Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase

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Edited by Joan S. Brugge, Harvard Medical School, Boston, MA, and approved February 14, 2011 (received for review October 29, 2010)

Sustained and complete inhibition of HER3 and its output to PI3K/Akt are required for the optimal antitumor effect of therapeutic inhibitors of the HER2 oncogene. Here, we show that, after inhibition of the HER2 tyrosine kinase with lapatinib, there is PI3K/Akt and FoxO3a-dependent up-regulation of HER3 mRNA and protein. Up-regulated HER3 was then phosphorylated by residual HER2 activity, thus partially maintaining P-Akt and limiting the antitumor action of lapatinib. Inhibition of HER3 with siRNA or a neutralizing HER3 antibody sensitized HER2+ breast cancer cells and xenografts to lapatinib both in vitro and in vivo. Combined blockade of HER2 and HER3 inhibited pharmacodynamic biomarkers of PI3K/Akt activity more effectively than each inhibitor alone. These results suggest that because of HER3-mediated compensation, current clinical inhibitors of HER2 and PI3K/Akt will not block the PI3K pathway completely. They also suggest that therapeutic inhibitors of HER3 should be used in combination with HER2 inhibitors and PI3K pathway inhibitors in patients with HER2- and PI3K-dependent cancers.

The HER (ErbB) transmembrane receptor tyrosine kinase family is comprised of four members: EGF receptor (ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). HER2 is amplified in approximately 25% of human breast cancers (1) and is associated with poor prognosis (2). HER2/HER3 heterodimers are the most transforming of this receptor network (3). HER3, which lacks intrinsic kinase activity (4), is able to potently activate the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway (5) via its six docking sites for the p85 adaptor subunit of PI3K (6). HER2-mediated transformation of mammary epithelial cells has been attributed to a large degree to activation of the PI3K–Akt survival pathway. Trastuzumab, a monoclonal antibody directed against the ectodomain of HER2, and the EGFR/HER2 tyrosine kinase inhibitor (TKI) lapatinib are approved for the treatment of HER2-overexpressing breast cancer. Although these therapies work by different mechanisms, it has been proposed that, to exert an antitumor effect, they should inhibit phosphorylation of HER3 and disable the PI3K/Akt pathway (7, 8).

The HER3 coreceptor plays an essential role in HER2-mediated transformation, tumor progression, and drug resistance. In HER2-dependent cells, loss of HER3 results in reduced signaling through PI3K and cell proliferation (9, 10), suggesting that HER2 may be dependent on HER3 to drive growth and survival of breast cancer cells. As it applies to drug resistance, inhibition of HER2 phosphorylation by TKIs targeting EGFR and HER2 in HER2+ breast cancer cells is followed by feedback upregulation of activated HER3, thus limiting the inhibitory effect of HER TKIs (11, 12). These studies point to a central role for HER3 in the survival of HER2+ cells that potentially limit the full action of HER2 antagonists.

Results

Inhibition of the HER2 Tyrosine Kinase Is Followed by Up-Regulation of HER3 and P-HER3. We hypothesized that sustained and complete inhibition of HER3 and its output to PI3K/Akt is required for the maximal antitumor effect of HER2 inhibitors. Thus, we examined the temporal effect of the HER2 TKI lapatinib on active HER3 in BT474, SKBR3, and SUM225 cells, all *HER2* gene amplified and an IC_{50} to lapatinib ≤0.1 μM (13). A time course with a clinically achievable dose of lapatinib (1 μM; ref. 14) showed time-dependent up-regulation of HER3 protein (Fig. 1*A*), beginning at 4 h and increasing through 48 h. Immunoprecipitation of HER3 from BT474 and SKBR3 cell lysates followed by HER3, HER2, and P-Tyr immunoblot revealed coprecipitation of HER3 and HER2 at baseline and prompt loss of P-Tyr in the HER3 pulldown upon treatment with lapatinib. Recovery of HER3 tyrosine phosphorylation was detectable in HER3 antibody pulldowns at and beyond 13 h simultaneous with a modest amount of coprecipitated HER2 (Fig. 1*B*). Site-specific antibodies revealed HER3 phosphorylation at Y1197 and Y1289, two of the six p85 binding sites in HER3 (6), even though phosphorylated P-HER2 and P-EGFR were undetectable. Recovery of Y1197 P-HER3 correlated with partial recovery of T308 P-Akt and S240/244 P-S6, but not S473 P-Akt (Fig. 1*C*).

We next confirmed up-regulation of HER3 in primary tumor sections from patients with HER2+ breast cancer treated with lapatinib. In this trial, lapatinib was given as a single agent for 6 wk (15). Eight matched pre- and posttreatment (2 wk) biopsies with evaluable tumor material were available. Sections from formalin-fixed paraffin-embedded tumor blocks were subjected to immunohistochemistry (IHC) with an antibody against total HER3. The percent and intensity of tumor cell staining was calculated as a histoscore. On wk 2 of therapy, HER3 levels increased 135% above pretherapy levels ($n = 8$; $P = 0.03$, Mann–Whitney *U*; Fig. 1*D*). In addition, we found an increase in both HER3 mRNA and protein 6 h after oral administration of one dose of lapatinib to mice bearing BT474 xenografts. There was inhibition of T308 and S473 P-Akt at 6 h after treatment, correlating with up-regulation of HER3 protein and RNA (Fig. S1 *A* and *B*). However, after 6 h, the effects of lapatinib on HER3 protein and RNA levels diminished, likely because of the half-life of lapatinib in vivo, as evidenced by increased P-Akt. These data suggest that, upon inhibition of the HER2 kinase and partial re-

Author contributions: J.T.G. and C.L.A. designed research; J.T.G., M.G.O., C.R., N.D.G.-I., V.S., A.C., and B.D. performed research; J.T.G., M.G.O., N.D.G.-I., R.S.C., E.M., H.C.M., J.C., and C.L.A. analyzed data; W.P. and J.C. contributed new reagents/analytic tools; and J.T.G. and C.L.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1016140108/-/DCSupplemental.

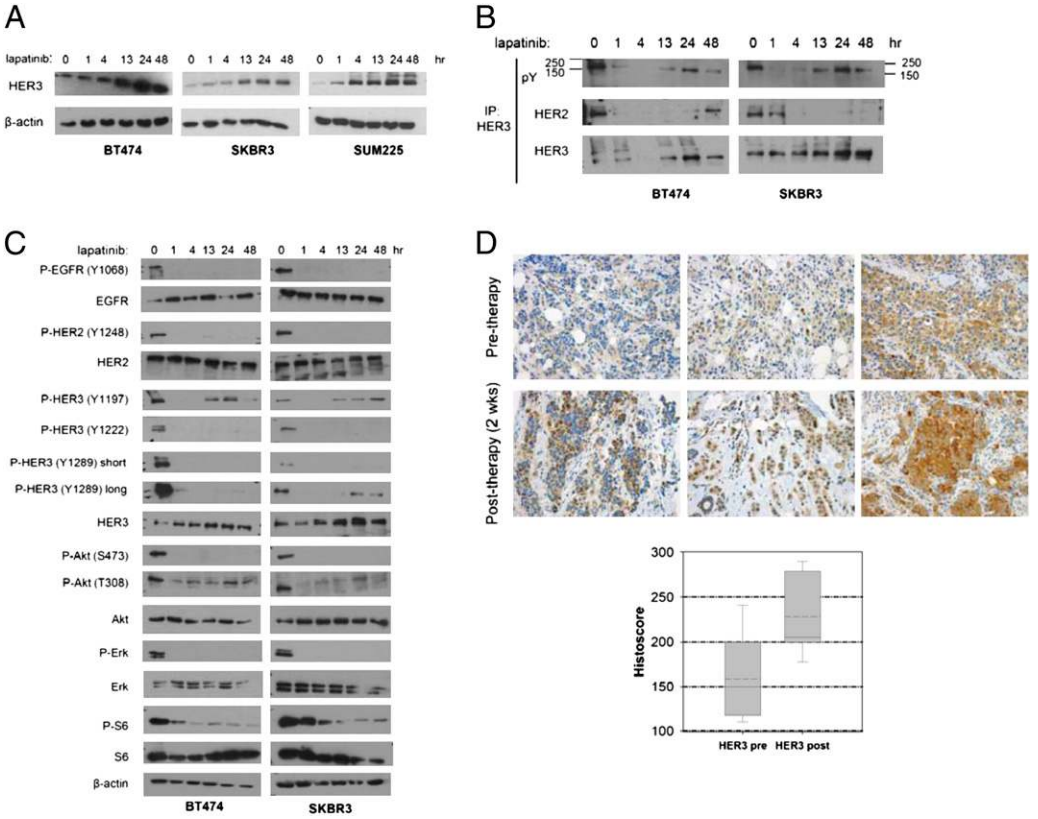

Fig. 1. Inhibition of HER2 results in feedback up-regulation of active HER3 and downstream signaling. (*A*) BT474, SKBR3, and SUM225 cells were treated with 1 μM lapatinib for the times indicated. Lapatinib and medium were replenished at 24 h. Whole cell lysates were prepared as described in *Materials and Methods* and separated in a 7% SDS gel, followed by immunoblot with the indicated antibodies. (*B*) Lysates from BT474 and SKBR3 cells treated with lapatinib for 1–48 h were precipitated with a HER3 antibody followed by immunoblot with P-Tyr, HER2, and HER3 antibodies. Molecular masses (in kDa) are indicated to the left of the P-Tyr immunoblot. (*C*) The same cell lysates from *B* were subjected to SDS/PAGE followed by immunoblot with the indicated antibodies. (*D*) Representative matched pre- and posttherapy sections of formalin-fixed core biopsies from HER2+ tumors treated for 2 wk with lapatinib and subjected to HER3 IHC. Box plots showing the expression at baseline and after 2 wk of treatment in paired tumor samples ($n = 8$) from a tissue microarray. Boxes indicate 90% of values. Dashed lines indicate mean values, and solid lines indicate median value. External lines indicate the complete range. Increased HER3 staining ($P = 0.038$) was observed in the posttreatment tumor sections compared with the pretreatment tumor sections.

sponse to therapy, HER2-overexpressing cells up-regulate HER3 protein levels and partially maintain PI3K/Akt activity.

Up-Regulation of HER3 Is Dependent on PI3K/Akt and FoxO3a. We next determined HER3 RNA levels with and without lapatinib. By real-time quantitative PCR (qPCR) both BT474 and SKBR3 cells showed marked up-regulation of HER3 mRNA beginning at 4 and 24 h after the addition of lapatinib (Fig. 2*A*), consistent with the increase in HER3 mRNA seen in BT474 xenografts (Fig. S1*B*). In addition siRNA-mediated knockdown of HER2 in BT474 and SKBR3 cells increased HER3 mRNA levels (Fig. S2). These data suggest that signaling downstream of HER2 represses transcription of HER3. Hence, inhibition of HER2 kinase activity would derepress HER3 transcription resulting in up-regulation of HER3 mRNA.

Treatment with BEZ235, a dual inhibitor of PI3K and mTOR (Fig. 2 *A* and *B*), or with 5J8, an allosteric inhibitor of Akt1/2 (Fig. S3 *A* and *B*), up-regulated HER3 mRNA and protein levels compared with controls. Knockdown of Akt1, Akt2, and Akt3 siRNA also resulted in up-regulation of HER3 mRNA (Fig. S3 *C* and *D*). Additionally, transfection of a plasmid containing constitutively active myristoylated (Myr) Akt into BT474 and SKBR3 cells reduced steady-state HER3 mRNA levels (0.76 and 0.83, respectively) compared with cells transfected with a control plasmid. In cells treated with lapatinib, Myr-Akt reduced HER3 mRNA levels >50% compared with controls (Fig. 2*C*). Myr-Akt also reduced lapatinib-induced up-regulation of total HER3 and P-HER3 in BT474 cells at 24 h. In SKBR3 cells, Myr-Akt by itself down-regulated HER3 and, in doing so, also reduced the levels of HER3 and P-HER3 following treatment with the TKI (Fig. S4*A*). Furthermore, transfection of Myr-Akt counteracted lapatinib-induced growth inhibition (Fig. S4*B*). Taken together, these data suggest that Akt represses transcription of HER3.

The inhibition of HER2 and PI3K/Akt in BT474 cells with lapatinib derepresses the transcription factor FoxO3a (16). Akt regulates the subcellular localization of FoxO3a by phosphorylation, thereby preventing FoxO3a from translocating to the nucleus and regulating transcription (17). There was an increase of nuclear FoxO3a at 1 and 4 h after addition of lapatinib (Fig. 3*A* and Fig. S5*A*), which was corroborated by immunoblot analysis of cytosolic and nuclear fractions of BT474 and SKBR3 cells (Fig. S5*B*). Increased FoxO3a mRNA levels upon treatment with lapatinib have been shown (18). Based on genomic sequence analysis of the 5 kb upstream of the 5′ *HER3* gene, there are three ATAAACA putative FoxO3a binding sites at −4915, −3297, and −2509 relative to the HER3 transcriptional start site. Thus, to determine whether FoxO3a is involved in lapatinib-induced increase in HER3 transcription, we transfected BT474 cells with FoxO3a or control siRNAs and examined HER3 mRNA levels by qPCR. Down-regulation of FoxO3a markedly reduced both basal and lapatinib-induced increase in HER3 RNA levels in BT474 cells (Fig. 3*B*). To determine FoxO protein binding to the HER3 promoter, we conducted chromatin immunoprecipitation (ChIP) with FoxO antibodies in cells with

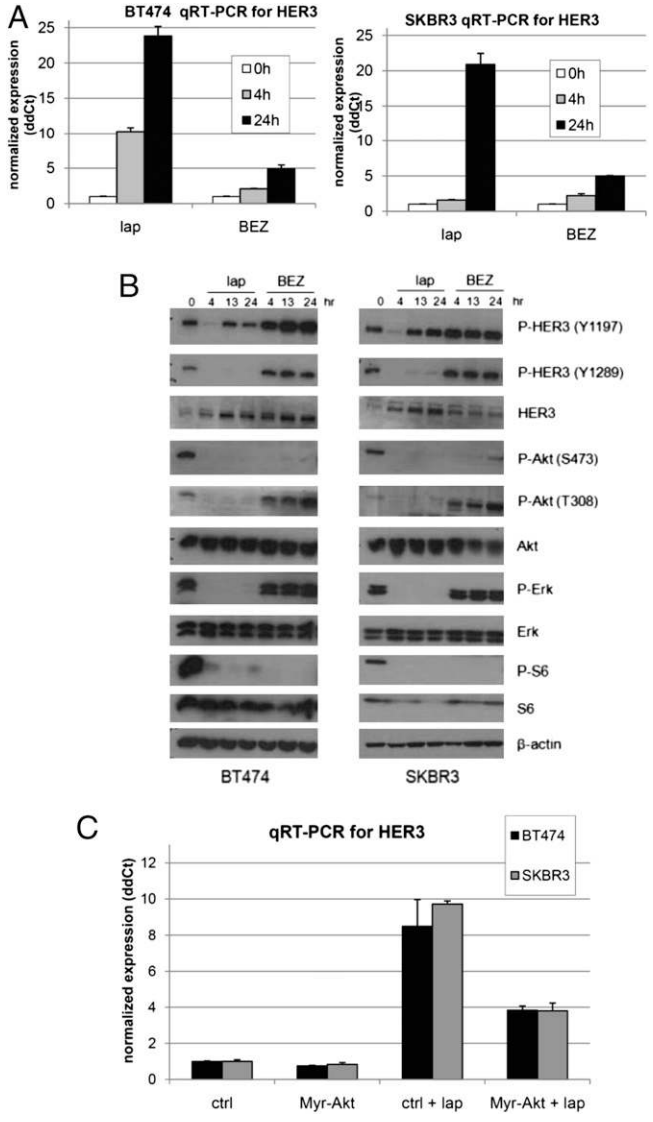

Fig. 2. Inhibition of HER2 and PI3K/Akt up-regulates HER3 transcription. (*A*) BT474 and SKBR3 cells were treated with lapatinib or BEZ235 over a time course. Total RNA was extracted and subjected to real-time qPCR for HER3 as described in *Materials and Methods*. Data were normalized to untreated cells. (*B*) Cells were treated with lapatinib or BEZ235 for the indicated times. Whole cell lysates were prepared and separated by 7% SDS/PAGE, followed by immunoblot with the indicated antibodies. (*C*) Cells were transiently transfected with a vector encoding myristoylated Akt (Myr-Akt) or an empty vector (ctrl) overnight followed by the addition of DMSO or lapatinib. After 24 h, cells were harvested, and HER3 RNA was quantitated by qPCR as indicated in *Materials and Methods*. Each bar represents the mean ± SEM of normalized HER3 expression for each treatment group (n = 3). y axis scale indicates $\Delta\Delta C_T$.

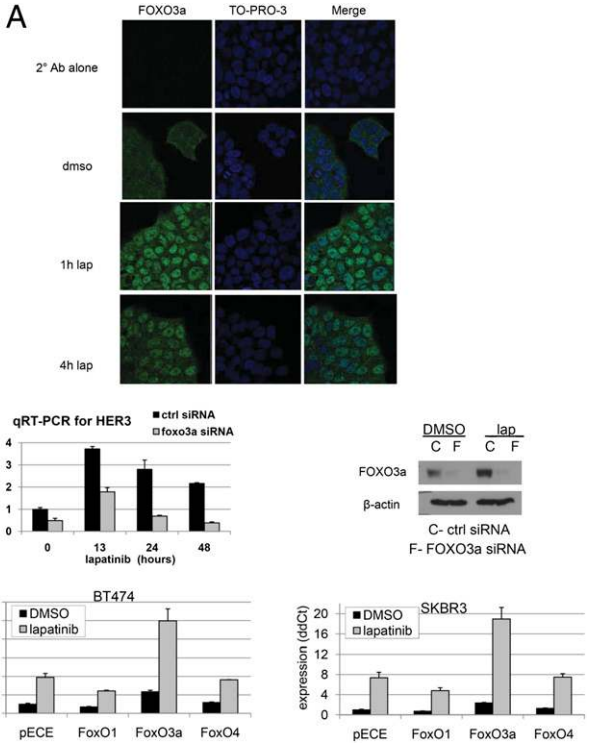

Fig. 3. Increased nuclear FoxO3a upon inhibition of HER2 modulates HER3 RNA. (*A*) Immunofluorescence showing enhanced nuclear FOXO3a in lapatinib-treated BT474 cells. (*B Left*) BT474 cells were transfected with siRNA oligonucleotides targeting FoxO3a or a control sequence (ctrl siRNA). The next day, the cells were changed to medium with lapatinib or medium alone. Fresh medium and lapatinib were replenished at 24 h. Cells were harvested at the indicated times and RNA extracted and subjected to real-time qPCR for HER3. Data were normalized to control cells. (*Right*) FoxO3a immunoblot of lysates from BT474 cells 48 h after transfection with control or FoxO3a siRNA and 24 h after the addition of lapatinib or DMSO. (*C*) BT474 (*Left*) and SKBR3 (*Right*) cells were transfected with vectors encoding FoxO3a, FoxO1, FoxO4, or empty vector (pECE) overnight followed by the addition of DMSO or lapatinib for 24 h. Each bar represents the mean ± SEM of normalized HER3 expression for each vector (n = 3). y axis scale is different for both cell lines and indicates $\Delta\Delta C_T$.

lapatinib and cells without lapatinib. We PCR-amplified the DNA contained in FoxO pulldowns using primers specific to putative FoxO binding sites in the HER3 promoter. In both cells, there was an increase in the PCR product using all three HER3 promoter primers upon treatment with lapatinib. IgG pulldowns were used as controls (Fig. S5*C*).

To examine whether this effect on HER3 transcription was specific to FoxO3a, we transiently transfected vectors encoding the empty vector pECE, FoxO1, FoxO3a, and FoxO4. Because FoxO1 and FoxO4 are Myc-tagged and a FoxO4 antibody was unavailable, we used FoxO3a, FoxO1, and Myc antibodies to confirm transfection efficiency (Fig. S5*D*). Only FoxO3a up-regulated HER3 RNA in both cell lines. Treatment with lapatinib increased HER3 RNA levels in cells transfected with empty vector alone (pECE). However, the combination of lapatinib and FoxO3a, but not with FoxO1 and FoxO4, increased HER3 levels 10-fold and >19-fold in BT474 and SKBR3 cells, respectively (Fig. 3*C*).

Genetic and Pharmacological Inhibition of HER3 Sensitizes to Lapatinib. The compensatory up-regulation of total HER3 and partial maintenance of P-HER3 and P-Akt upon inhibition of HER2 suggested that combined inhibition of HER2 and HER3 would synergistically inhibit tumor cell viability. Therefore, we transfected BT474 and SKBR3 cells with HER3 or mismatch siRNA, treated them with lapatinib, and assessed their viability as measured by TUNEL. HER3 knockdown alone did not induce apoptosis. However, it increased at least threefold the proportion of apoptotic BT-474 and SKBR-3 cells induced by treatment with lapatinib (Fig. 4*A*). HER3 levels were undetectable 2 d after transfection. In BT474 cells, the combination of lapatinib and HER3 siRNA inhibited P-HER3, P-Akt, and P-Erk better than each alone (Fig. 4*B*). Similar results were obtained in BT474 cells growing in 3D Matrigel and in cells plated in monolayer (Fig. S6 *A* and *B*).

To study the applicability of these results, we next tested the combination of lapatinib with a HER3 neutralizing monoclonal antibody currently in clinical development. AMG-888 binds to

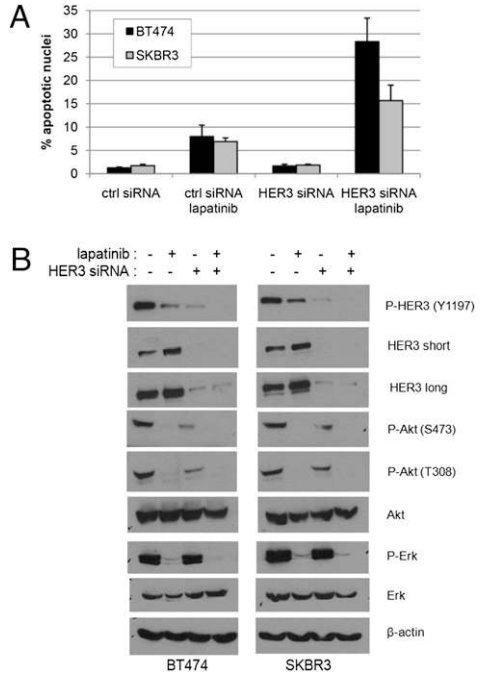

Fig. 4. Genetic inhibition of HER3 sensitizes HER2+ tumor cells to lapatinib. (*A*) BT474 and SKBR3 cells were seeded in six-well plates in 10% FCS and transfected by siRNA oligonucleotides targeting HER3 or a control sequence (ctrl siRNA). The next day, cells were changed to serum-free medium with or without lapatinib. Adherent and floating cells were collected 48 h later and subjected to TUNEL assay as described in *Materials and Methods*. Each bar represents the mean ± SEM of cells with apoptotic nuclei for each treatment group ($n = 3$). (*B*) Immunoblot of lysates from BT474 and SKBR3 cells 2 d after transfection with either control or HER3 siRNA.

mouse and human HER3, blocks heregulin-induced HER3 phosphorylation, reduces HER3 levels at the cell surface, and inhibits growth of lung, breast, and pancreatic xenografts in nude mice (19, 20). Treatment with AMG-888 dampened the early recovery of Y1197 P-HER3 in lapatinib-treated SKBR3 and of Y1197 and Y1289 P-HER3 and S473 P-Akt in MDA453 cells and enhanced lapatinib-induced apoptosis in BT474, SKBR3, and MDA453 cells (Fig. S6 *C* and *D*). In BT474 and MDA453 cells grown in 3D Matrigel, acini formation was not significantly affected by AMG-888 alone, whereas cells treated with the combination of lapatinib and AMG-888 displayed a statistically significant reduction in acini area compared with cells treated with lapatinib and control IgG_1 (Fig. S6*E*). These data suggest that, although pharmacologic inhibition of HER3 may not be an effective single agent therapy in HER2+ breast cancer, blockade of HER3 at the cell surface using an anti-HER3 antibody might be an effective approach to optimize the antitumor action of the HER2 antagonist.

Pharmacological Inhibition of HER3 Sensitizes to Lapatinib in Vivo. We tested whether the addition of AMG-888 would sensitize BT474 xenografts to lapatinib. Mice bearing established BT474 xenografts were randomized to therapy with vehicle, lapatinib, AMG-888, or the combination of both drugs for 28 d. AMG-888 as a single agent had no activity compared with control mice. Lapatinib inhibited growth of established BT474 xenografts. Tumors treated with the combination did not grow during treatment and exhibited a statistical reduction in volume compared with the lapatinib and control arms starting at 3 wk of therapy (Fig. 5*A*). This result was confirmed in a second experiment (Fig. S7*A*). There was no apparent drug-related toxicity in any of the treatment arms.

Finally, we examined pharmacodynamic biomarkers of target inactivation after 28 d of treatment. Partial down-regulation of Y1221/2 P-HER2 was observed in tumors treated with lapatinib and AMG-888 and with lapatinib alone compared with controls (Fig. S7*B*). Treatment with the combination, but not with lapatinib alone, reduced the intensity of total HER3 staining as well as detectable levels of Y1289 P-HER3 at the tumor cell membrane (Fig. 5*B*). This reduction in HER3 and P-HER3 correlates with the ability of AMG-888 to down-regulate HER3 from the cell surface (20). In addition, there was a statistical decrease in S473 P-Akt staining and an increase in nuclear FoxO3a staining in tumors treated with the combination compared with vehicle-treated controls. Furthermore, treatment with the combination induced a more potent anti-proliferative effect compared with the other treatment arms as measured by BrdU incorporation (Fig. S7*B*).

Recovery of P-HER3 Is Dependent on HER2. Immunoprecipitation with a P-Tyr antibody followed by immunoblot for HER2 and HER3 revealed robust phosphorylation of HER2 and HER3 at baseline. Treatment with 1 μM lapatinib promptly eliminated detectable recovery of phosphorylated HER2 and HER3. However, there was recovery of HER3 but not HER2 at 13 and 24 h of lapatinib treatment in the P-Tyr pulldowns from BT474 and SKBR3 cells (Fig. 6*A*), suggesting the engagement of an-

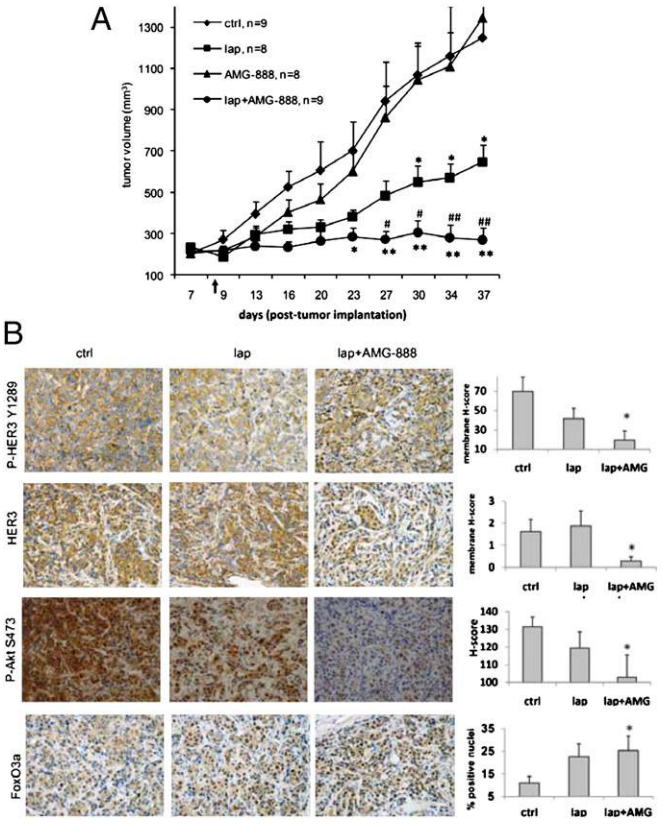

Fig. 5. Inhibition of HER3 sensitizes cells to lapatinib in vivo. (*A*) Athymic mice were injected with BT474 cells and treated with vehicle, lapatinib, AMG-888, or the combination as indicated in *Materials and Methods*. Treatment was administered for 4 wk. Tumors were measured overtime with calipers. Each data point represents the mean tumor volume ± SEM ($n = 8$–9). Black arrow indicates start of when treatment. $*P < 0.05$, $**P < 0.01$ vs. control, $\#P < 0.05$, $\#\#P < 0.01$ vs. lapatinib. (*B*) IHC analysis of Y1289 P-HER3, total HER3, FoxO3a, and S473 P-Akt in tumor sections. (*Left*) Representative images from control tumors, lapatinib-treated tumors, and lapatinib-and-AMG-888-treated tumors. (*Right*) Quantitative comparison of membrane histoscore (P-HER3 and HER3), histoscore (P-Akt), or percent FoxO3a-positive nuclei. $*P \leq 0.05$ compared with control by Student *t* test.

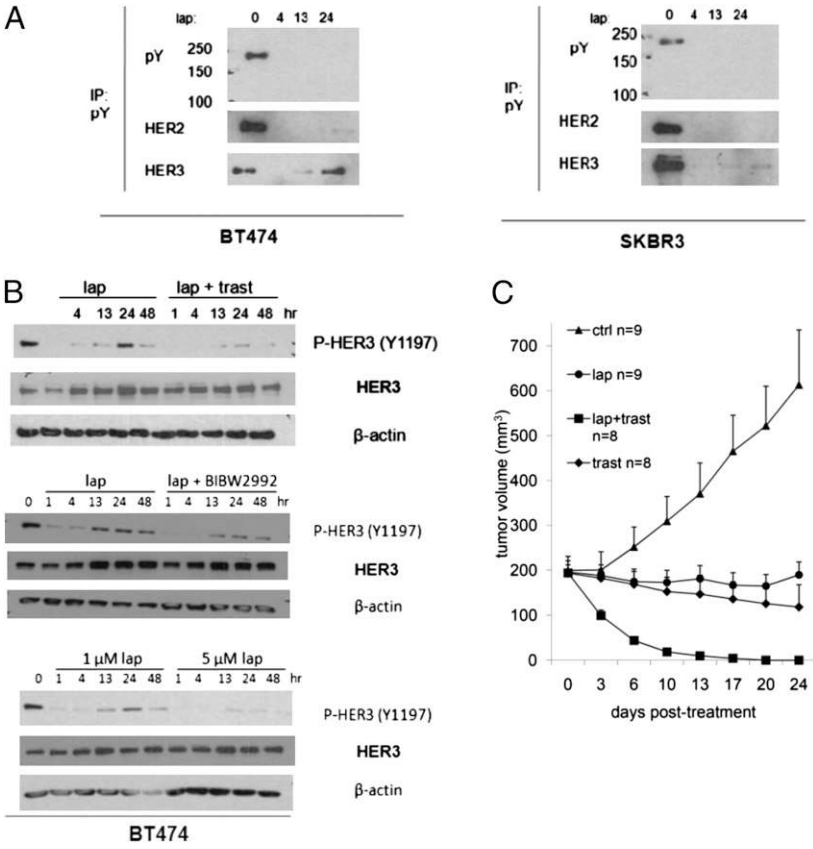

Fig. 6. Recovery of P-HER3 is dependent on HER2. (*A*) BT474 and SKBR3 cells were treated with lapatinib for the times indicated. Cell lysates were precipitated with a P-Tyr antibody followed by P-Tyr, HER2, and HER3 immunoblot. Molecular weights are indicated to the left of P-Tyr immunoblot. (*B*) Cells were treated with lapatinib ± trastuzumab or BIBW2992. (*Lower*) 1 and 5 μM lapatinib were compared side-by-side over 1–48 h. Drugs and fresh medium were replenished every 24 h. Whole cell lysates were subjected to immunoblot analysis with Y1197 P-HER3, HER3, and β-actin antibodies. (*C*) Athymic mice were injected with BT474 cells treated with vehicle, lapatinib, trastuzumab, or the combination as indicated in Methods. Treatment was administered for 24 d. Each data point represents the mean tumor volume ± SEM (n = 8–9).

other tyrosine kinase, which, in turn, may phosphorylate HER3. Therefore, by using small-molecule inhibitors, we examined tyrosine kinases other than HER2 that can potentially maintain P-HER3 upon treatment with lapatinib. Kinases that have been implicated in the phosphorylation of HER3 include HER2, EGFR, Src (21), MET (22), and FGFR2 (23). We added SU11274, dasatinib, gefitinib, and SU5402, TKIs of MET, Src, EGFR, and FGFR, respectively, to lapatinib-treated cells. None of these small molecules blocked the recovery of HER3 phosphorylation as measured by immunoblot with the Y1197 P-HER3 antibody (Fig. S8*A*).

We next considered the possibility of incomplete inhibition of the HER2 kinase after treatment with lapatinib to explain the recovery of HER3 phosphorylation. To test this, we used the HER2 antibodies trastuzumab and pertuzumab. Trastuzumab blocks the phosphorylation of HER3 that results from ligand-independent HER2–HER3 interactions, whereas pertuzumab inhibits phosphorylation of HER3 that follows ligand-induced HER2–HER3 dimerization. We also used the irreversible EGFR/HER2 TKI BIBW-2992, a small-molecule inhibitor that covalently binds Cys^{805} in HER2 and Cys^{773} in EGFR. Because lapatinib, a reversible ATP-competitive TKI, and BIBW2992 bind different conformations of the HER2 receptor, we speculated that a combination of TKIs at pharmacologically achievable concentrations may result in a more complete inhibition of HER2 and thus abrogate the up-regulation of P-HER3.

The addition of trastuzumab or BIBW2992 but not pertuzumab to lapatinib-treated cells markedly dampened the recovery of Y1197 P-HER3 in both cell lines (Fig. 6*B* and Fig. S8*B*). Of note, treatment with trastuzumab did not have an effect on P-Akt, P-Erk, or P-S6 or affected HER3 RNA levels (Fig. S9 *A* and *B*). We propose that the effect of trastuzumab on dampening recovery of P-HER3 in lapatinib-treated cells is due to its reported ability to disrupt HER2-HER3 dimers (8). Compared with 1 μM lapatinib, a suprapharmacological dose of 5 μM also abrogated recovery of P-HER3 (Fig. 6*B*). Furthermore, in mice bearing established BT474 xenografts, the combination of lapatinib and trastuzumab completely eliminated eight of eight tumors after <3 wk of treatment, whereas single agent lapatinib or trastuzumab did not induce complete tumor regression (Fig. 6*C*). These results are consistent with the possibility that the recovery of HER3 phosphorylation depends on a trastuzumab-sensitive ligand-independent HER2–HER3 interaction and that a more complete inhibition of the HER2 tyrosine kinase is required to completely eliminate the recovery of HER3 phosphorylation and maximize the antitumor action of HER2 inhibitors.

Discussion

We hypothesized that sustained and complete inhibition of HER3 and its output to PI3K/Akt is required for the maximal antitumor effect of HER2 inhibitors. We demonstrate herein that inhibition of the HER2 kinase with lapatinib results in time-dependent upregulation of the HER3 protein using both breast cancer cell lines (Fig. 1*A*) and core biopsies of HER2+ tumors from patients (Fig. 1*D*). These results imply HER3 expression is under negative regulation by signaling downstream of the HER2 network. Indeed, inhibition of PI3K/Akt resulted in upregulation of HER3 transcription (Fig. 2 and Fig. S3). These results are consistent with the observation of heregulin-induced downregulation of HER3 mRNA in ovarian cancer cells with low HER2 levels. In this report, downregulation of HER3 mRNA was blocked by pertuzumab and by the PI3K inhibitor GDC-0941 (24). In addition, we identified a role of FoxO3a, a downstream target of Akt, in the regulation of HER3 expression. Knockdown of FoxO3a reduced levels of both basal and lapatinib-induced increase in HER3 RNA, and transient transfection of FoxO3a, but not FoxO1 or FoxO4, increased HER3 RNA levels.

We hypothesized that by blocking the compensatory reengagement of HER3, combined targeting of HER2 and HER3 will be synergistic against HER2-dependent tumors. Indeed, RNAi-mediated knockdown of HER3 or treatment with the HER3 neutralizing antibody AMG-888 sensitized HER2+ cells to lapa-

tinib-induced apoptosis (Fig. 4 and Fig. S6). Similar results were observed with pharmacological inhibitors of HER3 in vivo. The combination of AMG-888 and lapatinib was markedly more effective than lapatinib alone at inhibiting P-HER3 and HER3 levels, P-Akt, the cytosolic retention of FoxO3a, and xenograft growth (Fig. 5 and Fig. S7). Because the maximally tolerated doses of lapatinib used did not completely inhibit P-HER2 (Fig. S7*B*), we speculate that the residual HER2 kinase activity was enough to maintain HER3 phosphorylation. By downregulating HER3 levels below a necessary threshold, AMG-888 enhanced the inhibition of PI3K induced by lapatinib as supported by the greater reduction in P-Akt and larger increase in nuclear FoxO3a in tumors treated with the combination (Fig. 5*B*).

The reemergence of P-HER3 in lapatinib-treated cells/tumors also suggested that, in addition to HER2, other tyrosine kinases present in the HER2+ cells can also phosphorylate HER3. Inhibitors to kinases implicated in transactivation of HER3 such as EGFR, Met, Src, and FGFR did not block recovery of P-HER3 (Fig. S8). Shi et al. recently demonstrated HER3 has some residual kinase activity, albeit at approximately 1000-fold less activity compared to active EGFR (25). We therefore cannot discount the possibility that HER3 autophosphorylation could play a role in the recovery of phosphorylated HER3 upon lapatinib treatment. The irreversible, covalent HER2 inhibitor BIBW2992 and trastuzumab but not pertuzumab blocked HER3 phosphorylation in lapatinib-treated cells. Similar results were obtained using a supraphamacological concentration of lapatinib (Fig. 6*B* and Fig. S8*B*). Moreover, the combination of lapatinib and trastuzumab resulted in complete tumor regressions in vivo (Fig. 6*C*). These results suggest that the rescue of HER3 phosphorylation depends on a ligand-independent interaction between HER2 and HER3. The inability of trastuzumab to potently inhibit PI3K/Akt and, in turn, derepress transcription HER3 mRNA (Fig. S9) underscores a possible advantage of trastuzumab over lapatinib as it applies to induction of compensatory feedback. On the other hand, the complementary mechanisms of action of both HER2 antagonists strongly support their use together: by trastuzumab blocking recovery of P-HER3 in lapatinib-treated cells, the combination disables signaling by the HER2 network better that each drug alone. In summary, this study points to cellular mechanisms of compensation that limit the antitumor effect of HER2 antagonists potentially leading to drug-resistant cancers. They provide a preclinical rationale and basis for the evaluation of HER3 inhibitors in combination with anti-HER2 and anti-PI3K therapies early in the natural history of HER2- and PI3K-dependent cancers.

Materials and Methods

Cells, Plasmids, and Reagents. All cells were from the American Type Culture Collection (ATCC). Cell culture and plasmid transfection were carried out according to standard procedures (details provided in *SI Materials and Methods*).

Immunoprecipitation, Immunoblot, and Immunofluorescence. Immunoprecipitation, immunoblot, and immunofluorescence analysis were performed using standard protocols (details provided in *SI Materials and Methods*).

Human Tumor Biopsies and Immunohistochemistry. After informed consent, patients with newly diagnosed HER2-overexpressing breast cancer were enrolled in an IRB-approved trial at Baylor College of Medicine. Lapatinib was administered at 1,500 mg/day p.o. for 6 wk. Core biopsies were obtained at baseline before treatment and after 14 d of therapy. Specific details on HER3 IHC staining are provided in *SI Materials and Methods*.

Transfection of siRNA, ChIP, and RT-PCR. Cell transfection, ChIP, and RT-PCR were performed using standard protocols (details provided in *SI Materials and Methods*).

3D Growth and Terminal TUNEL Assays. These experiments are detailed in *SI Materials and Methods*.

Studies with Xenografts. All mouse experiments used in this study were approved by the Institutional Animal Care Committee of Vanderbilt University. These experiments are detailed in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work was supported by R01 Grants CA80195 (to C.L.A.), CA121210 (to W.P.), and K25 CA127349 (to H.C.M.), American Cancer Society (ACS) Clinical Research Professorship Grant CRP-07-234 (to C.L.A.), The Lee Jeans Translational Breast Cancer Research Program (C.L.A.), Breast Cancer Specialized Program of Research Excellence (SPORE) P50 CA98131, National Cancer Institute Small Animal Imaging Resource Program (SAIRP) U24 CA126588, and Vanderbilt-Ingram Cancer Center Support Grant P30 CA68485. J.T.G. is a postdoctoral research fellow partially supported by Grant T32DK007563 and ACS 118813-PF-10-070-01-TBG and DOD BC093376 postdoctoral fellowship awards. This research was also supported by Stand Up to Cancer/American Association from Cancer Research Dream Team Translational Cancer Research Grant SU2C-AACR-DT0209.

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