Trastuzumab-resistant cells rely on a HER2-PI3K-FoxO-survivin axis and are sensitive to PI3K inhibitors

Anindita Chakrabarty¹, Neil E. Bhola², Cammie Sutton², Ritwik Ghosh², María Gabriela Kuba³, Bhuvanesh Dave⁴, Jenny C. Chang⁴, Carlos L. Arteaga¹,²,⁵

Departments of ¹Cancer Biology, ²Medicine, ³Pathology, and ⁵Breast Cancer Research Program, Vanderbilt-Ingram Cancer Center; Vanderbilt University School of Medicine, Nashville, TN 37232; ⁴Methodist Cancer Center, Methodist Hospital Research Institute, Houston, TX 77030

Corresponding author:

Carlos L. Arteaga

Division of Hematology-Oncology, VUMC

2220 Pierce Avenue, 777 PRB

Nashville, TN 37232-6307

Tel. 615-936-3524; Fax 615-936-1790

carlos.arteaga@vanderbilt.edu
Abstract

The antibody trastuzumab is approved for treatment of patients with HER2 (ERBB2)-overexpressing breast cancer. A significant fraction of these tumors are either intrinsically resistant or acquire resistance rendering the drug ineffective. The development of resistance has been attributed to failure of the antibody to inhibit phosphatidylinositol-3 kinase (PI3K), which is activated by the HER2 network. Herein, we examined the effects of PI3K blockade in trastuzumab-resistant breast cancer cell lines. Treatment with the pan-PI3K inhibitor XL147 and trastuzumab reduced proliferation and pAKT levels, triggering apoptosis of trastuzumab-resistant cells. Compared to XL147 alone, the combination exhibited a superior antitumor effect against trastuzumab-resistant tumor xenografts. Further, treatment with XL147 and trastuzumab reduced the cancer stem cell (CSC) fraction within trastuzumab-resistant cells both in vitro and in vivo. These effects were associated with FoxO-mediated inhibition of transcription of the anti-apoptosis gene survivin (BIRC5) and the CSC-associated cytokine IL-8. RNAi-mediated or pharmacological inhibition of survivin restored sensitivity to trastuzumab in resistant cells. In a cohort of patients with HER2-overexpressing breast cancer treated with trastuzumab, higher pre-treatment tumor levels of survivin RNA correlated with poor response to therapy. Together, our results suggest that survivin blockade is required for therapeutic responses to trastuzumab and that by combining trastuzumab and PI3K inhibitors CSCs can be reduced within HER2+ tumors, potentially preventing acquired resistance to anti-HER2 therapy.
Introduction

The HER2 oncogene encodes a transmembrane receptor tyrosine kinase (RTK) that is amplified in approximately 20% of invasive breast cancers (1). HER2 gene amplification in breast cancer is associated with increased cell proliferation and motility, tumor invasion and metastasis, accelerated angiogenesis, decreased apoptosis, and resistance to anti-cancer therapy (2). This translates into shorter disease-free and overall survival in patients (3). In HER2-overexpressing cells, HER2 dimerizes with its co-receptor HER3 which, in turn, directly couples to the p85 regulatory subunit of PI3K and activates the PI3K-AKT survival pathway (4-6).

Trastuzumab, a humanized antibody directed against the extracellular domain of the HER2 receptor is approved for the treatment of HER2-overexpressing breast cancer (7). Mechanisms of action of the antibody include endocytosis and downregulation of HER2, inhibition of ligand-independent HER2-HER3 dimers with subsequent inhibition of PI3K-AKT, induction of cell-cycle arrest and apoptosis. In addition, trastuzumab engages Fc receptor-expressing immune effector host cells to induce antibody-dependent, cell-mediated cytotoxicity (ADCC) (reviewed in (8)).

Although patients with metastatic HER2+ breast cancer respond clinically to single agent trastuzumab or in combination with chemotherapy, virtually all patients eventually adapt to the anti-HER2 therapy and progress (reviewed in (9)). One of the major proposed mechanisms of adaptation or resistance to trastuzumab involves aberrant activation of the PI3K-AKT pathway by i) loss of the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (10) and ii) activating mutations in PIK3CA, the gene encoding the p110α catalytic subunit of PI3K (11). The dependence of HER2-overexpressing breast cancer cells on the PI3K-AKT pathway together with several genetic and epigenetic alternations in the PI3K pathway...
associated with trastuzumab resistance suggest that early use of PI3K pathway inhibitors should be useful in preventing or delaying clinical resistance to trastuzumab. Indeed, several PI3K inhibitors have been shown to block growth of preclinical models of trastuzumab resistance (12, 13) and are currently the focus of clinical development in patients with breast cancers [reviewed in (14)].

In this study, we used breast cancer models of trastuzumab resistance with different modes of aberrant PI3K pathway activation to examine the effects of ATP-mimetic, small molecule inhibitors of PI3K either alone or in combination with trastuzumab both in vitro and in vivo. Treatment with the pan-PI3K inhibitor XL147 (15) with or without trastuzumab reduced proliferation and pAKT levels and induced apoptosis of trastuzumab-resistant cells. The combination potently inhibited trastuzumab-resistant xenografts established in athymic mice. Treatment with XL147 alone or in combination with trastuzumab modulated the cancer stem cell (CSC) fraction, which has been causally associated with drug resistance and tumor recurrences (16). Pharmacological and RNAi-based approaches suggested this was at least in part due to derepression of FoxO-mediated transcription which, in turn, downregulated expression of interleukin-8 (IL-8) and the anti-apoptosis protein survivin. Finally, in patients with HER2-overexpressing breast cancer treated with trastuzumab, higher pre-treatment tumor levels of survivin RNA correlated with poor response to therapy. Taken together, these data suggest that 1) trastuzumab-resistant cells continue to rely on HER2-PI3K-FoxO-survivin axis for survival, and 2) modulation of this axis with a combination of PI3K and HER2 inhibitors may abrogate or delay the development of resistance to anti-HER2 therapy.

Materials and Methods
Cell lines, reagents, inhibitors, plasmids, and viral vectors. All cell lines were from the American Tissue Culture Collection (ATCC; Rockland, MD), maintained in ATCC-recommended media plus 10% FBS (Gibco) and authenticated by short tandem repeat profiling using Sanger sequencing (March 2011). HR5 and HR6 cells were derived from previously described BT474 xenografts with acquired resistance to continuous treatment with trastuzumab (17). WST-1 reagent and Caspase-Glo 3/7 assay kit were obtained from Roche Applied Science and Promega Corporation, respectively. The following inhibitors were used: lapatinib and BEZ235 (LC Laboratories), BKM120 (Active Biochem), YM155 (Selleck Chemicals), trastuzumab (VUMC Pharmacy), and XL147 (Exelixis). The FHRE-Luc reporter plasmid (Addgene plasmid#1789) has been described in (18). The renilla Luc pRL-SV40 and human survivin cDNA-pJP1520 were obtained from Promega and Labome/DNASU plasmid repository, respectively. Retrovirus and stable cell line production were described in (19). Adenoviruses encoding beta-galactosidase/LacZ, non-phosphorylatable FKHRL1/FoxO3(AAA) mutant and dominant negative/dnFoxO3, both GFP-tagged, were from Vector Biolabs. Adenovirus infection was performed as described in (20).

Cell proliferation, immunoprecipitation, immunoblot analysis, RNA interference and real-time qPCR. These methods were performed as described in (21). Human FoxO1 and FoxO3a siRNAs were described in (22). The p110α, p110β and survivin siRNA duplexes were obtained from Ambion and Cell Signaling Technology. Primers for qPCR were obtained from Qiagen.

Mammosphere and ALDH assays. For mammosphere assay 0.5-1x10^4 cells/well were seeded in 6-well Ultra-low attachment plates (Costar) in DMEM-F12 + 20 ng/ml EGF + 1x B-27 serum-
free supplement (Invitrogen). Imaging and quantitation of colonies were done in GelCount colony counter (Oxford Optronix). Mammospheres were dissociated by trypsin digestion and 1000 cells/well were seeded in 6-well plates in IMEM/10% serum (Fig. 4J). Focus/colony formation was assessed 7 days later. Colonies were fixed and stained with methanol-crystal violet, and imaged. ALDH assay was performed with ALDEFLUOR kit (Stemcell Technologies) and subsequent FACS analysis in a 3 laser LSRII (BD Biosciences).

**Dual luciferase reporter assay.** Cells were transfected with 10 μg FHRE-Luc (18) plus 100 ng pRL-SV40 with FuGENE 6 (Promega) and treated overnight with trastuzumab, XL147, or both. Dual luciferase assay was performed with the Dual-Luciferase Reporter Assay System (Promega).

**Proteome Profiler™ human angiogenesis and phospho-RTK arrays.** Angiogenesis (ARY007) and pRTK (ARY001) arrays were performed according to manufacturer’s instructions (R&D Systems).

**Xenograft studies.** These were approved by the Institutional Animal Care Committee of Vanderbilt University. A 17β-estradiol pellet (Innovative Research of America) was injected subcutaneously (s.c.) in the dorsum of 4- to 5-week old athymic female mouse (Harlan Sprague Dawley, Inc.). Next day, HR6 cells (≈3x10^6) mixed 1:1 with Matrigel (BD Biosciences) were injected s.c. into the right flank of each mouse. Tumor diameters were measured twice a week and volume in mm^3 calculated by the formula: volume = width^2 x length/2. Mice with ≥200 mm^3 tumors were randomized to vehicle, trastuzumab (30 mg/kg twice a week i.p.), XL147 (100
mg/kg daily p.o.), or combination treatments for 28 days. Immunostaining and histoscore (H-score) analysis of formalin-fixed tumor sections harvested on day 28 were performed as described in (22).

Results

Trastuzumab-resistant cells remain dependent on PI3K. We treated a panel of trastuzumab-resistant HER2 gene-amplified human breast cancer cells with the pan-PI3K inhibitor XL147 (15) and the MEK inhibitor CI-1040 (23), either alone or in combination with trastuzumab. The HR5 and HR6 cell lines, derived from BT474 xenografts grew in presence of trastuzumab in vivo and overexpress EGFR/HER3 ligands (17). The HCC1954 and SUM190 cell lines contain a mutation in the catalytic domain (H1047R) of PIK3CA and HCC1569 cells are PTEN null (22, 24). Treatment with XL147 + trastuzumab but not CI-1040 + trastuzumab inhibited monolayer (Fig. 1A) and 3D growth (Fig. 1B) in all resistant lines. CI-1040 alone was inactive against all cell lines whereas growth of 3/5 resistant lines (HR5, HR6 and HCC1569) was inhibited by XL147, suggesting they depend on the PI3K/AKT pathway. The combination of XL147 and trastuzumab induced cell death and growth arrest as supported by immunoblot analysis of cleaved caspase 3 and PARP (apoptosis), and CDK inhibitor p27Kip1 (cell-cycle arrest) (Fig. 1C). This was further confirmed by enhanced caspase 3/7 activity following treatment with XL147 + trastuzumab compared to each inhibitor alone (Fig. 1D). The PI3K dependence of trastuzumab-resistant cells was also supported by siRNA-mediated knockdown of the p110α and p110β subunits of PI3K (Fig. S1D). Compared to the cells transfected with control siRNA and treated with trastuzumab, knockdown of both p110α and p110β resulted in greater inhibition of cell growth in both monolayer and in 3D (Fig. S1A-B) as well as apoptosis measured by activation of caspase 3/7 (Fig. S1C).
We next examined the effect of XL147, trastuzumab, and the combination on activated AKT, a main downstream target of PI3K (Fig. 2A). In all cell lines, treatment with XL147 alone or in combination with trastuzumab reduced pAKT<sup>S473</sup> and pAKT<sup>T308</sup> levels (Fig. 2A). Inhibition of PI3K/AKT is counteracted by compensatory feedback activation of receptor tyrosine kinases (RTKs), including HER3 (22, 25). In HER2-dependent breast cancer cells and xenografts, this compensation is reduced by co-treatment with trastuzumab or the HER2 TKI lapatinib (22). To determine whether a similar phenomenon occurs in trastuzumab-resistant cells, we hybridized lysates of cells treated with XL147 ± trastuzumab with arrays representing 42 different phosphorylated RTKs. The combination of XL147 + trastuzumab resulted in a modest reduction in pEGFR, pHER2 and pHER3 levels compared to XL147 alone (Fig. 2B; arrows).

In all 3 trastuzumab-resistant lines, treatment with both inhibitors reduced phosphorylation of VEGFR1 and/or VEGFR3 (Fig. 2B; longer exposure in Fig. S2A). Related to this finding, trastuzumab has been shown to act as an anti-angiogenic agent by affecting different pro-angiogenic proteins including VEGF and angiopoietin (26). Thus, we examined the effect of XL147 ± trastuzumab on the expression of 55 angiogenic growth factors by hybridizing HR6 and HCC1954 cell lysates with protein arrays. In both cell lines, only the combination of XL147 + trastuzumab, but not each drug alone, reduced the expression of VEGF (Fig. 2C). Several other pro-angiogenic factors including angiopoietin-1 and -2, IL8, and Chemokine ligand-4 were also downregulated in a cell line-specific manner (longer exposure in Fig. S2B-C). Of note, VEGF has a central role in breast cancer development and progression and its expression is regulated by the PI3K pathway (27). Real-time PCR with RNA from HR6 and HCC1954 cells showed a reduction in VEGFA RNA following treatment with XL147 alone or in a combination with trastuzumab (Fig. 2D). Similar data were obtained with the pan-PI3K inhibitor BKM120 (28).
and the PI3K/TOR inhibitor BEZ235 (29), either alone or in combination with trastuzumab (Fig. 2D), suggesting the effect of XL147 on VEGF transcription was PI3K pathway-specific and not an off target drug effect.

**Treatment with PI3K inhibitor and trastuzumab reduces the drug-resistant cancer stem cell (CSC) fraction.** It has been proposed that the clinical efficacy of trastuzumab is due to its ability to target CSCs within trastuzumab-sensitive tumors (30, 31). CSC activity is studied *in vitro* by primary mammosphere formation (attachment-independent growth) and analysis of specific cell surface markers (32). Aldehyde dehydrogenase (ALDH) positivity (ALDH+) correlates with HER2+ subtypes independent of ER status (30, 33). More recently the inflammatory cytokine IL8 has also been directly associated with mammosphere formation and ALDH positivity in breast CSCs (34). Thus, we measured the sensitivity of the CSC fraction to combined inhibition of PI3K and HER2 by mammosphere formation, ALDH activity and IL-8 expression. Treatment with trastuzumab alone reduced mammosphere formation, ALDH activity, and IL8 mRNA expression in trastuzumab-sensitive BT474 cells (Fig. S3A). The trastuzumab-resistant HR5 and HR6 cell lines contained a higher proportion of CSCs as indicated by greater ability to form mammospheres, enhanced ALDH activity, and higher levels of IL8 mRNA and protein (Fig. S3B). In these cells, the combination of XL147 + trastuzumab reduced mammosphere formation (Fig. 3A), IL8 mRNA (Fig. 3B) and ALDH activity (Fig. S3C). Treatment of HCC1954 cells with BKM120 and BEZ235, each alone and in combination with trastuzumab, reduced mammosphere formation, ALDH activity and IL8 expression, suggesting the effects were specified to the PI3K pathway (Fig. 3C-E). Finally, RNAi of p110α also
reduced the CSC fraction in HCC1954 cells as measured by a decrease in mammosphere growth and ALDH activity (Fig. S3E-G).

In epithelial and endothelial cells, FoxO1 and FoxO3 have been implicated as suppressors of IL8 transcription (35, 36). FoxO1 and FoxO3 activities are regulated by AKT-mediated phosphorylation. When AKT is inactive, FoxO factors are hypophosphorylated and predominantly nuclear where they modulate the transcription of target genes (37). We speculated that in trastuzumab-resistant cells, the combination of XL147 and trastuzumab, but not each drug alone, would optimally suppress PI3K-AKT resulting in FoxO-mediated suppression of IL-8 transcription. Indeed, the combination of XL147 and trastuzumab induced maximal activity of a FoxO3 promoter-reporter transfected into HCC1954 and HR6 cells compared to each inhibitor alone (Fig. 3F). Furthermore, transfection of FoxO1 and FoxO3 siRNA duplexes into trastuzumab-resistant HR5 and HCC1954 cells resulted in a 2-and 3.5-fold upregulation of IL8 mRNA, respectively (Fig. 3G).

**Treatment with PI3K inhibitor and trastuzumab reduces survivin expression.** To investigate mediators of the apoptosis induced by the combination of XL147 and trastuzumab in drug-resistant cells (Fig. 1C, D), we next examined levels of the pro-apoptotic molecule BIM and the anti-apoptotic molecules XIAP, survivin, BCL2, and MCL1. By immunoblot analysis, only survivin, a member of the inhibitor of apoptosis/IAP family of anti-apoptotic proteins (38), was downregulated in all cell lines upon treatment with XL147 + trastuzumab (Fig. 4A). Protein levels correlated with a change in survivin mRNA levels (Fig. 4B). Steady-state levels of survivin mRNA were similar between trastuzumab-sensitive (BT474) and -resistant (HR5 and HR6) cells (Fig. 4C, left panel; qPCR)). However, in antibody-sensitive cells, treatment with
trastuzumab inhibited survivin mRNA (qPCR) and protein (immunoblot) levels (Fig. 4C, right panel) whereas in the resistant cells, the antibody had no effect (Fig. 4A-B). In HCC1954 cells, BKM120 and BEZ235 each in combination with trastuzumab also reduced survivin mRNA levels (Fig. 4D), suggesting that survivin transcription is regulated by the PI3K pathway.

FoxO factors inhibit survivin gene transcription through direct interaction with its promoter (39, 40). Accordingly, siRNA knockdown of FoxO1 and FoxO3 in trastuzumab-resistant cells resulted in a 2-fold increase in survivin mRNA (Fig. 4E). Further, in BT474 cells, RNAi of FoxO1/3 counteracted the XL147 + trastuzumab-mediated suppression of survivin protein levels (Fig. 4F). In addition, overexpression of a constitutively active mutant of FoxO3, where the three AKT phosphorylation sites had been substituted with Ala [FoxO3(AAA)], reduced survivin protein levels (Fig. 4G) and BT474 cell growth and mammosphere formation (Fig. 4I, J). In a reverse experiment, transduction of a dominant negative FoxO3, where the C-terminal transactivation domain has been deleted (dnFoxO3), resulted in a modest increase in survivin protein levels in BT474 cells (Fig. 4H). These data suggest that survivin expression is regulated by PI3K-FoxO in HER2-overexpressing breast cancer cells.

RNAi of FoxO1/3 partially rescued the reduction of IL8 levels upon treatment with the combination of XL147 + trastuzumab (Fig. 4F). Conversely, BT474 cells transduced with the constitutively active FoxO3 mutant exhibited reduced IL8 expression (Fig. 4G) whereas dominant negative FoxO3 had the opposite effect (Fig. 4H).

Downregulation of survivin restores sensitivity to trastuzumab. Based on the results shown in Fig. 4, we speculated that downregulation of survivin is required for the apoptosis induced by the combination of XL147 + trastuzumab in cells resistant to the antibody. Indeed,
downregulation of survivin with RNAi (Fig. 5B) or with YM155, a small molecule inhibitor of survivin transcription (41), in trastuzumab-resistant cells resulted in growth inhibition (Fig. 5A and C: top panel) and apoptosis (Fig. 5C: bottom panel and Fig. S4A, B, D). A second survivin siRNA oligonucleotide produced comparable levels of knock-down, apoptosis, and growth inhibition in HCC1954 cells (Fig. S4E-F). Conversely, overexpression of survivin cDNA in drug-sensitive BT474 cells attenuated trastuzumab-mediated growth inhibition (Fig. S4G).

Survivin has been reported to play a role in maintenance of CSCs (42, 43). Thus, we next tested if genetic and/or pharmacological inhibition of survivin would have an effect on the CSC fraction within trastuzumab-resistant cells. In HR5 and HCC1954 cells, RNAi-mediated knockdown of survivin decreased mammosphere formation; this was further reduced when the RNAi oligonucleotides were combined with trastuzumab (Fig. 5D). Treatment with YM155 alone or in combination with trastuzumab also reduced mammosphere formation (Fig. 5E). These effects correlated temporally with reduction in ALDH activity (Fig. S4H, I).

**Combination of PI3K inhibitor and trastuzumab inhibits growth of trastuzumab-resistant xenografts.** Based on its superior ability to decrease survivin levels, we proposed that the combination of trastuzumab + XL147 would be a more potent inhibitor of trastuzumab-resistant xenograft growth compared to the PI3K inhibitor alone. Athymic mice bearing HR6 xenografts of $\geq$200 mm$^3$ were randomized to therapy with vehicle, XL147, trastuzumab, or the combination of both inhibitors. Each drug alone modestly delayed HR6 tumor growth whereas the combination of both inhibitors induced a marked anti-tumor effect (Fig. 6A). We next examined pharmacodynamic biomarkers of drug target inactivation after 28 days of treatment by immunohistochemistry (IHC), qRT-PCR, and immunoblot analyses. AKT activity has been
shown to correlate directly with both cytoplasmic and nuclear levels of pAKT$^{S473}$ (44). Consistent with the antitumor effect observed, only the combination of XL147 + trastuzumab reduced both cytoplasmic and nuclear pAKT levels (Fig. 6B: top panels). CD31 positivity (indicative of reduced vessel formation/angiogenesis) and ALDH1 levels by IHC and IL8 mRNA levels by qPCR were also lower in tumors treated with the combination (Fig. 6B: bottom panels and 6D). Finally, only the combination reduced survivin levels as measured by immunoblot analysis of tumor lysates (Fig. 6C). These results were further verified in mice bearing HCC1954 xenografts treated with vehicle, trastuzumab, XL147 and the combination of both drugs for one week. There was a noticeable decrease IL8 and survivin expression (Fig. S5) in tumors treated with the combination compared to tumors treated with each drug alone.

To support the clinical relevance of these data, we sought to determine whether steady-state and/or treatment-induced changes in survivin mRNA levels correlate with the response to trastuzumab in patients. In this study, patients with HER2+ breast cancer were treated with trastuzumab for 3 weeks, followed by a combination of trastuzumab + docetaxel for 12-weeks prior to surgery (45). Survivin mRNA was measured by microarray analysis in RNA extracted from tumor core biopsies obtained before therapy and from the surgical specimen after completion of the 4-month treatment. Evaluable matched microarray data were available in 13 patients. Five of 13 patients exhibited a response to treatment defined as absence of any invasive cancer or only residual cancer of <0.1 cm in diameter in the surgical specimen (45). There was a significant reduction in survivin mRNA levels in the post-treatment compared to the pre-treatment biopsies (p=0.0062; Fig. 6E). Furthermore, pre-treatment biopsies from patients exhibiting no response expressed significantly higher levels of survivin mRNA compared to those patients who responded clinically (p=0.026; Fig. 6F) These data suggest that baseline
levels and treatment-induced changes in survivin expression can potentially serve as a predictive biomarker of anti-HER2 therapy action in patients with HER2-overexpressing breast cancer.

**Discussion**

The antitumor action of trastuzumab depends in part on its ability to downregulate the PI3K-AKT signaling pathway. Persistent activation of this pathway has been shown to confer resistance to trastuzumab [reviewed in(8)]. Despite the increasing availability of therapeutic inhibitors of the PI3K/AKT/TOR pathway (reviewed in(14)) and abundant preclinical data linking this pathway with drug resistance, few mechanistic studies have examined the role of these inhibitors in trastuzumab-refractory breast cancer cells. We report herein the cellular, biochemical, and molecular effects of the ATP-competitive, reversible PI3K inhibitor XL147 against a panel of trastuzumab-resistant breast cancer cell lines and xenografts. The superior anti-proliferative and anti-tumor action of XL147 in combination with trastuzumab concurred with its ability to promote cell death and cell cycle arrest (Fig. 1 and 6A) and reduce AKT phosphorylation both *in vitro* (Fig. 2A) and *in vivo* (Fig. 6B).

HER2-PI3K-AKT signaling potently induces expression of the pro-angiogenic factor VEGF (2). Treatment with trastuzumab reduces tumor VEGF *in vivo* and this effect has been proposed to be central to the antitumor action of the antibody against HER2-dependent xenografts (26). We observed that, in drug-resistant xenografts, the combination of XL147 and trastuzumab reduced VEGF protein content and RNA expression, VEGF receptor phosphorylation, and blood vessel formation more potently than each drug alone (Fig. 2B-D and
6B), suggesting that PI3K-dependent enhanced angiogenesis is associated with resistance to trastuzumab.

Metastatic tumor relapses are characterized by rapidly proliferating, drug-resistant cancers associated with a high mortality rate. An increasing body of evidence suggests that survival of a small population of cells with stem-like properties may be responsible for these tumor recurrences after an initial response to anticancer therapy. This population, interchangeably called ‘cancer stem cells’ (CSCs) or ‘tumor-initiating cells’ (TICs), retains the capacity to self-renew and regenerate the total bulk of a heterogeneous tumor comprised mostly of non-stem cells. Therefore, in order to achieve cures, both CSCs and non-CSCs within a given tumor should be eliminated (46). It has been proposed that in HER2+ tumors, PI3K/AKT signaling increases the CSC fraction required for tumor progression (30). Indeed, treatment with trastuzumab reduced the CSC fraction in drug-sensitive BT474 cells (Fig. S3A) and xenografts (Fig. 6B). Conversely, this was not observed in antibody-resistant HR5, HR6 and HCC1954 cells (Fig. 3 and S3C). However, treatment with the PI3K inhibitor and trastuzumab reduced CSCs in trastuzumab-resistant tumors (Fig. 3, 6B and S3C-G) while decreasing expression of IL8, a cytokine that depends on FoxO transcription factors (Fig. 3B,G, 4F-H, and 6D) and that promotes maintenance of CSCs (47). These data suggest that the reduction of CSCs is associated with restoration of sensitivity to the anti-HER2 therapy.

Cell death induced by anticancer therapies is triggered by drug-induced modulation of endogenous levels of pro- and anti-apoptotic proteins. In the case of anti-HER2 therapies, this is the result of drug-induced downregulation of the PI3K/AKT and RAS/MEK/ERK pathways (48). The pro-apoptotic effects of the combination of XL147 and trastuzumab in trastuzumab-resistant cells and xenografts correlated temporally with transcriptional inhibition of survivin, a member
of the inhibitor of apoptosis family. High survivin expression has been associated with high nuclear grade, negative hormone receptor status, HER2 and VEGF overexpression, and worse disease-free or overall survival in breast cancer (49). In this study, inhibition of survivin function with either siRNA or a small molecule inhibitor reduced CSCs and non-CSCS in trastuzumab-resistant cells (Fig. 5 and S4), further suggesting a causal association of survivin expression with drug resistance. Similar to the effect on IL-8, the transcriptional repression of survivin also depended on FoxO factors (Fig. 4). Finally, high levels of survivin mRNA in HER2+ tumors correlated with a poor clinical response to trastuzumab-containing neoadjuvant therapy (Fig. 6F). These data suggest that in HER2+ tumors with high levels of survivin, a more sustained and comprehensive inhibition of the HER2/PI3K axis, perhaps with the addition of a PI3K pathway inhibitor, will be required to suppress expression of this anti-apoptotic protein. In sum, we conclude that acquired resistance to anti-HER2 therapies and subsequent metastatic progression of HER2-overexpressing cancers can be significantly ameliorated by early combinations of drugs that simultaneously target the HER2 receptor and the PI3K pathway.

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References


18.


**Figure legends**

**Figure 1.** XL147 but not CI-1040 inhibits trastuzumab-resistant cells. A, breast cancer cell lines sensitive or resistant to trastuzumab (lesions in the PI3K pathway are indicated within parentheses on top of each panel) were treated with DMSO (Ctrl), XL147 (6 μM), CI-1040 (0.5 μM), trastuzumab (10 μg/ml) alone or XL147 + trastuzumab and CI-1040 + trastuzumab for 5-days. Cell viability was measured by the WST-1 assay. Each bar, mean ± SE of four replicates. B, cells were grown in matrigel with or without inhibitors as in A and photographed (4x magnification) on day 11. C, cells were treated with or without XL147, trastuzumab, or both for 24 h and harvested for immunoblot analysis. D, cells were treated with XL147 (6 μM), trastuzumab (10 μg/ml) or both for 24 h (HR5, HR6) or 48 h (HCC1569 and HCC1954) prior to performing the Caspase-Glo 3/7 assay. Results were expressed as percent over DMSO control (straight line is drawn at 100%). Each bar, mean ± SE of 8 replicates.

**Figure 2.** Combination therapy inhibits signal transduction and expression of angiogenic factors. In both A and B, cell lines were harvested after a 24-h treatment with XL147 (6 μM: HR5, HR6, SUM190, HCC1569 and 10 μM: HCC1954), trastuzumab (10 μg/ml), or both inhibitors. A, cell lysates were subjected to immunoblot analysis with the indicated antibodies. B, cell lysates (500 μg) were hybridized with pRTK arrays. Arrows highlight pRTKs modulated by treatments.
indicated at the left of the panels. C, HR6 and HCC1954 cells were treated with DMSO, XL147, trastuzumab, or both as in A. Each lysate (500 μg) was hybridized with angiogenesis arrays. VEGF (circled) was downregulated following treatment with XL147 + trastuzumab. D, qRT-PCR for VEGFA mRNA in trastuzumab-resistant cells treated with DMSO, trastuzumab, XL147 or BKM120 (1 μM), and BEZ235 (250 nM), either alone or in the indicated combinations for 48 h. Each bar, mean ± SE of triplicates.

**Figure 3.** PI3K inhibition modulates the CSC fraction in trastuzumab-resistant cells. A, mammosphere assay was performed with cells treated with 6 μM XL147, 10 μg/ml trastuzumab, or both. Total mammosphere volume was measured on day 13 as described in Methods. Each bar, mean ± SD of two replicates. B, IL8 qPCR was performed on RNA isolated from HR6 and HCC1954 cells treated with XL147, trastuzumab, or XL147 + trastuzumab for 24 and 48 h, respectively. Each bar, mean ± SE of triplicates. C, average mammosphere volume in HCC1954 cells treated with trastuzumab (10 μg/ml), BKM120 (1 μM), BEZ235 (250 nM), BKM120 + trastuzumab, or BEZ235 + trastuzumab for 7 days. Each bar, mean ± SE of three wells. D, percent ALDH activity in HCC1954 cells treated with the indicated inhibitors alone or in combination for 48 h. Each bar, mean ± SD of duplicates. E, HCC1954 cells were treated with trastuzumab, BEZ235, or BEZ235 + trastuzumab for 48 h prior to RNA isolation and subsequent IL8 qPCR. Each bar represents the mean ± SE of triplicates. F, luciferase activity was measured in HCC1954 and HR6 cells transiently transfected with a FoxO promoter-reporter (FHRE-Luc) and treated with DMSO, XL147 (6 μM: HR6; 10 μM: HCC1954), trastuzumab (10 μg/ml), or XL147 + trastuzumab. Each bar, mean ± SE of 6 replicates. G, HR5 and HCC1954 cells were transfected with control or FoxO1 and FoxO3 siRNA oligonucleotides as described in (22).
Three days later, the cells were harvested for RNA extraction followed by determination of IL8, FoxO1 and 3 mRNA levels by qPCR. Each bar, mean ± SE of triplicates.

**Figure 4.** Combination of PI3K inhibitor and trastuzumab reduces survivin expression. A-B, cells were treated with XL147 (6 μM), trastuzumab (10 μg/ml), or both inhibitors for 24 h (HR6) or 48 h (HR5, HCC1569 and HCC1954) and subjected to immunoblot analysis for pro- or anti-apoptotic proteins (A) and qPCR for survivin mRNA (B). In B, each bar represents the mean ± SE of triplicates. C, survivin expression in trastuzumab-sensitive vs. -resistant cells. Left panel: qPCR for survivin mRNA in BT474, HR5, and HR6; right panel: immunoblot (top) and qPCR (bottom) for survivin in BT474 cells treated with XL147, trastuzumab, or XL147 + trastuzumab for 48 h. Each bar, mean ± SE of triplicates. D, qPCR for survivin mRNA in HCC1954 cells treated with trastuzumab, BKM120, BEZ235, BKM120 + trastuzumab, or BEZ235 + trastuzumab for 48h (same doses as in Fig. 3). Each bar, mean ± SE of triplicates. E, HR5 and HCC1954 cells were transfected with control or FoxO1 and FoxO3 siRNA duplexes for 3 days, followed by survivin qPCR. Each bar, mean ± SE of triplicates. F, BT474 cells were transfected with control or FoxO1 and FoxO3 siRNA duplexes and treated with 6 μM XL147 + 10 μg/ml trastuzumab for 48 h prior to immunoblot analyses. G-J, BT474 cells were infected with recombinant adenoviruses expressing GFP-tagged constitutively active FoxO3 (AAA) (G, I, J) or dominant negative DNFoxO3 (H). Control cells were infected with adenovirus encoding beta-galactosidase/LacZ (Ad-CMV-b-Gal). G, H, six days post-infection, cells were harvested and lysates prepared for immunoblot analysis. I, eight days post-infection, cells were trypsinized and counted as indicated in Methods (each bar, mean ± SE of triplicates). J, twenty-four h after infection, cells were plated in a mammosphere assay and imaged after 7 days (each bar, mean ± SE of triplicates).
SE of triplicates; left panel). Mammospheres were dissociated by trypsin digestion and single cells were plated in monolayer in low density. After 7 days, tumor cell foci/colonies were stained with crystal violet and imaged with 4x magnification (right panel).

**Figure 5.** Downregulation of survivin restores sensitivity to trastuzumab. A, HR5 and HR6 (left panel) and HCC1569 and HCC1954 (right panel) cells were transfected with control or survivin siRNA duplexes and treated with 10 μg/ml trastuzumab. Cells were counted after 6- (HR5 and HR6) or 4- (HCC1569 and HCC1954) days of treatment. Each bar, mean ± SE of 3 wells. B, immunoblot analysis of control or survivin siRNA transfected cells ± trastuzumab as indicated. C, growth inhibitory and pro-apoptotic effects of survivin transcriptional inhibitor in HCC1954 cells. Cells were treated with YM155 (50 nM), trastuzumab (10 μg/ml), or both inhibitors and harvested for cell counting on day 5 (top panel) or immunoblot analysis for PARP cleavage after 24 h (bottom panel). Each bar, mean ± SE of triplicates. D and E, effects of survivin knockdown with either siRNA (D) or YM155 (E) on CSCs within trastuzumab-resistant cells. D, average mammosphere volume in HCC1954 and HR5 cells transfected with control or survivin siRNA and treated with trastuzumab for 6 days. Each bar, mean ± SD of duplicates. E, average mammosphere volume in HCC1954 cells treated with YM155, trastuzumab, or YM155 + trastuzumab for 6 days. Each bar, mean ± SE of triplicates.

**Figure 6.** PI3K inhibitor + trastuzumab inhibit growth of trastuzumab-resistant xenografts. A, volumes of HR6 xenografts established in athymic mice treated with vehicle (Ctrl), XL147, trastuzumab, or XL147 + trastuzumab. Each data point represents the log transformed value of mean tumor volume ± SE over the time indicated in the X axis. B, H-score analysis of IHC sections for pAKT S473, CD31 and ALDH1. Each bar, mean ± SE; *p<0.05, unpaired t-test. C,
immunoblot analysis for survivin expression in tumor lysates collected on day 28. D, qPCR analysis for IL8 mRNA in HR6 xenografts harvested on day 28. Each bar, mean ± SE of 3 wells. 

E-F, survivin mRNA expression in thirteen HER2+ paired breast tumors from patients treated with neoadjuvant trastuzumab + chemotherapy as described in Results. E, comparison between the pre- and post-treatment survivin RNA levels in primary tumor biopsies. Each bar, mean ± SE (n=13); *p<0.05; paired t-test. F, survivin mRNA levels in pre-treatment biopsies of tumors that responded (R) vs. those that did not respond (NR) to neoadjuvant trastuzumab. Each bar, mean SE; *p<0.05; Mann Whitney test.
Figure. 1
Figure. 2
Figure 3
Figure. 4
Figure. 5
Figure. 6
Trastuzumab-resistant cells rely on a HER2-PI3K-FoxO-survivin axis and are sensitive to PI3K inhibitors


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