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Trastuzumab-Resistant Cells Rely on a HER2-PI3K-FoxO-Survivin Axis and Are Sensitive to PI3K Inhibitors

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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 phosphoinositide 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 with XL147 alone, the combination exhibited a superior antitumor effect against trastuzumab-resistant tumor xenografts. Furthermore, 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 antiapoptosis gene survivin (*BIRC5*) and the CSC-associated cytokine interleukin-8. RNA interference–mediated or pharmacologic inhibition of survivin restored sensitivity to trastuzumab in resistant cells. In a cohort of patients with HER2-overexpressing breast cancer treated with trastuzumab, higher pretreatment 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. *Cancer Res; 73(3); 1190–200. ©2012 AACR.*

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 anticancer therapy (2). This translates into shorter disease-free and overall survival in patients (3). In HER2-overexpressing cells, HER2 dimerizes with its coreceptor HER3 that, in turn, directly couples to the p85 regulatory subunit of phosphoinositide 3-kinase (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, and 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 ref. 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 ref. 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*; ref. 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 ref. 14).

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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). Pharmacologic and RNA interference (RNAi)-based approaches suggested this was, at least in part, due to derepression of FoxO-mediated transcription that, in turn, downregulated expression of interleukin (IL)-8 and the antiapoptosis protein survivin. Finally, in patients with HER2-overexpressing breast cancer treated with trastuzumab, higher pretreatment tumor levels of survivin RNA correlated with poor response to therapy. Taken together, these data suggest that (i) trastuzumab-resistant cells continue to rely on HER2-PI3K-FoxO-survivin axis for survival, and (ii) 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), 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 previously (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 previously (19). Adenoviruses encoding β-galactosidase/LacZ, non-phoshorylatable FKHRL1/FoxO3(AAA) mutant and dominant-negative/dnFoxO3, both GFP-tagged, were from Vector Biolabs. Adenovirus infection was conducted as described previously (20).

Cell proliferation, immunoprecipitation, immunoblot analysis, RNA interference, and real-time quantitative PCR

These methods were conducted as described previously (21). Human FoxO1 and FoxO3a siRNAs were described previously (22). The p110α, p110β, and survivin siRNA duplexes were obtained from Ambion and Cell Signaling Technology. Primers for quantitative PCR (qPCR) were obtained from Qiagen.

Mammosphere and aldehyde dehydrogenase assays

For mammosphere assay, 0.5×10^4 to 1×10^4 cells/well were seeded in 6-well Ultra-low attachment plates (Costar) in Dulbecco's Modified Eagles' Media (DMEM):F12 + 20 ng/mL EGF + 1× 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 1,000 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. Aldehyde dehydrogenase (ALDH) assay was conducted with ALDEFLUOR kit (Stemcell Technologies) and subsequent fluorescence-activated cell-sorting (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 conducted with the Dual-Luciferase Reporter Assay System (Promega).

Proteome Profiler human angiogenesis and phospho-RTK arrays

Angiogenesis (ARY007) and pRTK (ARY001) arrays were conducted according to manufacturer's instructions (R&D Systems).

Xenograft studies

These were approved by the Institutional Animal Care Committee of Vanderbilt University (Nashville, TN). A 17β-estradiol pellet (Innovative Research of America) was injected subcutaneously in the dorsum of 4- to 5-week-old athymic female mouse (Harlan Sprague Dawley, Inc.). Next day, HR6 cells ($\sim 3 \times 10^6$) 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 = $width^2 \times length/2$. Mice with ≥ 200 mm^3 tumors were randomized to vehicle, trastuzumab (30 mg/kg twice a week intraperitoneally), XL147 (100 mg/kg daily *per os*), or combination treatments for 28 days. Immunostaining and histoscore (*H*-score) analysis of formalin-fixed tumor sections harvested on day 28 were conducted as described previously (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

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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 3-dimensional growth (Fig. 1B) in all resistant lines. CI-1040 alone was inactive against all cell lines, whereas growth of 3 of 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 $p27^{Kip1}$ (cell-cycle arrest; ref. Fig. 1C). This was further confirmed by enhanced caspase-3/7 activity following treatment with XL147 + trastuzumab compared with 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 (Supplementary Fig. S1D). Compared with 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 3-dimensional (Supplementary Fig. S1A and S1B) as well as apoptosis measured by activation of caspase-3/7 (Supplementary 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 $pAKT^{S473}$ and $pAKT^{T308}$ levels (Fig. 2A). Inhibition of PI3K/AKT is counteracted by compensatory feedback activation of RTKs, including HER3 (22, 25). In HER2-dependent breast cancer cells and xenografts, this compensation is reduced by cotreatment with trastuzumab or the HER2 tyrosine kinase inhibitor (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 with XL147 alone (Fig. 2B; arrows).

In all 3 trastuzumab-resistant lines, treatment with both inhibitors reduced phosphorylation of VEGFR1 and/or

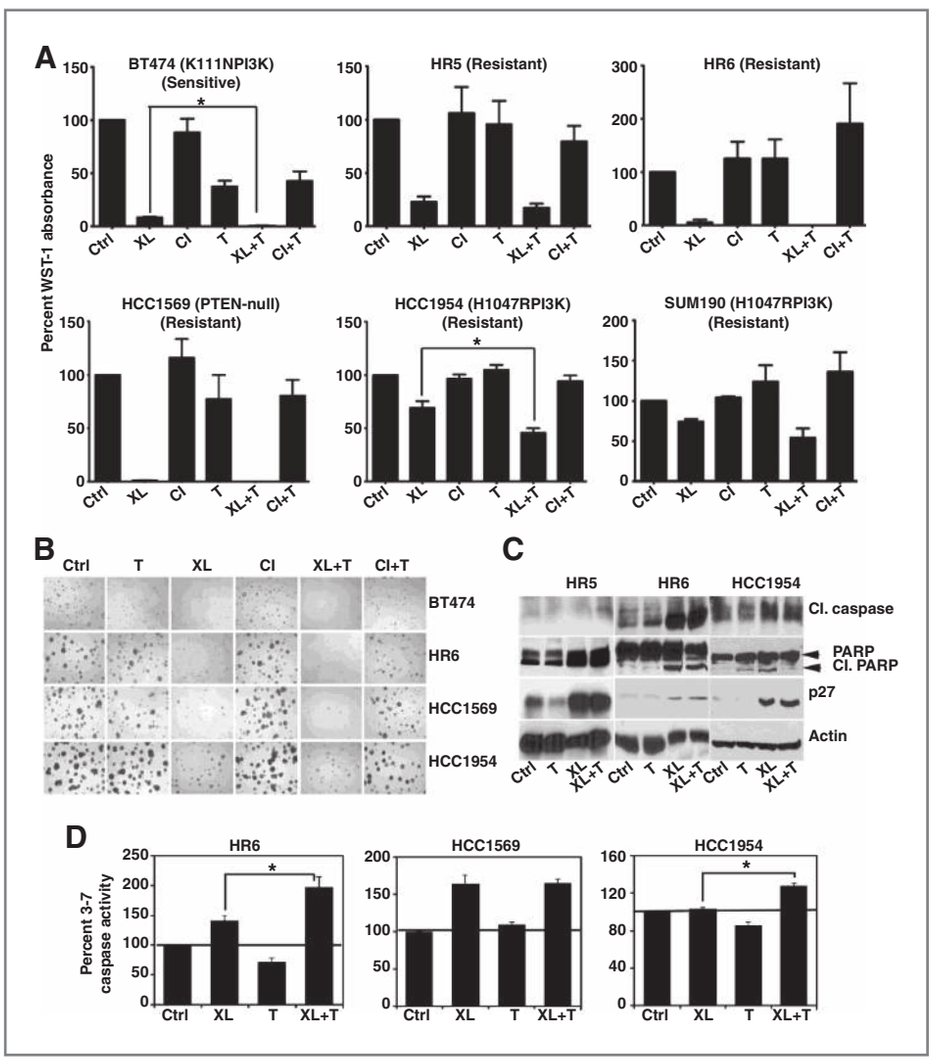

Figure 1. XL147 but not CI-1040 inhibits trastuzumab-resistant cells. A, breast cancer cell lines sensitive or resistant to trastuzumab (lesions in the PI3K pathway are indicated within parentheses on top of each panel) were treated with dimethyl sulfoxide (Ctrl), XL147 (6 μmol/L), CI-1040 (0.5 μmol/L), 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 represents mean ± SE of 4 replicates. *, $P < 0.05$; paired *t* test. 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 hours and harvested for immunoblot analysis. D, cells were treated with XL147 (6 μmol/L), trastuzumab (10 μg/mL), or both for 24 hours (HR5 and HR6) or 48 hours (HCC1569 and HCC1954) before conducting the Caspase-Glo 3/7 assay. Results were expressed as percent over dimethyl sulfoxide control (straight line is drawn at 100%). Each bar represents mean ± SE of 8 replicates. *, $P < 0.05$; paired *t* test.

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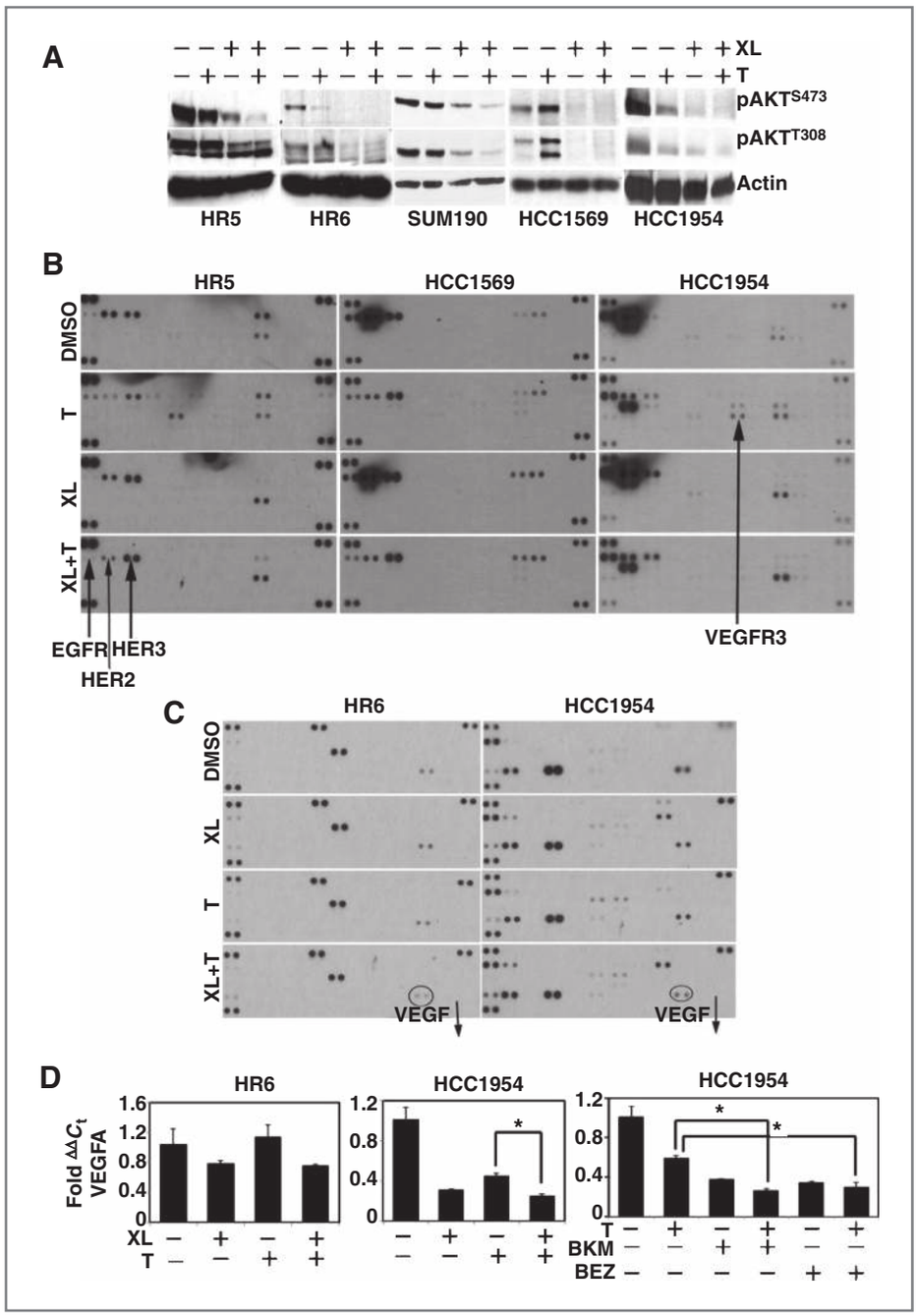

Figure 2. Combination therapy inhibits signal transduction and expression of angiogenic factors. A and B, cell lines were harvested after a 24-hour treatment with XL147 (6 μmol/L HR5, HR6, SUM190, HCC1569 and 10 μmol/L HCC1954), trastuzumab (10 μg/mL), or both inhibitors. A, cell lysates were subjected 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. C, HR6 and HCC1954 cells were treated with dimethyl sulfoxide (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 μmol/L), and BEZ235 (250 nmol/L), either alone or in the indicated combinations for 48 hours. Each bar represents mean ± SE of triplicates. *, $P < 0.05$; paired *t* test.

VEGFR3 (Fig. 2B; longer exposure in Supplementary Fig. S2A). Related to this finding, trastuzumab has been shown to act as an antiangiogenic agent by affecting different proangiogenic 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 proangiogenic factors including angiopoietin-1 and -2, IL-8, and Chemokine ligand-4 were also downregulated in a cell line–specific manner (longer exposure in Supplementary Fig. S2B and S2C). 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/mTOR inhibitor BEZ235 (29), either alone or in combination with trastuzumab

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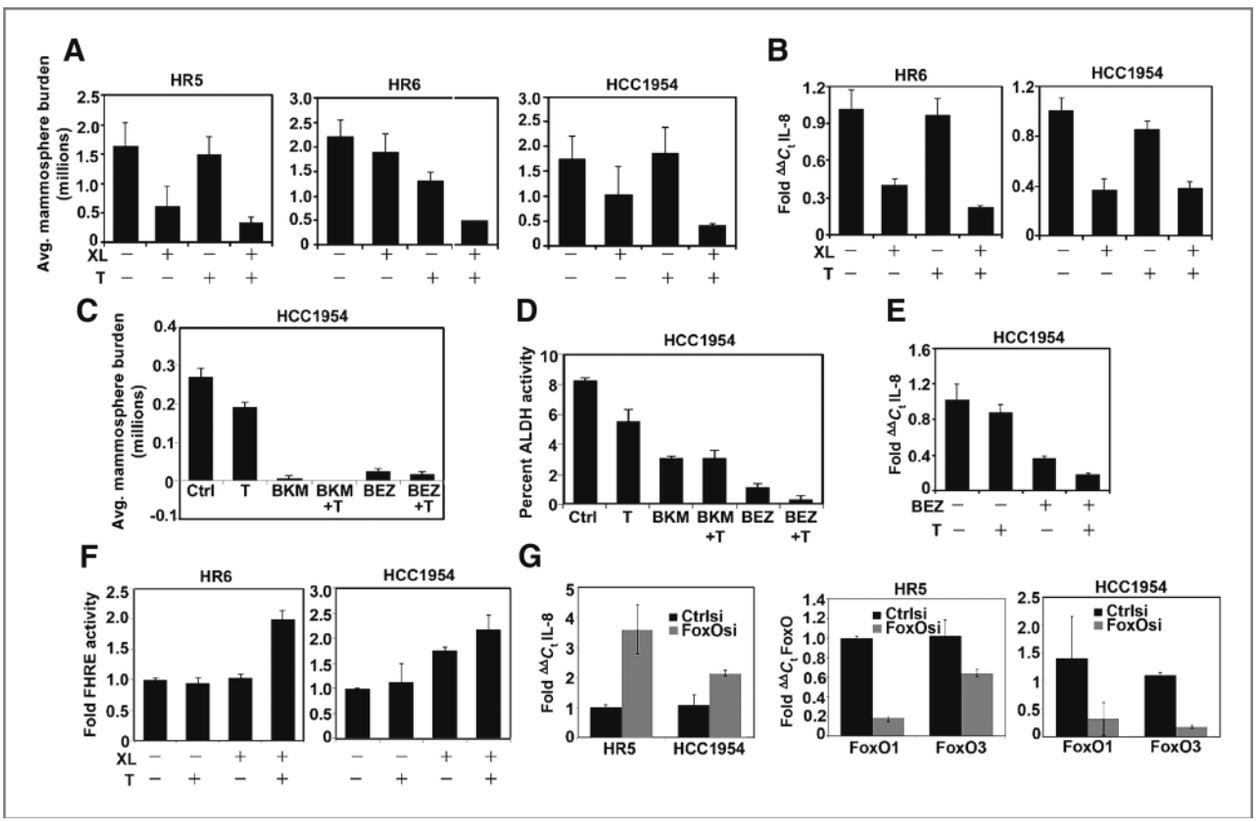

Figure 3. PI3K inhibition modulates the CSC fraction in trastuzumab-resistant cells. A, mammosphere assay was conducted with cells treated with 6 μmol/L XL147, 10 μg/mL trastuzumab, or both. Total mammosphere volume was measured on day 13 as described in Materials and Methods. Each bar represents mean ± SD of 2 replicates. B, IL-8 qPCR was carried out on RNA isolated from HR6 and HCC1954 cells treated with XL147, trastuzumab, or XL147 + trastuzumab for 24 and 48 hours, respectively. Each bar represents mean ± SE of triplicates. C, average mammosphere volume in HCC1954 cells treated with trastuzumab (10 μg/mL), BKM120 (1 μmol/L), BEZ235 (250 nmol/L), BKM120 + trastuzumab, or BEZ235 + trastuzumab for 7 days. Each bar represents mean ± SE of 3 wells. D, percentage of ALDH activity in HCC1954 cells treated with the indicated inhibitors alone or in combination for 48 hours. Each bar represents mean ± SD of duplicates. E, HCC1954 cells were treated with trastuzumab, BEZ235, or BEZ235 + trastuzumab for 48 hours before RNA isolation and subsequent IL-8 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 μmol/L: HR6; 10 μmol/L: HCC1954), trastuzumab (10 μg/mL), or XL147 + trastuzumab. Each bar represents mean ± SE of 6 replicates. G, HR5 and HCC1954 cells were transfected with control or FoxO1 and FoxO3 siRNA oligonucleotides as described previously (22). Three days later, the cells were harvested for RNA extraction followed by determination of IL-8, FoxO1, and 3 mRNA levels by qPCR. Each bar represents mean ± SE of triplicates.

(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 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). ALDH positivity (ALDH+) correlates with $HER2^+$ subtypes independent of estrogen receptor (ER) status (30, 33). More recently, the inflammatory cytokine IL-8 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 IL-8 mRNA expression in trastuzumab-sensitive BT474 cells (Supplementary 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 IL-8 mRNA and protein (Supplementary Fig. S3B). In these cells, the combination of XL147 + trastuzumab reduced mammosphere formation (Fig. 3A), IL-8 mRNA (Fig. 3B), and ALDH activity (Supplementary Fig. S3C). Treatment of HCC1954 cells with BKM120 and BEZ235, each alone and in combination with trastuzumab, reduced mammosphere formation, ALDH activity, and IL-8 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 (Supplementary Fig. S3E–S3G).

In epithelial and endothelial cells, FoxO1 and FoxO3 have been implicated as suppressors of IL-8 transcription (35, 36).

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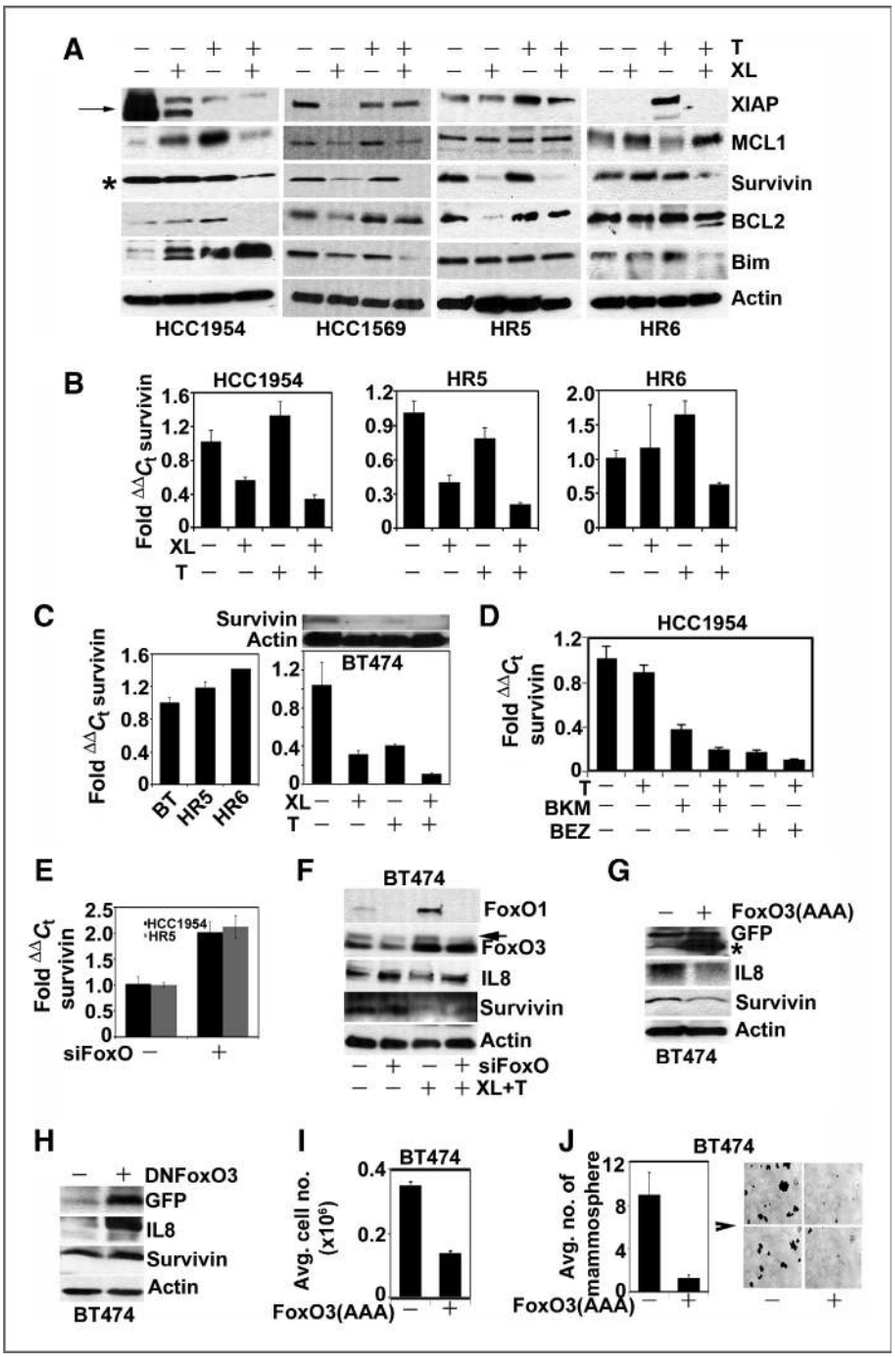

Figure 4. Combination of PI3K inhibitor and trastuzumab reduces survivin expression. A and B, cells were treated with XL147 (6 μmol/L), trastuzumab (10 μg/mL), or both inhibitors for 24 hours (HR6) or 48 hours (HR5, HCC1569, and HCC1954) and subjected to immunoblot analysis for pro- or antiapoptotic proteins (A) and qPCR for survivin mRNA (B). B, each bar represents the mean ± SE of triplicates. C, survivin expression in trastuzumab-sensitive versus -resistant cells. Left, qPCR for survivin mRNA in BT474, HR5, and HR6; right, immunoblot (top) and qPCR (bottom) for survivin in BT474 cells treated with XL147, trastuzumab, or XL147 + trastuzumab for 48 hours. Each bar represents mean ± SE of triplicates. D, qPCR for survivin mRNA in HCC1954 cells treated with trastuzumab, BKM120, BEZ235, BKM120 + trastuzumab, or BEZ235 + trastuzumab for 48 hours (same doses as in Fig. 3). Each bar represents 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 represents mean ± SE of triplicates. F, BT474 cells were transfected with control or FoxO1 and FoxO3 siRNA duplexes and treated with 6 μmol/L XL147 + 10 μg/mL trastuzumab for 48 hours before 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 β-galactosidase/LacZ (Ad-CMV-b-Gal). G and H, six days postinfection, cells were harvested and lysates prepared for immunoblot analysis. I, eight days postinfection, cells were trypsinized and counted as indicated in Materials and Methods (each bar represents mean ± SE of triplicates). J, twenty-four hours after infection, cells were plated in a mammosphere assay and imaged after 7 days (each bar represents mean ± SE of triplicates; left). 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 ×4 magnification (right).

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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 with 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 IL-8 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 and D), we next examined levels of the proapoptotic molecule BIM and the antiapoptotic molecules X-linked inhibitor of apoptosis protein (XIAP), survivin, BCL2, and MCL1. By immunoblot analysis, only survivin, a member of the inhibitor of apoptosis/IAP family of antiapoptotic 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; qPCR). However, in antibody-sensitive cells, treatment with trastuzumab inhibited survivin mRNA (qPCR) and protein (immunoblot) levels (Fig. 4C, right), whereas in the resistant cells, the antibody had no effect (Fig. 4A and 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). Furthermore, 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 3 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 and 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 IL-8 levels upon treatment with the combination of XL147 + trastuzumab (Fig. 4F). Conversely, BT474 cells transduced with the constitutively active FoxO3 mutant exhibited reduced IL-8 expression (Fig. 4G), whereas dominant-negative FoxO3 had the opposite effect (Fig. 4H).

Downregulation of survivin restores sensitivity to trastuzumab

On the basis of 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) and apoptosis (Fig. 5C, bottom and Supplementary Fig. S4A, B, and D). A second survivin siRNA oligonucleotide produced comparable levels of knockdown, apoptosis, and growth inhibition in HCC1954 cells (Supplementary Fig. S4E and S4F). Conversely, overexpression of survivin cDNA in drug-sensitive BT474 cells attenuated trastuzumab-mediated growth inhibition (Supplementary Fig. S4G).

Survivin has been reported to play a role in maintenance of CSCs (42, 43). Thus, we next tested whether genetic and/or pharmacologic 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 (Supplementary Fig. S4H and S4I).

Combination of PI3K inhibitor and trastuzumab inhibits growth of trastuzumab-resistant xenografts

On the basis of 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 with 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 antitumor effect (Fig. 6A). We next examined pharmacodynamic biomarkers of drug target inactivation after 28 days of treatment by immunohistochemistry (IHC), real-time qPCR (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). CD31 positivity (indicative of vessel formation/angiogenesis) and ALDH1 levels by IHC and IL-8 mRNA levels by qPCR were also lower in tumors treated with the combination (Figs. 6B, bottom, and D). 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

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of both drugs for 1 week. There was a noticeable decrease IL-8 and survivin expression (Supplementary Fig. S5) in tumors treated with the combination compared with 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 before 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 posttreatment compared with the pretreatment biopsies ($P = 0.0062$; Fig. 6E). Furthermore, pretreatment biopsies from patients exhibiting no response expressed significantly higher levels of survivin mRNA than 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.

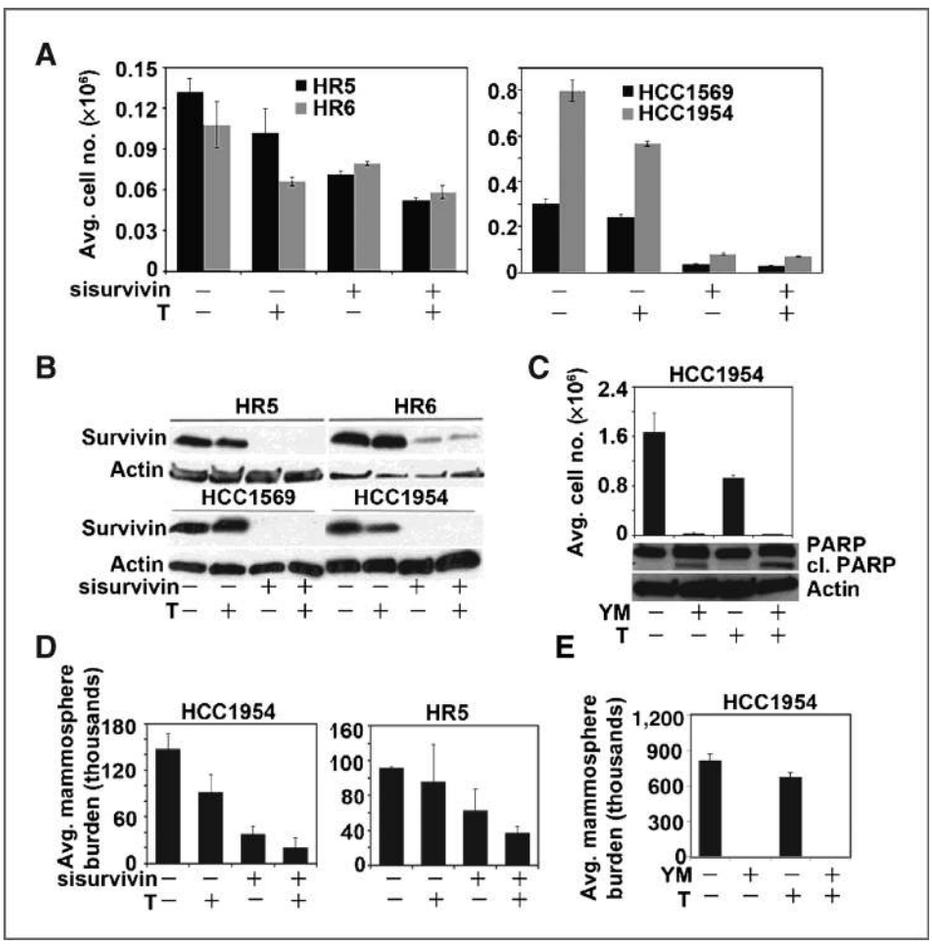

Figure 5. Downregulation of survivin restores sensitivity to trastuzumab. A, HR5 and HR6 (left) and HCC1569 and HCC1954 (right) cells were transfected with control or survivin siRNA duplexes and treated with 10 μg/mL trastuzumab. Cells were counted after 6 (HR5 and HR6) or 4 (HCC1569 and HCC1954) days of treatment. Each bar represents mean ± SE of 3 wells. B, immunoblot analysis of control or survivin siRNA–transfected cells ± trastuzumab as indicated. C, growth inhibitory and proapoptotic effects of survivin transcriptional inhibitor in HCC1954 cells. Cells were treated with YM155 (50 nmol/L), trastuzumab (10 μg/mL), or both inhibitors and harvested for cell counting on day 5 (top) or immunoblot analysis for PARP cleavage after 24 hours (bottom). Each bar represents 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 represents mean ± SD of duplicates. E, average mammosphere volume in HCC1954 cells treated with YM155, trastuzumab, or YM155 + trastuzumab for 6 days. Each bar represents mean ± SE of triplicates.

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Persistent activation of this pathway has been shown to confer resistance to trastuzumab (reviewed in ref. 8). Despite the increasing availability of therapeutic inhibitors of the PI3K/AKT/TOR pathway (reviewed in ref. 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 antiproliferative and antitumor action of XL147 in combination with trastuzumab concurred with its ability to promote cell death and cell-cycle arrest (Figs. 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 proangiogenic 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 (Figs. 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 CSCs or "tumor-initiating cells" (TIC), retains the capacity to self-renew and regenerate the total bulk of a heterogeneous tumor comprised mostly of non-stem cells. Therefore, 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

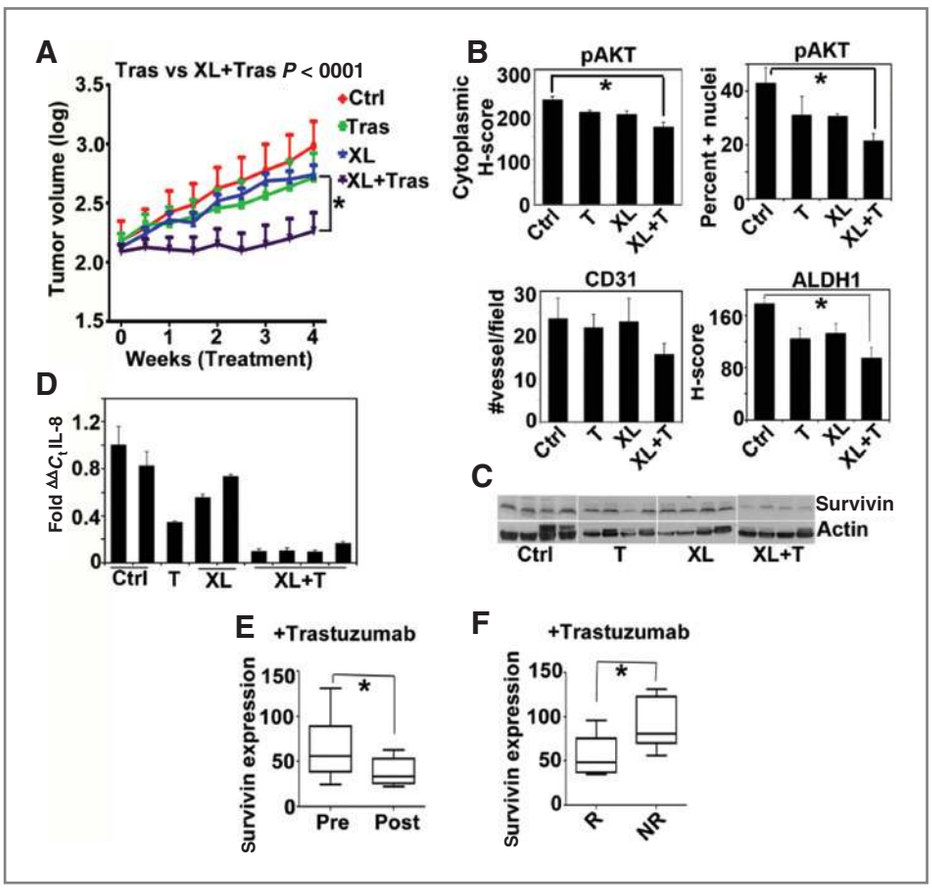

Figure 6. PI3K inhibitor + trastuzumab inhibit growth of trastuzumab-resistant xenografts. A, volumes of HR6 xenografts established in athymic mice treated with vehicle (Ctrl), XL147, trastuzumab, or XL147 + trastuzumab. Each data point represents the log-transformed value of mean tumor volume ± SE over the time indicated in the *x*-axis. B, *H*-score analysis of IHC sections for pAKT^{S473}, CD31, and ALDH1. Each bar represents 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 IL-8 mRNA in HR6 xenografts harvested on day 28. Each bar represents mean ± SE of 3 wells. E and F, survivin mRNA expression in 13 HER2$^+$ paired breast tumors from patients treated with neoadjuvant trastuzumab + chemotherapy as described in Results. E, comparison between the pre- and posttreatment survivin RNA levels in primary tumor biopsies. Each bar represents mean ± SE ($n = 13$). *, $P < 0.05$; paired *t* test. F, survivin mRNA levels in pretreatment biopsies of tumors that responded (R) versus those who did not respond (NR) to neoadjuvant trastuzumab. Each bar represents mean ± SE. *, $P < 0.05$; Mann–Whitney test.

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fraction required for tumor progression (30). Indeed, treatment with trastuzumab reduced the CSC fraction in drug-sensitive BT474 cells (Supplementary Fig. S3A) and xenografts (Fig. 6B). Conversely, this was not observed in antibody-resistant HR5, HR6, and HCC1954 cells (Fig. 3 and Supplementary Fig. S3C). However, treatment with the PI3K inhibitor and trastuzumab reduced CSCs in trastuzumab-resistant tumors (Figs. 3 and 6B and Supplementary Fig. S3C–S3G) while decreasing expression of IL-8, a cytokine that depends on FoxO transcription factors (Figs. 3B and 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 antiapoptotic 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 proapoptotic 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 IAP 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 Supplementary Fig. 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 antiapoptotic 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Chakrabarty, J.C. Chang
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.E. Bhola, C.R. Sutton, R. Ghosh, M.G. Kuba, J.C. Chang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Chakrabarty, N.E. Bhola, C.R. Sutton, R. Ghosh, B. Dave
Writing, review, and/or revision of the manuscript: A. Chakrabarty, J.C. Chang, C.L. Arteaga
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.R. Sutton
Study supervision: A. Chakrabarty, C.L. Arteaga
Other: Carried out experiments, A. Chakrabarty

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Trastuzumab-Resistant Cells Rely on a HER2-PI3K-FoxO-Survivin Axis and Are Sensitive to PI3K Inhibitors

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