K complex. The findings presented suggest that a combination of trastuzumab and the PI3K inhibitor GDC-0941 warrants clinical evaluation in patients who have not been treated with a HER2-directed therapy in the metastatic setting. Moreover, patients who progress on trastuzumab or lapatinib (a HER2-directed tyrosine kinase inhibitor) may derive benefit from additional therapy that includes the PI3K inhibitor GDC-0941.

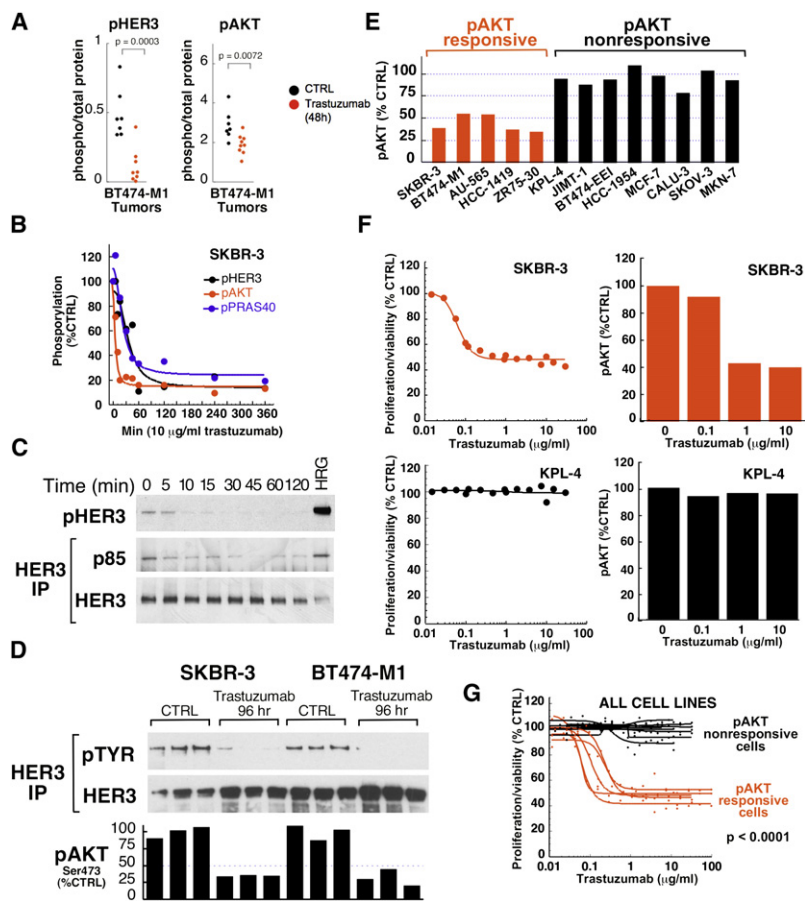


Figure 1. Trastuzumab-Induced Inhibition of Proliferation Correlates with Inhibition of the HER3/PI3K/AKT Pathway

(A) Pre-established BT474-M1 tumor xenografts were treated with 20 mg/kg trastuzumab or control antibody. Tumors were harvested after 48 hr, homogenized, and analyzed for pHER3 (Tyr1289) and total HER3 by western blot. pAKT (Ser473) and total AKT were analyzed by using ELISA. The statistical significance was determined by using Dunnett's test.

(B) SKBR-3 cells were treated with 10 μ g/ml trastuzumab for indicated times. pHER3 (Tyr1289), pAKT (Ser473), and pPRAS40 (Thr246) were analyzed by using western blot or ELISA (pAKT). See Figure S1A for original western blots.

(C) SKBR-3 cells were treated with 10 μ g/ml trastuzumab for indicated times or with heregulin (HRG) for 15 min. The p85 subunit of PI3K was detected from HER3 immunoprecipitates by western blot. pHER3 (Tyr1289) was blotted directly from the lysates.

(D) SKBR-3 and BT474-M1 cells were treated with 10 μ g/ml trastuzumab for 96 hr. pHER3 was analyzed by HER3 immunoprecipitation, followed by detection with HRP-conjugated p-Tyr antibody. pAKT (Ser473) was analyzed by using ELISA.

(E) HER2-overexpressing cancer cells and MCF-7 cells were treated with or without 10 μ g/ml trastuzumab for 2 hr, lysed, and subjected to pAKT ELISA (Ser473). Cell lines were grouped as pAKT responsive (red columns) and nonresponsive (black columns) according to the level of pAKT inhibition by trastuzumab. 25% inhibition was used as a cutoff for the response.

(F) Trastuzumab dose response of pAKT-responsive (SKBR-3) and -nonresponsive (KPL-4) cells. pAKT (Ser473) was analyzed by ELISA after 2 hr of treatment. Proliferation was analyzed after 6 days of treatment.

(G) Trastuzumab dose response (see [E] for cell lines). Proliferation was analyzed after 6 days of treatment. Grouping into pAKT responsive (red data points) and nonresponsive (black data points) is described in (E). The statistical significance was determined by using a t test from the values achieved with 1 μ g/ml trastuzumab.

effects in HER2-positive breast cancer cell lines (Holbro et al., 2003; Ram et al., 2000). We recently confirmed and extended upon these observations by using a combination of RNA silencing of HER receptor expression, a tumor model with inducible HER3 knockdown, and the analysis of HER3 activation status in HER2-positive breast cancer specimens (Lee-Hoeflich et al., 2008). Based on these findings, we conclude that constitutively active HER2 is dependent on HER3 for mediating its effects, and that EGFR plays little or no role in driving the biology of HER2-positive breast cancer.

Previously, we reported that pertuzumab binding to the extracellular domain II of HER2 (Franklin et al., 2004) efficiently blocks ligand-induced HER2/HER3 dimerization (Agus et al., 2002). In contrast, trastuzumab binds to subdomain IV of HER2, which results in the downregulation of PI3K/AKT signaling in tumor cells that overexpress HER2 in the absence of ligands. An objective of the present study was to examine the molecular basis for trastuzumab's effect on ligand-independent HER2/HER3/PI3K signaling. In addition, we investigated GDC-0941, a potent and selective PI3K inhibitor (Folkes et al., 2008) in model systems that were sensitive and resistant to trastuzumab.

RESULTS

Trastuzumab Treatment of Tumors Inhibits the HER3/PI3K/AKT Pathway In Vivo

To examine whether trastuzumab treatment results in dephosphorylation of HER3 and AKT in vivo, we treated mice harboring established BT474-M1 tumors with 20 mg/kg trastuzumab for 48 hr. Tumor lysates were subjected to western blot (pHER3) and ELISA (pAKT) analysis. Treatment with trastuzumab (Figure 1A) significantly reduced the level of pHER3 (Tyr1289) in the tumors ($p = 0.0003$, Dunnett's test). Similarly, pAKT (Ser473) was significantly reduced in trastuzumab-treated tumors ($p = 0.0072$). Together, these data demonstrate that trastuzumab treatment leads to inhibition of the HER3/PI3K/AKT pathway in vivo.

Kinetics of HER3/PI3K/AKT Pathway Inhibition by Trastuzumab

Although trastuzumab treatment is reported to decrease pHER3, pAKT, and PI3K activity in HER2-amplified cells (Lane et al., 2000; Yakes et al., 2002), the kinetics and contribution of this inhibition

to the antiproliferative effect are unclear. Yakes et al. (2002) describe a robust initial increase in pHER3 after treatment for 1 hr with trastuzumab, followed by slow dephosphorylation after 12–24 hr. In contrast, we detect a rapid dephosphorylation of HER3 (Tyr1289), visible already after 10 min of treatment with trastuzumab in SKBR-3 and BT474-M1 cells. The degree of inhibition plateaus at 80% after 60 min and was maintained for 6 hr (Figure 1B; Figure S1A, available online). HER3 dephosphorylation was also reflected in loss of total phosphotyrosine and thus is not specific for Tyr1289 (Figure S1B). Basal pHER3 is not derived from ligand activation based on the following: (1) no exogenous ligand is used in the experiments; (2) the cells do not express HER3 ligands; (3) extended serum starvation does not affect pHER3. Trastuzumab is also reported to decrease HER2 phosphorylation (Lane et al., 2000). In contrast, we did not detect inhibition of HER2 phosphorylation (Figure S1B). Dephosphorylation of HER3 led to an immediate dissociation of the p85 subunit of PI3K from HER3 (Figure 1C). AKT dephosphorylation (Ser473, Thr308) followed similar kinetics. Maximal inhibition was reached in 15–60 min (Figure 1B; Figures S1A and S1B). Dephosphorylation of the AKT substrate PRAS40 (Thr246) proceeded to a similar magnitude of inhibition and with parallel kinetics (Figure 1B). Sergina et al. (2007) reported that small-molecule EGFR tyrosine kinase inhibitors decrease HER3 and AKT phosphorylation, but fail to maintain dephosphorylation after long-term incubation. In contrast, inhibition of HER3 and AKT phosphorylation was sustained after 4 days of treatment with trastuzumab (Figure 1D). In conclusion, trastuzumab causes an immediate and potent inhibition of the HER3/PI3K/AKT pathway that is maintained for at least 4 days. The kinetics and magnitude of pathway inhibition suggest that the effect has an important role in the trastuzumab response.

Trastuzumab-Induced Growth Inhibition Correlates with HER3/PI3K/AKT Pathway Inhibition In Vitro

We next analyzed whether the strong effect on the HER3/PI3K/AKT pathway is predictive of antiproliferative responses in high-HER2-expressing cell lines. We selected a set of breast cancer cells known to overexpress HER2 (SKBR-3, BT474-M1, AU-565, HCC-1419, ZR75-30, KPL-4, JIMT-1, BT474-EEI, and HCC-1954), a low-HER2-expressing breast cancer cell line (MCF-7), and HER2-overexpressing lung, ovarian, and gastric cancer cell lines (CALU-3, SKOV-3, and MKN-7, respectively). Treatment of the cells with trastuzumab for 2 hr caused a strong and dose-dependent (Figures 1E and 1F) decrease in AKT phosphorylation (60%–45%) in five HER2-overexpressing breast cancer cell lines (SKBR-3, BT474-M1, AU-565, HCC-1419, and ZR75-30). No AKT dephosphorylation was detected in other cell lines (Figure 1E). All of the cell lines express detectable levels of HER3 (Figure S2).

Inhibition of proliferation corresponded with the ability of trastuzumab to inhibit pAKT. In SKBR-3 cells, trastuzumab treatment inhibited both pAKT and proliferation by 50%–60%; in contrast, no inhibition was detected in either pAKT or proliferation in KPL-4 cells (Figure 1F). Cell lines were grouped as pAKT responsive (>25% pAKT inhibition by 10 μ g/ml trastuzumab) and pAKT nonresponsive (<25% pAKT inhibition by 10 μ g/ml trastuzumab). Inhibition of proliferation strongly correlated with the ability of

trastuzumab to inhibit pAKT ($p < 0.0001$; t test at 1 μ g/ml trastuzumab; Figure 1G).

All HER-family receptors are capable of activating the MEK/ERK pathway (Hynes and Lane, 2005). Moreover, trastuzumab was reported to inhibit ERK phosphorylation (Yakes et al., 2002). We treated two trastuzumab-sensitive cell lines (SKBR-3, BT474-M1) with the allosteric MEK inhibitor PD0325901 (Solit et al., 2006). Although the MEK inhibition resulted in dephosphorylation of ERK, the inhibitor had a minimal effect on cell proliferation (Figure S3), suggesting that the inhibitory effect of trastuzumab on the MEK/ERK pathway is not sufficient for inhibition of proliferation. This result is consistent with previous similar experiments with BT474 xenografts (Solit et al., 2006).

In conclusion, the ability of trastuzumab to inhibit in vitro proliferation correlates with HER3/PI3K/AKT pathway inhibition, suggesting that the pathway inhibition is required for the antiproliferative effects and indicating that activating modifications involving the PI3K pathway are a likely and important cause of trastuzumab resistance.

The PI3K Inhibitor GDC-0941 Inhibits the Proliferation of Both Trastuzumab-Sensitive and -Insensitive Cells

To further investigate whether the HER3/PI3K/AKT pathway is controlling the proliferation and survival of HER2-amplified cells, we treated all cell lines with GDC-0941. GDC-0941 inhibits p110 α , p110 β , and p110 δ subunits of PI3K with strong selectivity over other assayable kinases (Folkes et al., 2008). Treatment with 250 nM GDC-0941 for 2 hr resulted in 40%–85% inhibition of pAKT in all cell lines tested (Figure 2A). No substantial difference was observed in the degree of AKT dephosphorylation between trastuzumab-sensitive and -insensitive cells after GDC-0941 treatment. The effect of GDC-0941 on cell proliferation/viability was tested after 3 days of GDC-0941 treatment. Inhibition of the PI3K/AKT pathway by GDC-0941 was reflected as a dose-dependent reduction in cell proliferation/viability (Figure 2B). GDC-0941 inhibited the growth of both trastuzumab-sensitive and -insensitive cells (Figures 2B and 2C). The IC₅₀ values for GDC-0941 ranged between 150 and 950 nM and did not correlate with trastuzumab sensitivity ($p = 0.47$; t test; Figure 2D). These results confirm that PI3K/AKT pathway activity is directly linked to the proliferation of HER2-overexpressing cells.

Trastuzumab Inhibits the Ligand-Independent HER2/HER3 Interaction

Our recent studies confirmed that the complex of HER2 and HER3 is a critical oncogenic unit in HER2-positive breast cancer cell lines (Lee-Hoeflich et al., 2008). However, the initial effect of trastuzumab on the HER2/HER3 complex is unclear. Previous studies demonstrated that trastuzumab does not induce downregulation of HER2 (Austin et al., 2004). Furthermore, trastuzumab does not reduce HER2 phosphorylation (Figure S1B). Our previous report on the effect of trastuzumab on HER2/HER3 association focused on ligand-activated HER2/HER3 dimerization (Agus et al., 2002). Using standard immunoprecipitation methods (Agus et al., 2002) (Figure 3A, right panel), we were initially unable to detect a direct HER2/HER3 interaction in the absence of ligand stimulation. We hypothesized that heregulin (HRG)/HER3/HER2 complexes could be more stable or abundant than the ligand-independent HER3/HER2 complexes that were

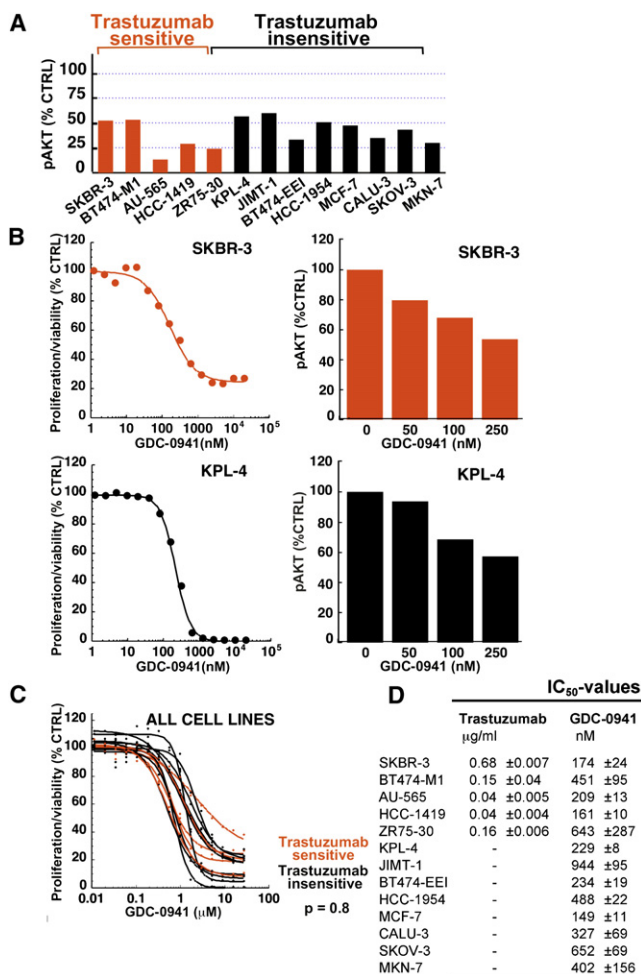


Figure 2. The PI3K Inhibitor GDC-0941 Inhibits the Growth of Both Trastuzumab-Sensitive and -Insensitive HER2-Amplified Cells

(A) Cells were treated with or without 250 nM GDC-0941 for 2 hr, lysed, and subjected to pAKT ELISA (Ser473). Cell lines were grouped as trastuzumab-sensitive (red columns) and -insensitive (black columns) according to proliferation inhibition by trastuzumab (Figure 1G). 25% inhibition was used as a cutoff for the sensitivity.

(B) GDC-0941 dose response of trastuzumab-sensitive (SKBR-3) and -insensitive (KPL-4) cells. pAKT (Ser473) was analyzed by ELISA after 2 hr of treatment. Proliferation/viability was analyzed after 3 days of treatment.

(C) GDC-0941 dose response (see [A] for cell lines). Proliferation/viability was analyzed after 3 days of treatment. Grouping to trastuzumab-sensitive (red) and -insensitive cells (black) are described in (A). The statistical significance was determined by using a *t* test from the values achieved with 500 nM GDC-0941.

(D) Trastuzumab and GDC-0941 IC₅₀ values (± standard error) from the proliferation/viability assays (Figures 1G and [C]).

formed as a result of HER2 overexpression. It is likely that breast cancers that overexpress HER2 may be activated in both a ligand-independent and -dependent manner. To increase the sensitivity of the analysis, we utilized a reversible chemical cross-linking procedure with 3,3'-dithiobis[sulfosuccinimidyl]propionate (DTSSP). This reagent crosslinks extracellular proteins through amino groups. Even weakly interacting protein complexes can then be coimmunoprecipitated. Protein complex compo-

nents are identified by western blots after SDS-PAGE in reducing conditions, which reverses the crosslinking. Ligand-independent HER2/HER3 complexes were readily detected in SKBR-3 cells by using DTSSP crosslinking (Figure 3A, left panel). The HER2/HER3 interaction was significantly increased when cells were pre-treated with the HER3 ligand HRG (Figure 3A). Consistent with previous reports, pertuzumab efficiently inhibited ligand-induced HER2/HER3 dimerization, whereas trastuzumab had only a minor effect (Figure 3A, right panel). Pertuzumab is a therapeutic antibody that binds the extracellular domain II of HER2 and blocks the association of HER2 with HER3 when cells are stimulated with HER3 ligand (Agus et al., 2002). Interestingly, in the absence of HRG, the abilities of the antibodies to disrupt the ligand-independent HER2/HER3 complex were reversed: the amount of HER3 associating with HER2 was clearly reduced when cells were treated with trastuzumab (Figure 3A, left panel). Pertuzumab caused a minor decrease in HER3 in complex with HER2 (Figure 3A, left panel).

Next, we performed a series of coimmunoprecipitation experiments in the absence of the crosslinker. For this, we modified the immunoprecipitation protocol by omitting the zwitterionic detergent (CHAPS) from the lysis buffer, by increasing the amount of lysate, and by utilizing a more sensitive chemiluminescent reagent. These modifications allowed us to detect ligand-independent HER2/HER3 dimer in the absence of chemical crosslinking. The kinetics of HER3 release from the HER2 complex corresponded with inhibition of the pHER3/pAKT pathway after trastuzumab treatment, occurring 5–15 min after trastuzumab addition (Figure 3B). Moreover, trastuzumab disrupted the HER2/HER3 interaction in a dose-dependent manner (Figure 3C), corresponding with the concentrations typically required for AKT dephosphorylation (Figure 1F) and inhibition of proliferation (Figure 1G).

We further hypothesized that if displacement of HER3 from HER2 is essential for the antiproliferative activity of trastuzumab, then interfering with the HER2/HER3 interaction should be proportional to inhibiting cell proliferation. As predicted, trastuzumab inhibited pHER3, pAKT, and the proliferation of BT474-M1 cells more efficiently than pertuzumab (Figures 3D–3F). Together, these results demonstrate that trastuzumab treatment disrupts ligand-independent HER2/HER3 association, which results in a rapid decrease of HER3 phosphorylation and PI3K/AKT pathway inactivation, ultimately leading to inhibition of cell proliferation.

Trastuzumab and GDC-0941 Synergistically Inhibit the PI3K/AKT Pathway and Cell Proliferation

Since both trastuzumab and GDC-0941 inhibit the PI3K/AKT pathway, combining these inhibitors might result in additive or synergistic effects on the proliferation of trastuzumab-sensitive cell lines. Treatment of BT474-M1 with 10 μg/ml trastuzumab caused an equal decrease in pAKT as 100 nM GDC-0941 treatment (63% and 63% inhibition, respectively; Figure 4A). Addition of trastuzumab to GDC-0941 caused a dose-dependent, enhanced decrease in pAKT (Figure 4A). The addition of 10 μg/ml trastuzumab resulted in a 67% reduction in pAKT compared to 50 nM GDC-0941 treatment alone.

Next, we evaluated whether the downstream AKT signaling components were affected. The addition of trastuzumab to

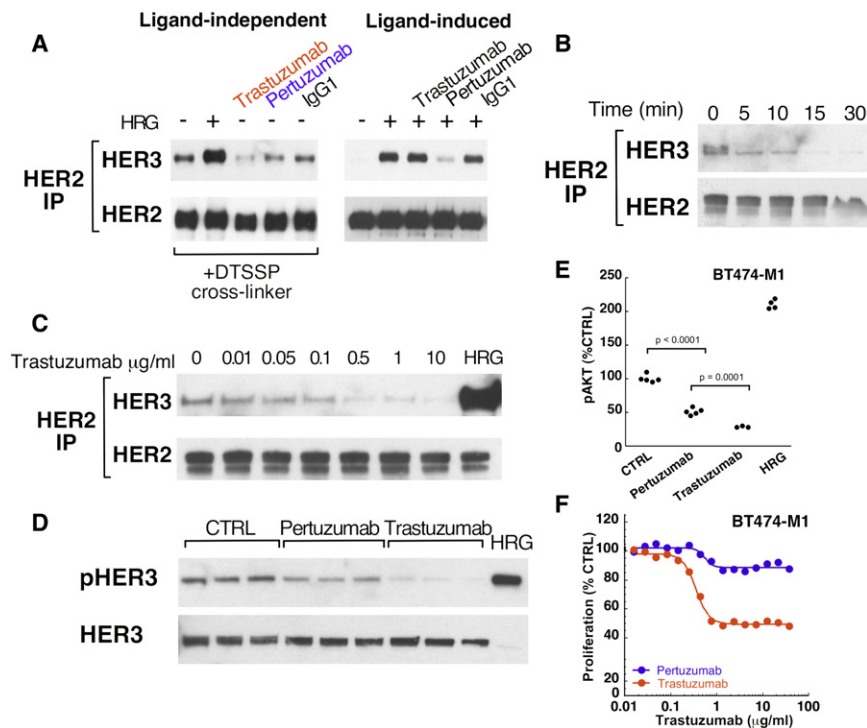


Figure 3. Trastuzumab Disrupts the Ligand-Independent HER2/HER3 Interaction

(A) SKBR-3 cells were treated with 100 nM anti-body (1 hr), followed by 0.5 nM heregulin (HRG, 12 min) when indicated. In the left panel, extracellular proteins were crosslinked with DTSSP. Lysates were immunoprecipitated by using anti-HER2 antibody (7C2). SDS-PAGE in reducing conditions reverses the crosslinking and allows for quantitative analysis of coimmunoprecipitated HER3 by western blot.

(B) BT474-M1 cells were treated with 10 $\mu\text{g/ml}$ trastuzumab. Lysates were immunoprecipitated by using anti-HER2 antibody (7C2) and analyzed for coimmunoprecipitating HER3 by western blot. (C) Trastuzumab dose response (100 min) in BT474-M1 cells was determined as in (B). HRG = 10 nM heregulin, 15 min.

(D and E) (D) BT474-M1 cells were treated with 10 $\mu\text{g/ml}$ antibodies for 2 hr, and pHER3 (Tyr1289) was analyzed by western blot. (E) pAKT (Ser473) was analyzed by ELISA. CTRL lanes represent nontreated samples. Human IgG1 treatment does not inhibit pHER3 or pAKT (data not shown). HRG = 10 nM heregulin, 15 min. The statistical significance was determined by using Dunnett's test.

(F) Proliferation of BT474-M1 was analyzed after 6 days of treatment with trastuzumab or pertuzumab.

GDC-0941 further decreased the phosphorylation of a direct AKT substrate, PRAS40 (Thr246), and a distal substrate, phospho-S6 ribosomal protein (Ser235/236), demonstrating that trastuzumab and GDC-0941 exhibit a pronounced combinatorial effect on downstream AKT signaling (Figure 4B). Sergina et al. (2007) recently reported a compensatory feedback from PI3K inhibition that results in elevated pHER3. A similar compensatory effect on pHER3 is seen when using GDC-0941 (Figure 4B). However, addition of trastuzumab efficiently blocks this compensatory effect (Figure 4B).

We next addressed whether enhanced PI3K/AKT pathway inhibition results in decreased cell proliferation/viability. In the absence of trastuzumab, the IC_{50} value for GDC-0941 in a 6-day treatment of BT474-M1 was 296 nM (Figure 4C). The addition of trastuzumab to GDC-0941 caused a dose-dependent decrease in cell proliferation/viability. Addition of 10 $\mu\text{g/ml}$ trastuzumab resulted in a 64% decrease in the concentration of GDC-0941 that is required to reach its IC_{50} of 106 nM. A similar combinatorial effect on pAKT and inhibition of proliferation was also observed with SKBR-3 cells (data not shown), but not when the trastuzumab-insensitive KPL-4 cells were treated (Figure S4). Proliferation data were analyzed by the established method of Chou and Talalay (1984) by using CalcuSyn software. The resulting combination index (C.I.) values were < 1 over most of the effect range of the drugs (fractional effect 0.3–0.8), demonstrating that the combination of trastuzumab and GDC-0941 inhibits proliferation synergistically in BT474 and SKBR-3 cells (Figure 4D). Together, these data show that combining trastuzumab and GDC-0941 enhances the inhibitory effect on AKT and its downstream targets, resulting in a synergistic effect on the proliferation of trastuzumab-sensitive breast cancer cells. Our results

also demonstrate that trastuzumab blocks the compensatory negative-feedback loop caused by GDC-0941 treatment (Figure 4B), providing a plausible molecular mechanism that promotes the synergistic effect observed with trastuzumab-GDC-0941 combination treatment.

Combination of Trastuzumab and GDC-0941 Induces Apoptosis of Breast Cancer Cells

To investigate the effect of the trastuzumab-GDC-0941 combination on apoptosis, BT474-M1 cells were treated for 48 hr with both inhibitors. Combining trastuzumab and GDC-0941 increased the accumulation of cleaved caspase-3 fragments, indicating activation of this key effector caspase (Figure 4E). Combining trastuzumab and GDC-0941 also resulted in an increase in the cleaved PARP 89 kDa fragment, a known response to caspase-3 activation (Figure 4E). The activity of caspases 3 and 7 was also increased when trastuzumab was added to GDC-0941 treatment (Figure 4F, left panel). Combining trastuzumab with 250 nM GDC-0941 increased caspase activity to a level similar to that detected with a 4-fold higher dose (1000 nM) of GDC-0941 alone. Importantly, this increase in caspase activity was reflected in the apoptotic index of these cells. The addition of 10 $\mu\text{g/ml}$ trastuzumab dramatically decreased the concentration of GDC-0941 required to induce apoptosis (Figure 4F, middle panel). A near equivalent level of apoptosis was detected when cells were treated with 100 nM GDC-0941 and trastuzumab than when treated with 1000 nM GDC-0941 alone. As expected, the increase in apoptosis was reflected in a decrease in cell viability after 48 hr (Figure 4F, right panel). A similar increase in caspase activity and apoptosis was observed with SKBR-3 cells (data not shown). Together, these data show that the combination

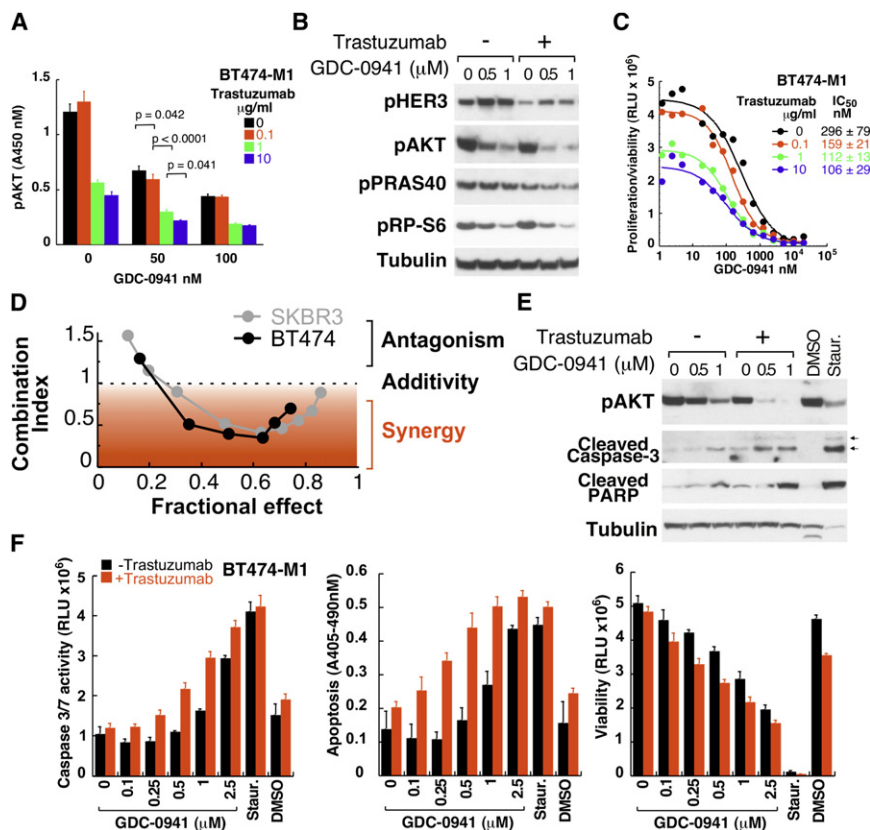


Figure 4. GDC-0941 and Trastuzumab Synergistically Inhibit Proliferation and Induce Apoptosis

(A) BT474-M1 cells were treated with the indicated GDC-0941 concentrations and 0, 0.1, 1, or 10 $\mu\text{g/ml}$ trastuzumab (black, red, green, and blue columns, respectively). pAKT (Ser473; $\pm\text{SD}$) was analyzed by ELISA after 2 hr of treatment. The statistical significance was determined by using Dunnett's test.

(B) BT474-M1 cells were treated with the indicated GDC-0941 concentrations in the presence (+) or absence (–) of 10 $\mu\text{g/ml}$ trastuzumab for 4 hr. pHER3 (Tyr1289), pAKT (Ser473), pPRAS40 (Thr246), phospho-S6 ribosomal protein (pRP-S6; Ser235/236), and tubulin were analyzed by western blot.

(C) BT474-M1 cells were treated with the indicated GDC-0941 concentrations and 0, 0.1, 1, or 10 $\mu\text{g/ml}$ trastuzumab (black, red, green, and blue dots, respectively). Cell proliferation/viability was analyzed and IC_{50} (\pm standard error) was determined after 6 days of treatment.

(D) The proliferation/viability of BT474 or SKBR-3 cells was analyzed after 3 days of treatment with the drug combinations. Combination index (C.I.) values were determined by using the method of Chou and Talalay (1984) (CalcuSyn software) for drug combinations with a fractional effect between 0.2 and 0.9 (inhibiting 20%–90% of proliferation/viability). C.I. values < 1 indicate drug synergy.

(E) BT474-M1 cells were treated with the indicated GDC-0941 concentrations in the presence (+)

or absence (–) of 10 $\mu\text{g/ml}$ trastuzumab for 48 hr. pAKT (Ser473), cleaved caspase-3 (arrowheads depict 19 and 17 kDa fragments), cleaved PARP, and tubulin were analyzed by western blot. Staurosporin (1 μM) was used as a positive control for apoptosis.

(F) BT474-M1 cells were treated with indicated GDC-0941 concentrations in the presence (red columns) or absence (black columns) of 10 $\mu\text{g/ml}$ trastuzumab for 48 hr. The Caspase-Glo 3/7 assay, the Cell Death Detection ELISA^{plus} assay, and the CellTiter-Glo Luminescent Cell Viability Assay were used to detect caspase 3/7 activity (left), apoptosis (middle), and cell viability (right). Bars represent mean \pm SD. Staurosporin (1 μM) was used as positive control for apoptosis.

of GDC-0941 with trastuzumab significantly lowers the GDC-0941 concentration required for reaching the threshold of caspase activation and apoptosis in trastuzumab-sensitive breast cancer cells. Therefore, trastuzumab treatment sensitizes HER2-amplified cells to PI3K inhibition and thus provides an additional level of tumor specificity for the PI3K inhibitor GDC-0941.

Activating PIK3CA Mutation Results in Independence from HER2/HER3 Signaling and Trastuzumab Resistance in MDA-MB-361.1 Cells

Mutations in *PIK3CA*, the gene encoding the catalytic p110 α subunit, can be found in 30% of breast tumors (Vogt et al., 2007). *PIK3CA* mutations are clustered in “hot spots” of kinase and helical domains, and 80% of the cancer-specific mutations lead to a single amino acid substitution: E542K, E545K, or H1047R (Vogt et al., 2007). These “hot spot mutations” increase the activity of the kinase, can transform cells, and are oncogenic in vivo. Activating *PIK3CA* mutations have also been suggested to confer trastuzumab resistance (Berns et al., 2007). The MDA-MB-361.1 breast cancer cell line harbors HER2 amplification and an activating *PIK3CA* mutation (E545K). Trastuzumab caused a similar decrease in pHER3 in MDA-MB-361.1 (Figure 5A) as was detected in trastuzumab-sensitive SKBR-3 cells

(Figure 1B). Dephosphorylation of HER3 also led to the dissociation of both p85 and p110 subunits of PI3K from HER3 (Figure 5B), again similar to the trastuzumab-sensitive SKBR-3 (Figure 1C). Despite the similar signaling changes seen upstream of PI3K upon trastuzumab treatment, no decrease in pAKT was detected (Figure 5C). Accordingly, MDA-MB-361.1 cells also show resistance to trastuzumab in cell proliferation assays (Figure 5D). However, GDC-0941 efficiently inhibited AKT phosphorylation (Figure 5E) and cell proliferation, as well as survival (IC_{50} of 443 nM; Figure 5F), confirming that MDA-MB-361.1 proliferation is dependent on the PI3K/AKT pathway. In contrast, the proliferation of MDA-MB-361.1 cells is not dependent on the HER2/HER3 oncogenic unit, as the cells retain proliferation capability when treated with HER2 or HER3 siRNA (Figure 5G). Proliferation of trastuzumab-sensitive cells, however, is abolished by siRNA knockdown of either HER2 or HER3 (Figure 5H) (Lee-Hoeflich et al., 2008). We also investigated the ability of GDC-0941 to inhibit proliferation of other HER2-amplified cells that harbor *PIK3CA* mutations. HCC-202, HCC-1954, and MDA-MB-453 cells express high levels of HER2 and have an activating *PIK3CA* mutation (E545K, H1047R, and H1047R, respectively). All three cell lines with a hot spot *PIK3CA* mutation were insensitive to trastuzumab, but sensitive to GDC-0941 (IC_{50} values < 500 nM; Figure S5). We returned to investigate the *PIK3CA* mutation

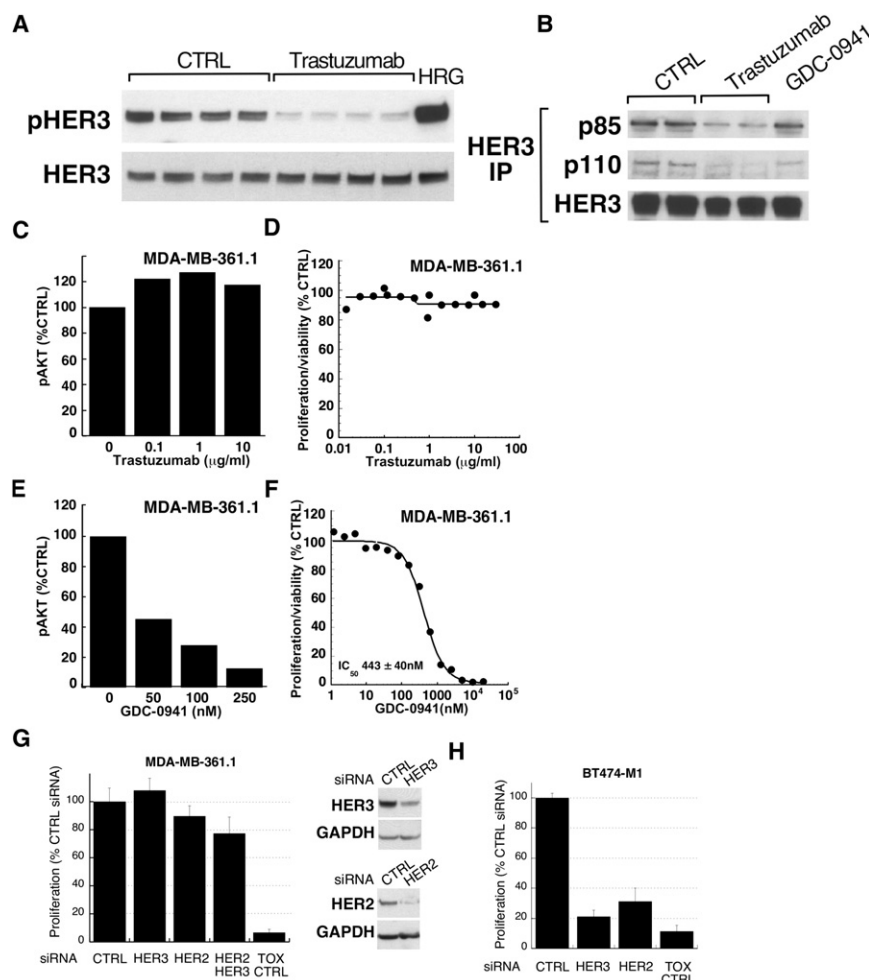


Figure 5. Activating PIK3CA Hot Spot Mutation Results in Independence from HER2/HER3 Signaling and Trastuzumab Resistance in MDA-MB-361.1 Cells

(A) MDA-MB-361.1 breast cancer cells, which harbor HER2 amplification and *PIK3CA* E545K mutation, were treated with 10 μ g/ml trastuzumab for 2 hr and analyzed for pHER3 (Tyr1289). CTRL lanes represent nontreated samples. Human IgG1 treatment does not inhibit pHER3 (data not shown). (B) The p85 and p110 subunits of PI3K were detected from MDA-MB-361.1 HER3 immunoprecipitates by western blot after a 2 hr treatment with 10 μ g/ml trastuzumab, human IgG1 (CTRL), or 250 nM GDC-0941.

(C–F) (C and E) MDA-MB-361.1 cells were treated with the indicated doses of (C) trastuzumab or (E) GDC-0941 for 2 hr, lysed, and subjected to pAKT ELISA (Ser473). (D and F) Proliferation of MDA-MB-361.1 was analyzed, and GDC-0941 IC_{50} (\pm standard error) was determined after (D) 6 days of trastuzumab or (F) 3 days of GDC-0941 treatment. (G and H) (G) MDA-MB-361.1 and (H) BT474-M1 cells were treated with pooled HER3, HER2, or non-targeting control (CTRL) siRNA for 48 hr. Proliferation (\pm SD) of cells was measured by using a [3 H]thymidine incorporation assay. Cytotoxic TOX transfection control siRNA was used as an indicator of successful transfection. Downregulation of HER2 and HER3 in MDA-MB-361.1 cells after siRNA treatment was detected by western blot.

status of the cell line panel used in Figure 1. Most of the trastuzumab-resistant cells carried the activating *PIK3CA* mutation or were PTEN null (Figure S2). In contrast, no hot spot mutations were detected in the trastuzumab-sensitive cell lines (Figure S2).

Together, these studies demonstrate that an activating hot spot mutation in *PIK3CA* uncouples PI3K activity from the HER2/HER3 oncogenic unit, which, in turn, renders these cell lines insensitive to trastuzumab treatment. Furthermore, trastuzumab-resistant, HER2-amplified breast cancer cells with PI3K mutations are sensitive to the PI3K inhibitor GDC-0941. It is likely that GDC-0941 would be useful in the treatment of breast cancer where activating *PIK3CA* mutations cause or contribute to trastuzumab resistance.

HER2-Amplified Breast Cancer Cells that Are Resistant to Trastuzumab Due to Loss of PTEN Are Sensitive to GDC-0941

PTEN loss occurs commonly in breast tumors, can lead to activation of the PI3K/AKT pathway, and has been suggested to cause trastuzumab resistance (Nagata et al., 2004). We addressed whether GDC-0941 can overcome trastuzumab resistance caused by PTEN loss. Transfection of SKBR-3 cells with PTEN siRNA resulted in a 76% reduction of PTEN protein and in a modest increase in pAKT (114% of CTRL siRNA treated

cells) after 48 hr (Figure 6A). Consistent with previous reports (Nagata et al., 2004), knockdown of PTEN resulted in trastuzumab resistance (Figure 6A). Although PTEN loss increased the GDC-0941 concentration required for inhibition of proliferation, complete inhibition was achieved with 1 μ M concentration, demonstrating that GDC-0941 inhibits the growth of SKBR-3 cells that were made resistant to trastuzumab due to PTEN loss. A similar result was achieved with BT474-M1 cells (data not shown). HCC-1569 is an HER2-amplified breast cancer cell line that does not express detectable amounts of PTEN (Figure S2). HCC-1569 cells are resistant to trastuzumab (Figure 6B). Treatment of HCC-1569 with GDC-0941 resulted in inhibition of AKT phosphorylation and inhibition of proliferation (IC_{50} of 313 nM) (Figure 6B). Together, the results show that GDC-0941 effectively inhibits both proliferation and the viability of HER2-amplified breast cancer cells that are resistant to trastuzumab due to PTEN loss, suggesting a clinical niche for this PI3K inhibitor.

GDC-0941 Inhibits the Growth of HER2-Amplified, Trastuzumab-Resistant Tumors with PIK3CA Mutation In Vivo

MDA-MB-361.1 cells were implanted in the mammary fat pads of nude mice. After tumor establishment, the mice were treated with 3 mg/kg weekly doses of trastuzumab or vehicle. After the third trastuzumab dose, it was apparent that the MDA-MB-361.1 tumors did not respond to trastuzumab in vivo (Figure 7A). No

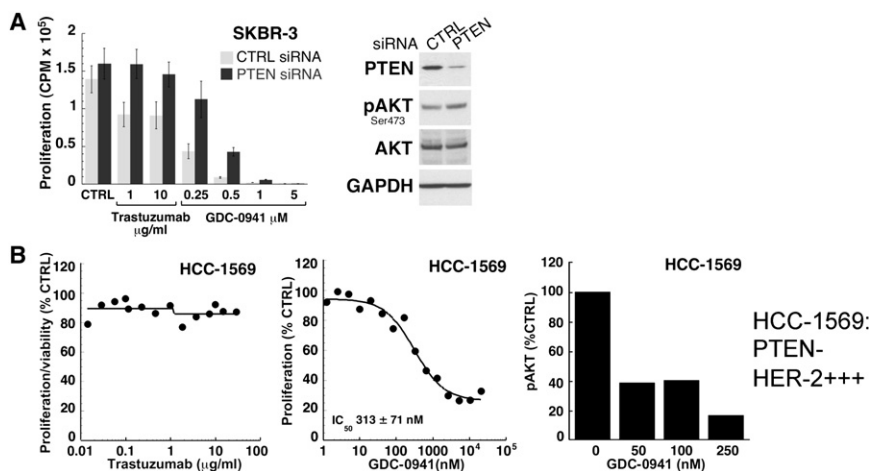


Figure 6. Breast Cancer Cells Resistant to Trastuzumab Due to PTEN Loss Are Sensitive to GDC-0941

(A) SKBR-3 cells were transfected with PTEN (black columns) or nontargeting control (gray columns) siRNA. After 24 hr, cells were treated with the indicated concentrations of trastuzumab or GDC-0941 for 48 hr, and proliferation (\pm SD) was measured by a [3 H]thymidine incorporation assay. PTEN and pAKT (Ser473) levels after siRNA treatment were detected by western blot.

(B) The trastuzumab and GDC-0941 dose response of HCC-1569 breast cancer cells. Cell proliferation was analyzed, and IC_{50} (\pm standard error) was determined after 6 days of trastuzumab or 3 days of GDC-0941 treatment. pAKT (Ser473) was analyzed by ELISA after 2 hr of treatment.

responses were observed even with a higher 30 mg/kg weekly trastuzumab dose, which causes complete responses in trastuzumab-sensitive models (data not shown). At day 17 of the trastuzumab treatment, both vehicle and trastuzumab groups were randomly divided in half. Daily oral administration of GDC-0941 at 150 mg/kg was delivered to half of the mice, whereas the other half received the formulation buffer of the PI3K inhibitor. Addition of GDC-0941 to the dose regimen stopped the growth of MDA-MB-361.1 tumors, whereas tumors continued to grow in vehicle-treated mice (Figure 7A). Response to treatment was monitored as time to tumor progression (the time when tumor volume reached twice the volume on day 17). GDC-0941 significantly delayed the tumor progression both alone and in combination with trastuzumab ($p = 0.0049$, $p = 0.0018$, respectively). The in vivo combination of trastuzumab and GDC-0941 did not result in a significant benefit over the single-agent GDC-0941 regimen. Increased PARP cleavage was detected in tumors treated for 7–24 hr with 100–150 mg/kg PI3K inhibitor, suggesting that GDC-0941 induces apoptosis in tumors (data not shown). In summary, these results demonstrate that GDC-0941 is effective in treating HER2-amplified, trastuzumab-resistant mammary tumors harboring PIK3CA mutations in vivo.

The combination of Trastuzumab and GDC-0941 Is Efficient in Treatment of Trastuzumab-Sensitive Tumors In Vivo

To address whether the trastuzumab and GDC-0941 combination would result in more efficient inhibition of tumor growth than the single agents, we treated BT474-M1 tumor xenografts with trastuzumab (20 mg/kg, iv, 2 \times /wk \times 3), GDC-0941 (100 mg/kg, po, qd \times 21), or a combination of the agents. As predicted by the in vitro studies, the combination of inhibitors was more efficient in inhibition of the BT474-M1 tumors than either of the single agents (Figure 7B).

DISCUSSION

Clinical observations indicate that not all patients benefit from trastuzumab-based therapies. Moreover, a subset of patients progress after an initial response. The nature of both innate and acquired resistance is an area of active research in both

the laboratory and the clinic. In the present work, we show that the antiproliferative activity of trastuzumab is directly linked to its ability to disrupt the constitutive, ligand-independent HER2/HER3/PI3K signaling complex that forms as a result of HER2

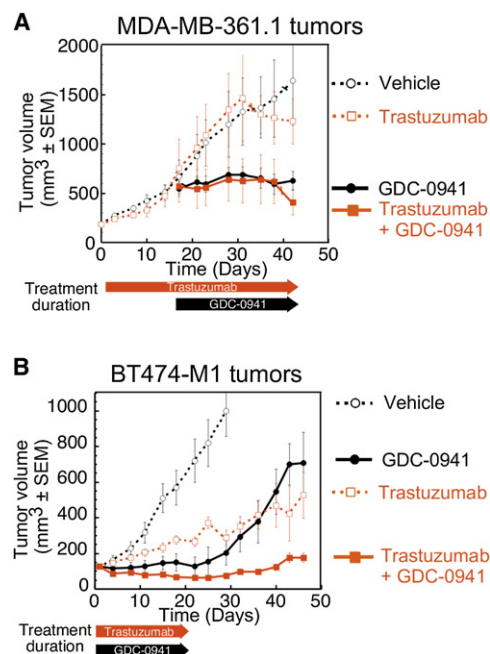


Figure 7. GDC-0941 Is Highly Efficacious Both in Treating Trastuzumab-Resistant Tumors and in Combination with Trastuzumab in Treating Trastuzumab-Sensitive Tumors In Vivo

(A) Pre-established MDA-MB-361.1 tumor xenografts were treated with weekly doses of trastuzumab (intraperitoneal; 3 mg/kg; red dotted line) or vehicle (black dotted line). After 17 days, mice were randomized, and half of the groups received additional GDC-0941 (daily oral gavage; 150 mg/kg \times 25; black and red solid lines). The remaining half was administered with vehicle (dotted red and black lines). Data are presented as mean tumor volume \pm SEM.

(B) Pre-established BT474-M1 tumor xenografts were treated with vehicle (black dotted line), trastuzumab (intravenous; 20 mg/kg; 2 \times /wk \times 3; red dotted line), GDC-0941 (daily oral gavage; 100 mg/kg \times 21; black line), or a combination of the agents (red line). Data are presented as mean tumor volume \pm SEM.

overexpression. Furthermore, we describe how PI3K mutations generate trastuzumab resistance and that trastuzumab-resistant cell lines commonly harbor activating “hot spot” PI3K mutations or are PTEN null. Additionally, we demonstrate that the selective PI3K inhibitor GDC-0941 significantly inhibits proliferation of both trastuzumab-sensitive and -insensitive cell lines.

Several studies have noted that trastuzumab treatment leads to a downregulation of AKT signaling (Lane et al., 2000; Yakes et al., 2002). We recently extended this observation by showing that HER2 and HER3 form the major oncogenic unit in HER2-positive breast cancer (Lee-Hoeflich et al., 2008). However, the molecular details concerning the mechanism by which trastuzumab disrupts constitutive AKT signaling remains elusive. In the present work, we provide direct evidence for the ability of trastuzumab to disrupt the oncogenic ligand-independent HER2/HER3/PI3K complex, resulting in rapid inhibition of AKT signaling and cell proliferation. Our results clarify several previously unclear areas of signaling changes caused by trastuzumab. We establish that inhibition of HER3 phosphorylation and the subsequent dissociation of p85, which, in turn, leads to downregulation of AKT signaling, is an immediate effect after trastuzumab treatment and correlates with the disruption of HER2/HER3 complexes. Previous studies have reported much slower kinetics for the inhibition of HER3/AKT signaling (Yakes et al., 2002), which was attributed to downregulation of HER2 from the cell surface (Hudziak et al., 1989). However, subsequent studies clearly indicate that trastuzumab treatment does not alter cell surface HER2 levels (Austin et al., 2004; Hommelgaard et al., 2004). We also fail to observe any transient increase in HER3 or HER2 phosphorylation after trastuzumab treatment that was reported in previous studies (Sarup et al., 1991; Yakes et al., 2002). Additionally, we do not observe the trastuzumab-mediated HER2 dephosphorylation that had been suggested as the mechanism of action for trastuzumab (Lane et al., 2000; Nagata et al., 2004; Sarup et al., 1991). We conclude from our results that the inhibition of PI3K/AKT signaling by trastuzumab is caused by disruption of ligand-independent HER2/HER3 association, which, in turn, is critical for the cytostatic activity of trastuzumab. Our model does not require trastuzumab-mediated activation of PTEN through src for trastuzumab to mediate its antiproliferative activity (Nagata et al., 2004). However, some of the previously described mechanisms of action, including the ability to inhibit HER2 shedding (Molina et al., 2001), may still be valid in some context.

Previously, we reported that pertuzumab, a therapeutic antibody that binds extracellular domain II of HER2 (Franklin et al., 2004), functions by blocking the association of HER2 with ligand-occupied HER3 (Agus et al., 2002). In contrast, trastuzumab, which binds to subdomain IV of HER2, had little effect on ligand-mediated HER2/HER3 association (Agus et al., 2002). In the present study, we demonstrate that HER2/HER3 complexes can be isolated from cell lines that overexpress HER2 without stimulating receptor dimerization by ligand. In the absence of HRG, trastuzumab is superior to pertuzumab in disrupting constitutive HER2/HER3 complexes. These findings raise the possibility that when HER2 is overexpressed and in the absence of ligand, the HER2/HER3 interaction is fundamentally different from the canonical ligand-induced, receptor-mediated dimerization model proposed by structural studies. The findings further

imply that the domain II-domain II interface may not mediate interactions in the ligand-independent receptor complex. The ability to detect HER2 in association with HER3 confirms and extends the notion that the oncogenic unit in HER2-positive breast cancer is a complex of HER2 and HER3 that signals constitutively to PI3K and AKT (Lee-Hoeflich et al., 2008). One hypothesis, supported by studies of Landgraf and colleagues, is that HER2/HER3 complexes exist in the membrane in microdomains, and that subsequent binding of monoclonal antibodies to HER2 accelerates the partitioning of HER2 away from these microdomains, thereby limiting access to HER3 (Kani et al., 2005). Trastuzumab, which binds near the transmembrane region in domain IV, appears to be more effective in accelerating this partitioning event. Experiments to test this hypothesis are currently ongoing.

It is clear that the oncogenic potential of HER2 in HER2-amplified breast cancer is primarily, if not solely, mediated by its ability to inappropriately activate HER3 and the PI3K pathway (schematically depicted in Figure 8A) (Lee-Hoeflich et al., 2008). In the current study, we demonstrate that trastuzumab can disrupt the ligand-independent HER2/HER3/PI3K complex in both trastuzumab-sensitive (Figure 8A) and -insensitive cells (Figure 8B). In the latter case, the breast cancer cell lines that harbor alterations in the PI3K pathway remain sensitive to GDC-0941, a highly specific and effective PI3K inhibitor (Figure 8B). Several properties that are critical for therapeutic agents differentiate GDC-0941 from previously tested PI3K inhibitors, including wortmannin and LY294002 (Bain et al., 2007; Hennessy et al., 2005; Nagata et al., 2004). First, GDC-0941 is highly selective for class IA PI3K (Folkes et al., 2008). Importantly, it also displays favorable pharmacokinetic and toxicological properties that enable therapeutic use in vivo (Folkes et al., 2008). Given this advantageous profile, GDC-0941 is currently undergoing early clinical testing for treatment of various advanced malignancies. A recent publication showed that a dual PI3K/mTOR inhibitor is able to inhibit growth of tumors with *PIK3CA* mutation. However, the study did not demonstrate whether PI3K/mTOR inhibitor overcomes trastuzumab resistance in vivo (Serra et al., 2008).

In trastuzumab-sensitive cells, trastuzumab and GDC-0941 inhibit proliferation and cell viability synergistically. Recently, it was reported that PI3K inhibition leads to a compensatory increase in HER3 phosphorylation, which was linked to AKT-mediated negative-feedback signaling (Sergina et al., 2007). In the present report, we observe that treatment of HER2-amplified breast cancer cells with GDC-0941 also results in negative feedback that is manifested by an increase in HER3 phosphorylation. When trastuzumab is given with GDC-0941, the feedback loop fails due to the disruption of the HER2/HER3 complex. As a consequence of feedback loop failure, trastuzumab and GDC-0941 are observed to be synergistic in cells that are sensitive to trastuzumab's antiproliferative effects.

Several clinical implications can be drawn from our studies. First, PI3K inhibitors, such as GDC-0941, could be used in combination with trastuzumab-based therapies, and, based on our current study, it is anticipated that the combination will be highly effective. Second, GDC-0941 may be effective in patients who progress on trastuzumab or lapatinib therapy. Third, patients whose tumors contain HER2 gene amplification and also harbor PI3K pathway alterations are candidates for GDC-0941 therapy

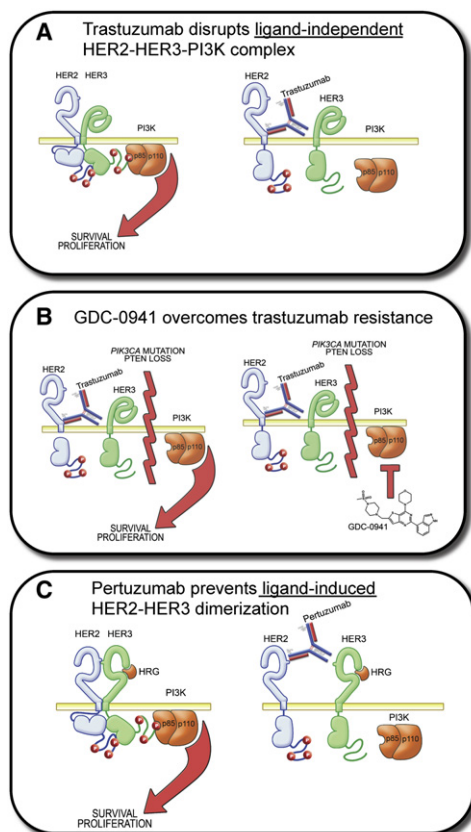


Figure 8. Schematic Representation of the Mechanism of Action of Trastuzumab and How PI3K Activation Leads to Trastuzumab Resistance

(A) Amplification of HER2 leads to ligand-independent HER2/HER3 interaction and HER3 phosphorylation. Phosphorylated HER3 activates the PI3K pathway promoting cell survival and proliferation. Trastuzumab disrupts the ligand-independent HER2/HER3 interaction, leading to rapid HER3 dephosphorylation and inhibition of the PI3K/AKT pathway, thereby inhibiting cell proliferation.

(B) PI3K mutation or PTEN loss results in HER2/HER3-independent signaling and trastuzumab resistance. However, a novel class IA PI3K small-molecule inhibitor, GDC-0941, inhibits both wild-type and mutant PI3K and overcomes trastuzumab resistance generated by PI3K mutations or PTEN loss.

(C) Ligand-induced HER2/HER3 dimerization can occur in both HER2-amplified and nonamplified cells. Pertuzumab is efficient in preventing ligand-induced HER2/HER3 dimerization.

and are not likely to optimally respond to trastuzumab-based regimens. Our mechanistic studies predict that these patients would also be refractory to HER2 tyrosine kinase inhibitors, such as lapatinib, in agreement with a previous report (Eichhorn et al., 2008). Fourth, our studies suggest that it is imperative to clinically validate whether patients who initially respond to trastuzumab or lapatinib therapy and then progress acquire PI3K pathway alterations. Only detailed molecular analysis of longitudinal biopsy specimens from the same patient's tumor undergoing these therapies will provide definitive data in support of this hypothesis.

In summary, this study characterizes a mechanism of action for trastuzumab whereby trastuzumab disrupts basal, ligand-independent HER2/HER3 interactions in HER2-amplified cells. The disruption of the constitutively active receptor complex

leads to rapid inhibition of the HER3/PI3K/AKT pathway and cell proliferation. We also show that the PI3K inhibitor GDC-0941 is highly efficacious both in combination with trastuzumab and in the treatment of trastuzumab-resistant cells and tumors.

EXPERIMENTAL PROCEDURES

Cell Lines

Breast cancer cell lines BT474-M1, BT474-EEI, and MDA-MB-361.1 are in vivo-passaged subclones of BT474 and MDA-MB-361 cells (American Type Culture Collection [ATCC], Manassas, VA). The generation of BT474 subclones has been previously described (Clynes et al., 2000; Lewis Phillips et al., 2008). For in vivo passaging of MDA-MB-361, the parental cell lines were grown as xenografts, and the tumors were regrafted once. A new cell line (MDA-MB-361.1) was generated from the in vivo-passaged tumors and was used in all in vitro and in vivo experiments. KPL-4 breast cancer cells were obtained from J. Kurebayashi (Kurebayashi et al., 1999), and MKN-7 gastric carcinoma cells were obtained from Mitsubishi Corp. (Tokyo, Japan). All other cell lines were obtained from ATCC or Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Cell lines were maintained in high-glucose DMEM:Ham's F-12 (50:50) supplemented with 10% FBS and 2 mM L-glutamine.

Analysis of HER3/PI3K/AKT Pathway Activation

Cells were lysed with nondenaturing lysis buffer including 1% Triton (Cell Signaling Technology [CST] Cat#9803; Danvers, MA). The activation of the HER3/PI3K/AKT pathway was detected by using antibodies against pHER3 (Tyr1289; CST), phospho-S6 ribosomal protein (Ser235/236; CST), and pPRAS40 (Thr246; Invitrogen/Biosource, Carlsbad, CA). AKT phosphorylation was detected by western blot or ELISA detecting phosphorylated Ser473 (CST). The association of PI3K with HER3 was analyzed by immunoprecipitation with agarose-conjugated HER3 antibody (sc-285; Santa Cruz, Santa Cruz, CA), followed by detection with p85 (Millipore/Upstate, Billerica, MA) or p110 antibody (clone EP383Y; Epitomics, Burlingame, CA).

For analysis of pHER3 and pAKT in vivo, tumor-bearing Rag2 mice (Taconic, Germantown, NY) were treated with 20 mg/kg (iv) trastuzumab or control antibody for 48 hr. Tumors were snap frozen and homogenized in cell lysis buffer (CST; Cat#9803). Ratios of pHER3 to total HER3 and pAKT to total AKT were determined by western blot and ELISA, respectively.

Receptor Crosslinking and HER2 Coimmunoprecipitation

SKBR-3 cells were incubated in growth medium with 10% fetal bovine serum and 10 mM HEPES (pH 7.2) for 1 hr at 37°C with the indicated treatment antibody. The cells were washed twice with ice-cold 50 mM HEPES (pH 7.2), 150 mM NaCl (HEPES/NaCl buffer) and were incubated with 2 mM 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTSSP) dissolved in HEPES/NaCl buffer for 60 min at 4°C. After the incubation with DTSSP, the cells were washed three times with ice-cold 25 mM Tris (pH 7.1), 150 mM NaCl and lysed in 1.0% v/v Triton X-100, 1.0% w/v CHAPS in RPMI with 10 mM HEPES (pH 7.2) supplemented with protease and phosphatase inhibitors. For HER2 immunoprecipitation, N-terminal anti-HER2 antibody 7C2 (Genentech; binds to extracellular domain I of HER2) was conjugated to Ultralink biosupport (Pierce, Rockford, IL) by incubation in 100 mM HEPES (pH 8.2), 150 mM NaCl buffer in 4°C overnight. The conjugated beads were washed, and free amines were blocked with 3 M ethanolamine (pH 9). In Figures 3B and 3C, cells were lysed with nondenaturing lysis buffer (1% Triton, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, protease and phosphatase inhibitors; CST; Cat#9803). Lysate (1 [Figure 3A] to 3 mg [Figures 3B and 3C]) was used for overnight immunoprecipitation in 4°C with 20 μ l 7C2 beads. After immunoprecipitation, 7C2 beads were washed twice with lysis buffer. SDS-sample buffer containing an additional 50 mM dithiothreitol was added to each precipitate, and the samples were boiled for 4 min. Coimmunoprecipitating HER3 was analyzed by western blot with C-terminal HER3 antibody (sc-285; Santa Cruz).

siRNA

siRNA was introduced into BT474-M1 cells (1.7×10^4 /well of 96-well plate) by reverse transfection by using Lipofectamine-2000 (Invitrogen) as previously

described (Lee-Hoeflich et al., 2008). For transfection of MDA-MB-361.1, 4×10^3 cells were grown in 96-well plates and transfected with Lipofectamine on the following day. Sets of four pooled siRNA oligos (50 nM) were used for PTEN, HER3, and nontargeting siRNA (siGENOME, siCONTROL; Dharmacon, Lafayette, CO). HER2 oligos (pool of four) have been previously described (Lee-Hoeflich et al., 2008). TOX transfection control siRNA (Dharmacon) was used as an indicator of successful transfection. Treatments were started 24 hr after transfection, and the effect of 48 hr of treatment on cell proliferation and protein expression was determined.

Cell Proliferation/Viability

Proliferation/viability of cells was detected by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). For the assay, 5×10^3 cells were plated on 96-well plates and incubated overnight for cell attachment. Cells were treated for 6 days with antibodies or 3 days with PI3K inhibitor before analysis. After transfection with siRNA, proliferation was detected by a [3 H]thymidine incorporation assay after 48 hr of treatment as previously described (Lee-Hoeflich et al., 2008). All measurements were performed in triplicate.

Determination of Drug Synergy

Cells were seeded at a density of 2.5×10^3 (BT474) or 1.5×10^3 (SKBR-3) cells per well of a 384-well plate. Concentration ranges were chosen to span the complete dose-response range of both drugs. All treatments were performed in quadruplicate. Cell proliferation/viability was determined after 3 days by using the Luminescent Cell Viability Assay (Promega). Multiple drug effect analysis was performed by using CalcuSyn software (Biosoft, Cambridge, United Kingdom), which quantitatively describes the interaction between two or more drugs (Chou and Talalay, 1984). This method assigns combination index (C.I.) values to each drug combination and defines drug synergy when a C.I. value is less than 1 or drug antagonism when a C.I. value is greater than 1. C.I. values were determined at 7–9 unique data points for each cell line, which corresponded to 20%–90% of cell growth inhibition relative to control.

Caspase Activation and Apoptosis

For detection of caspase activity and apoptosis, 1.5×10^4 cells were plated on 96-well plates and incubated overnight before 48 hr treatments and the Caspase-Glo 3/7 assay (Promega) or Cell Death Detection ELISA^{plus} assay (Roche; measures cytoplasmic histone-associated DNA fragments indicative of apoptosis). Cleaved caspase-3 (Asp175; 5A1) and cleaved PARP (Asp214) were detected with antibodies that do not recognize the respective full-length proteins (CST).

In Vivo Drug Efficacy

For MDA-MB-361.1 xenograft studies, NCR nude mice (Taconic) were supplemented with subcutaneous estrogen pellets (0.36 mg, 60-day release; Innovative Research of America). After 3 days, 5 million MDA-MB-361.1 cells were injected into the mammary fat pad in a 1:1 HBSS:matrigel suspension (BD matrigel; BD Biosystems, San Jose, CA). When tumor volumes reached 200–300 mm³, mice were randomly grouped for the treatment cohorts. For BT474-M1 xenograft studies, 1 mm³ tumor fragments were implanted subcutaneously in the flank of the mice (Fox Chase SCID, C.B-17/lcrHsd-Prkdc^{scid}, Harlan). When tumor volumes reached an average of 125 mm³, mice were randomized for treatment groups. Dosing and drug are described in the figure legends. Tumor volumes were calculated with the formula: (mm³) = (L × W²) × 0.5. All animal procedures followed protocols approved by the Institutional Animal Care and Use Committee (IACUC).

Statistical Analysis

All statistical analysis was performed by using JMP 7.0 software (SAS Institute Inc., Cary, NC).

SUPPLEMENTAL DATA

Supplemental Data include five figures and are available at [http://www.cell.com/cancer-cell/supplemental/S1535-6108\(09\)00109-3](http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00109-3).

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