Sustained inhibition of HER3 and EGFR is necessary to induce regression of HER2-amplified gastrointestinal carcinomas

Simonetta M. Leto¹,², Francesco Sassi², Irene Catalano¹,², Valter Torri³, Giorgia Migliardi¹,², Eugenia R. Zanella¹,², Mark Throsby⁴, Andrea Bertotti¹,²,⁵ #, and Livio Trusolino¹,² #

¹Department of Oncology, University of Turin Medical School, 10060 Candiolo, Turin, Italy
²Laboratory of Translational Cancer Medicine, Candiolo Cancer Institute – FPO IRCCS, 10060 Candiolo, Turin, Italy
³Laboratory of Methodology for Biomedical Research, Oncology Department, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, 20156 Milan, Italy
⁴Merus B.V., 3584 CH Utrecht, The Netherlands
⁵Istituto Nazionale di Biostrutture e Biosistemi, INBB, 00136 Rome, Italy

# Andrea Bertotti and Livio Trusolino contributed equally to this work as senior authors.

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Correspondence: Andrea Bertotti or Livio Trusolino; Laboratory of Translational Cancer Medicine, Candiolo Cancer Institute, Strada Provinciale 142, km 3.95, 10060 Candiolo (Torino), Italy; e-mails: andrea.bertotti@unito.it; livio.trusolino@unito.it; phone: +39 011 993.32.02; fax: +39 011 993.32.25

Running title: HER3-EGFR blockade in HER2-amplified gastrointestinal cancer.
TRANSLATIONAL RELEVANCE

About 20% of gastric cancers and 5% of KRAS wild-type colorectal carcinomas harbor HER2 amplification. Similar to evidence in mammary tumors, initial preclinical investigation has shown that HER2-amplified gastrointestinal (GI) carcinomas are more effectively contrasted by dual HER2 blockade with antibody-small molecule combinations than by single-agent therapy; however, a mechanistic appraisal of how such treatments impact HER2 signaling in GI cancer is lacking. We found that the major differential outcome of combination therapy with trastuzumab and lapatinib compared with either inhibitor was prolonged dephosphorylation of HER3 and EGFR, which resulted in more powerful and sustained neutralization of downstream signals. These effects were essentially mirrored by irreversible EGFR/HER2 inhibition by afatinib, which, alone, was sufficient to induce tumor shrinkage in vivo. These findings point to HER3 and EGFR as crucial co-extinction targets in HER2-amplified GI tumors and propose monotherapy with irreversible HER2 inhibitors as a viable alternative to dual HER2 blockade.

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ABSTRACT

Purpose: Preclinical studies in HER2-amplified gastrointestinal cancer models have shown that co-targeting HER2 with a monoclonal antibody and a small molecule is superior to monotherapy with either inhibitor, but the underlying cooperative mechanisms remain unexplored. We investigated the molecular underpinnings of this synergy to identify key vulnerabilities susceptible to alternative therapeutic opportunities.

Experimental Design: The phosphorylation/activation of HER2, HER3, EGFR (HERs) and downstream transducers was evaluated in HER2-overexpressing colorectal and gastric cancer cell lines by Western blotting and/or multiplex phosphoproteomics. The in vivo outcome of antibody-mediated HER2 blockade by trastuzumab, reversible HER2 inhibition by lapatinib, and irreversible HER2 inhibition by afatinib was assessed in patient-derived tumorgrafts and cell-line xenografts by monitoring tumor growth curves and by using antibody-based proximity assays.

Results: Trastuzumab monotherapy reduced HER3 phosphorylation, with minor consequences on downstream transducers. Lapatinib alone acutely inhibited all HER receptors and effectors but led to delayed rephosphorylation of HER3 and EGFR and partial restoration of ERK and AKT activity. When combined with lapatinib, trastuzumab prevented HER3/EGFR reactivation and caused prolonged inhibition of ERK/AKT. Afatinib alone was also very effective in counteracting the reinstatement of HER3, EGFR, and downstream signaling activation. In vivo, the combination of trastuzumab and lapatinib – or, importantly, monotherapy with afatinib – resulted in overt tumor shrinkage.

Conclusions: Only prolonged inhibition of HER3 and EGFR, achievable by dual blockade with trastuzumab and lapatinib or irreversible HER2 inhibition by single-agent afatinib, led to regression of HER2-amplified gastrointestinal carcinomas.

Keywords: Patient-derived Xenografts; Colorectal Cancer; Gastric Cancer; HER2/ERBB2; Combination Therapies.
INTRODUCTION

Amplification of the *HER2/ERBB2* gene leads to overexpression and constitutive activation of the encoded tyrosine kinase receptor in a subset of breast, gastric, and colorectal carcinomas (1). The arsenal of HER2 antagonists includes clinically approved monoclonal antibodies (trastuzumab, pertuzumab) and reversible ATP-mimetics (lapatinib) (2). Irreversible inhibitors, such as afatinib and neratinib, have received preclinical attention and are now being evaluated in clinical trials (3). In mammary tumors, trastuzumab is recommended in the adjuvant setting, following potentially curative surgical treatment (4). In the context of combination therapy, HER2 inhibition by trastuzumab and lapatinib produces higher rates of pathological complete response (in the neoadjuvant setting) and increased progression-free and overall survival (in the metastatic setting) compared with lapatinib alone (5, 6).

Results in HER2-positive gastric cancer (GC) are less satisfactory (7-9). Although trastuzumab has been approved for GC treatment because it has been shown to provide statistically significant advantage when added to standard-of-care chemotherapy (10), the margins of benefit remain limited (9); on a worse note, the combination of lapatinib with cytotoxics failed to determine any survival improvement (11). One reason for these shortcomings can be ascribed to the adoption of loose criteria for patient selection, with inclusion of cases harboring low or heterogeneous *HER2* copy number gains that likely diluted the enrichment for potential responders (12). This notwithstanding, the modest or null superiority of trastuzumab and lapatinib, respectively, over the chemotherapy backbone suggests that more powerful neutralization of HER2 signals by combinatorial or alternative approaches may be warranted to improve response (13). Indeed, preliminary evidence in *HER2*-amplified gastric cancer cell lines indicates that the association of trastuzumab and lapatinib exerts greater antitumor efficacy than either drug alone (14). We and others have recently demonstrated that *HER2* amplification is also the hallmark of a subset (5-10%) of metastatic colorectal carcinomas (mCRCs) refractory to EGFR blockade (15-17). Preclinical findings suggest that anti-HER2 monotherapy may be poorly effective also in this tumor setting (15).

The rationale for dual HER2 blockade in gastrointestinal (GI) carcinomas is rooted in previous experience with breast cancer, but remains substantially empirical. Here, we examine the signaling and biological consequences of trastuzumab and lapatinib treatments (alone and in combination) to identify key liabilities that, once inhibited, induce manifest tumor regression.
MATERIALS AND METHODS

Cell cultures, reagents, vectors, and viral infection
NCI-H508, NCI-N87, and BT-474 cells were purchased from ATCC and cultured in RPMI. OE-19 were purchased from Sigma and cultured in RPMI. DiFi cells (from J. Baselga, Memorial Sloan-Kettering Cancer Center, New York, NY) were cultured in F12. HDC-142, originally described in ref 18, were a gift from A. Bardelli (Candiolo Cancer Institute, Candiolo, Torino, Italy) and were cultured in DMEM/F12. The genetic identity of cell lines was authenticated by short tandem repeat profiling (Cell ID, Promega). Primary antibodies included: mouse anti-phosphotyrosine, mouse anti-HER3 (Millipore); rabbit anti-phospho-Tyr1068-EGFR (Abcam); rabbit anti-EGFR, rabbit anti-phospho-Tyr1248-HER2, rabbit anti-phospho-Tyr1289-HER3, rabbit anti-phospho-Ser473-AKT, rabbit anti-AKT, rabbit anti-phospho-Thr202/Tyr204-ERK, rabbit anti-ERK (Cell Signaling Technology); mouse anti-HER2, goat anti-actin (Santa Cruz); mouse anti-vinculin (Sigma Aldrich). The wild-type HER2 cDNA was subcloned into the pLVX-IRES-puro lentiviral vector (PT4063-5, Clontech). The HER3 lentiviral pLKO.1-puro shRNA vectors (for NCI-N87) and pLKO.1-neo shRNA vectors (for NCI-H508-HER2), as well as the non-targeting control vector, were purchased from Sigma (target sequences: shRNA_1-pLKO.1-puro and shRNA_1-pLKO.1-neo,
CCGGAGGTAGGTAGATATTGACTCGAGTCATATCTACTCTAACCTCTTTTTG; shRNA_2-pLKO.1-puro,
CCGGCCTTCATGTTGACTATAACTCGAGTTACACACGAGTTTTTTG; shRNA_2-pLKO.1-neo,
CCGGAAATCTCTACTCTACCATTGCTCGAGCAATGGTAGAGTAGAGAACTCTCTTTTTG). Lentiviral particles were produced by LipofectAMINE 2000 (Invitrogen)-mediated transfection of 293T cells. HER2-transduced cells were kept in culture in the presence of 20 µg/mL cetuximab to avoid the potential emergence of HER2-negative subclones.

Biological assays
Short-term proliferative responses were assessed with an ATP content assay as previously reported (19). For long-term proliferation assays, cells were seeded in 24-well plates (2 x10^4 cells per well), cultured in the absence or presence of 20 µg/mL cetuximab for 15 days, fixed, stained with crystal violet, and photographed. For soft agar assays, 10,000 cells were resuspended in complete medium containing 0.5% SeaPlaque agar and seeded onto 24-well plates containing a 1% agar underlay. Colonies were stained by incorporation of tetrazolium salts two weeks after seeding and quantitated using ImageJ. Lapatinib and trastuzumab were given at the indicated concentrations once weekly.
Immunoprecipitation and immunoblot analysis
Proteins were extracted with cold EB buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 5 mM EGTA) in the presence of phosphatase and protease inhibitors. HER2 immunoprecipitation was performed by incubating protein extracts with trastuzumab (Roche) and Protein A sepharose beads (GE Healthcare) for 1 hour at 4°C. Immunoprecipitated or total proteins were electrophoresed on precasted SDS-polyacrylamide gels (BioRad) and transferred onto nitrocellulose membranes (GE Healthcare). Nitrocellulose-bound antibodies were detected by the enhanced chemiluminescence system (Promega).

Phosphoproteomics
Phospho-ERK (Thr202/Tyr204; Thr185/Tyr187), phospho-p70S6K (Thr421/Ser424), phospho-GSK-3β (Ser9), and phospho-Akt (Ser473) were measured by Meso Scale Discovery (MSD) assays (kits K15115D and K15107D). MSD is a solid-phase multiarray technology in which multiple capture antibodies are immobilized onto single microplate wells. After incubation with protein extracts, detection is performed by quantitative electrochemiluminescence with reporter antibodies coupled with SULFO-TAG, an amine-reactive, N-hydroxysuccinimide ester that emits light upon electrochemical stimulation. HER2-HER3 heterodimers, total HER3, and phospho-HER3 (Tyr1289) were measured in formalin-fixed, paraffin-embedded specimens from end-of-treatment patient-derived tumourgrafts using a fluorescent antibody-based proximity assay (VeraTag, Monogram Biosciences) (20-22).

Patient-derived tumourgrafts and cell-line xenografts
Tumor implantation and expansion were performed as previously described (15, 23, 24). For cell-line xenografts, 5 × 10^6 NCI-N87 cells in 30% Matrigel were injected subcutaneously into the right flank of 6-week-old NOD (nonobese diabetic)/SCID (severe combined immunodeficient) mice. Established tumors (average volume 400 mm^3) were treated with the following regimens, either single-agent or in combination: cetuximab (Merck), 20 mg/kg, twice-weekly (vehicle: physiological saline); lapatinib (Sequoia Research Products) 100 mg/kg, daily (vehicle, 0.5% methylcellulose, 0.2% Tween-80); trastuzumab (Roche), 30 mg/kg once-weekly (vehicle: physiological saline); afatinib (Sequoia Research Products) 20 mg/kg, daily (vehicle: 2% hydroxypropyl beta cyclodextrin, 0.5% natrosol, 0.5% acetic acid). Tumor size was evaluated once-weekly by caliper measurements and the approximate volume of the mass was calculated using the formula 4/3π·(d/2)^2·D/2, where d is the minor tumor axis and D is the major tumor axis. All values for tumor growth curves were recorded blindly. Animal procedures were approved by the Ethical Commission of the Candiolo Cancer Institute and by the Italian Ministry of Health.
Real Time RT-PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen) and reverse-transcribed using High Capacity cDNA reverse transcription (Life Technologies). Results were normalized to the average of one or two housekeeper genes. The Taqman probes (Life Technologies) were the following: Hs00176538_m1 (HER3), Hs02800695_m1 (HPRT1); Hs00942570_g1 (CETN2); Hs00427621_m1 (TBP).

Statistical analysis

Statistical analyses for cell-line experiments were performed by two-tailed Student’s t-test or two-way ANOVA. For in vivo assays in xenografts or patient-derived tumorgrafts, statistical analyses were performed by repeated measures ANOVA. This latter analysis was performed after log-transformation of each individual tumor volume measurement at each time point. To standardize the effect by baseline value, the difference between tumor volume at treatment initiation and the volume at each timepoint along treatment was used. A generalized linear model procedure accounting for repeated measures was then employed to estimate the coefficient and relative standard error of treatment time and the interaction between therapies and time. The estimates of the effect were than back-transformed onto original scale. For all tests, the level of statistical significance was set at $P < 0.05$. 
RESULTS

Combination therapy with trastuzumab and lapatinib is superior to single-agent treatment in HER2-amplified gastrointestinal tumors in vivo

We have previously demonstrated that HER2-amplified patient-derived mCRC tumorgrafts shrink when treated with the anti-EGFR antibodies cetuximab or pertuzumab together with lapatinib (15). Because these combinations are not approved for clinical use and knowledge of their toxicity profiles is limited, we decided to test M051, the first HER2-amplified mCRC tumorgraft identified during systematic collection of consecutive mCRC samples, with lapatinib and trastuzumab, a dual treatment modality that is employed investigationally in HER2-positive breast cancer (4). Similar to findings with cetuximab or pertuzumab monotherapy (15), trastuzumab alone proved to be completely ineffective, resulting in rampant tumor growth with kinetics that almost overlapped those of the placebo arm. Single-agent lapatinib induced disease stabilization, which tended to turn into tumor progression over time. Notably, the combination of trastuzumab and lapatinib induced rapid and long-lasting tumor regression (Fig. 1A). Albeit less evident than in M051, a stronger antitumor activity for the combination was observed in three other patient-derived tumorgrafts that were established more recently from independent HER2-amplified mCRCs (M091, M147, and M155) (Supplementary Fig. S1). These findings are consistent with initial clinical observations: according to interim results from an ongoing clinical trial, dual blockade of HER2 by trastuzumab and lapatinib resulted in a 35% objective response rate and 78% disease control rate in heavily pretreated chemorefractory patients with HER2-amplified mCRC (25).

As a comparison, the same therapies were applied to mice bearing xenografts of the HER2-amplified gastric cancer cell-line NCI-N87. Again, and in agreement with previous observations (14), tumor shrinkage was achieved by combined trastuzumab and lapatinib, whilst monotherapy with either agent simply blocked tumor growth (Fig. 1B). The stronger effect of trastuzumab alone in NCI-N87 xenografts, when compared with the M051 tumorgrafts, was likely due to the higher extent of HER2 amplification (34 fold in NCI-N87 versus 24 fold in M051, as assessed by genomic qPCR).

In the absence of existing information on the mechanisms underlying the therapeutic cooperation of trastuzumab and lapatinib in GI tumors, we sought to investigate the signaling consequences of HER2 blockade by such inhibitors, alone and in combination, in molecularly pertinent cellular models.

HER2-overexpressing CRC cell lines are resistant to cetuximab and sensitive to the combination of trastuzumab and lapatinib

None of 151 immortalized CRC cell lines screened for HER2 gene amplification was found to be positive. We therefore generated HER2-overexpressing cell models by lentiviral transduction of
HER2 into three CRC cell lines (NCI-H508, DiFi, and HDC-142) reported to be sensitive to anti-EGFR antibodies (19, 26) (Fig. 2A, Supplementary Fig. S2A).

In line with results in patients and patient-derived tumorgrafts (15-17), all HER2 overexpressors proved to be resistant to cetuximab in short-term viability assays (Fig. 2B and Supplementary Fig. S2B). In long-term clonogenic assays, HER2 overexpressors were positively selected by continuous exposure to cetuximab (Fig. 2C, Supplementary Fig. S2C).

NCI-H508-HER2 cells were chosen for further studies thanks to their amenability to successful xenografting in immunocompromised mice. Consistent with the in vitro findings, NCI-H508-HER2 xenografts displayed overt resistance to cetuximab (Fig. 2D). Importantly, when challenged with HER2-targeted therapies, NCI-H508-HER2 xenografts exhibited tumor responses strictly in line with those observed in mCRC tumorgrafts featuring endogenous HER2 amplification/overexpression: the strongest effect was obtained by the combination of trastuzumab and lapatinib; trastuzumab monotherapy was ineffective; and lapatinib alone induced initial disease stabilization followed by resumption of tumor growth (Fig. 2E). These in vivo results were recapitulated in soft agar assays, with higher growth-inhibitory activity exerted by the combination therapy in both NCI-H508-HER2 (Fig. 2F) and NCI-N87 (Fig. 2G).

Lapatinib monotherapy leads to delayed reactivation of HER3 and EGFR, which is prevented by trastuzumab

To get new insight into the molecular mechanisms underlying the enhanced antitumor activity of dual trastuzumab-lapatinib therapy, we evaluated the activation status of HER receptors (HER2, HER3, and EGFR) upon treatment of NCI-H508-HER2 cells with increasing concentrations of the single agents or their combination. Trastuzumab monotherapy was unable to appreciably affect HER2 and EGFR phosphorylation (with only minor effects on HER2 at high antibody concentration), but dose-dependently decreased HER3 activation. On the contrary, lapatinib and combo potently inhibited phosphorylation of all HER receptors (Fig. 3A, Supplementary Fig. S3A, Supplementary Table S1).

To obtain quantitative data amenable to statistical analysis, we gauged the activation status of canonical HER downstream transducers by antibody-based phosphoproteomics. In particular, we assessed the phosphorylation levels of ERK1/2 (as a proxy of RAS pathway activity) and AKT, p70S6K and GSK-3β (as readouts of PI3K-dependent signals) using the Meso Scale Discovery (MSD) platform, a technology that enables multiplex analysis of phosphoprotein changes by quantitative electrochemiluminescence detection. In agreement with results on HER receptors, trastuzumab alone did not substantially affect the baseline levels of transducers’ activation, with only minor reductions in AKT phosphorylation and no discernible effects on the other signals (Fig. 3A, Supplementary Table S1). Conversely, lapatinib potently impaired the activity of ERK and AKT.
and (slightly) decreased the phosphorylation of p70S6K and GSK-3β (Fig. 3A, Supplementary Table S1). Again in coherence with data on receptors, the combination therapy was not superior to lapatinib alone in inducing downstream signal neutralization (Fig. 3A, Supplementary Table S1). Partially analogous results were obtained in NCI-N87 cells: on one side, trastuzumab specifically impaired HER3 phosphorylation, with no activity against EGFR, paradoxical hyperphosphorylation of HER2, and negligible repercussions on downstream signals; on the other, lapatinib and combo similarly depressed all HER receptors and transducers (Fig. 3A, Supplementary Fig. S3A, Supplementary Table S1). MSD data were independently confirmed in both cell lines by Western blot analysis of phospho-ERK and phospho-AKT levels (Supplementary Fig. S4A).

Dose-response assays after 2 hours of treatment did not reveal marked differences in HER-based signaling pathways that could account for the stronger effect of the combination therapy. We therefore carried out time-course experiments in NCI-H508-HER2 cells to assess potential variations in signal kinetics. Consistent with results in dose-response curves, the main effect of trastuzumab monotherapy was immediate and persistent mitigation of HER3 phosphorylation, whereas activation of HER2 and EGFR was dampened exclusively at late time points (Fig. 3B, Supplementary Fig. S3B, Supplementary Table S1). Of note, treatment with lapatinib acutely reduced phosphorylation of all HER receptors but caused a delayed recovery in the phosphorylation of HER3 and EGFR and, to a lesser extent, HER2 itself (Fig. 3B, Supplementary Fig. S3B, Supplementary Table S1). HER3 rephosphorylation was accompanied by increased protein expression (Fig. 3B, Supplementary Fig. S3B). In this extended temporal window, the advantage of the trastuzumab-lapatinib association was evident: when added to lapatinib, trastuzumab prevented lapatinib-induced rephosphorylation of HER receptors, in particular, that of HER3 and EGFR (Fig. 3B, Supplementary Fig. S3B, Supplementary Table S1).

Importantly, the therapy-induced changes in HER3 expression and phosphorylation observed in NCI-H508-HER2 cells were confirmed in mCRC patient-derived tumorgrafts (M051) in vivo. Antibody-based proximity assays, performed with the VeraTag technology on end-of-treatment material, revealed increased HER3 content and higher representation of HER2/HER3 heterodimers following treatment with lapatinib alone, with no reduction in HER3 phosphorylation. The addition of trastuzumab to lapatinib counteracted the formation of HER2/HER3 heterodimers and potently depressed HER3 phosphorylation (Fig. 3C, Supplementary Table S1).

At the level of downstream signals, evaluated by both MSD-based phosphoproteomics (Figure 3B, Supplementary Table S1) and conventional Western blot analysis (Supplementary Fig. S4B), trastuzumab exerted overall minor responses and lapatinib caused a short-lived neutralization of the RAS/ERK and PI3K/AKT pathways, which was followed by partial restoration of signal activity paralleling receptors’ rephosphorylation (Fig. 3B, Supplementary Fig. S4B, Supplementary Table S1). Again, the combination therapy counteracted the deferred resumption of ERK and AKT.
phosphorylation and induced durable signal abrogation, mainly for the AKT pathway (Fig. 3B, Supplementary Fig. S4B, Supplementary Table S1).

Prolonged exposure to lapatinib led to protein upregulation of HER3, rephosphorylation of HER receptors, and partial re-activation of ERK and AKT also in NCI-N87 (Fig. 3B, Supplementary Fig. S3B, Supplementary Fig. S4B, Supplementary Table S1). In accordance with results in NCI-H508-HER2, co-treatment of NCI-N87 with trastuzumab blunted lapatinib-induced HER3 and EGFR rephosphorylation and induced a more sustained inhibition of downstream effectors, in particular AKT (Fig. 3B, Supplementary Fig. S3B, Supplementary Fig. S4B, Supplementary Table S1). Curiously, NCI-N87 cells featured delayed rephosphorylation of HER3 and EGFR not only in response to lapatinib, but also following treatment with trastuzumab (Fig. 3B, Supplementary Fig. S3B, Supplementary Table S1). We did not further explore this issue.

Altogether, these findings point to re-activation of HER3 and EGFR, with the ensuing restoration of downstream transducers, as a mechanism that could limit the efficacy of anti-HER2 monotherapy in GI tumors and suggest that higher therapeutic benefit may be achieved by prolonged inhibition of HER receptors.

Delayed reactivation of EGFR and HER3 can be prevented by EGFR/HER2 irreversible inhibition

Phosphorylation of HER2 resumed after prolonged exposure to lapatinib (Fig. 3B, Supplementary Fig. S3B). This suggests that the compound did not maintain full inhibitory activity over time, leading to rephosphorylation of HER3 (encouraged by HER3 overexpression) and EGFR. Importantly, different from catalytically inert HER3, reactivated EGFR could reciprocally contribute to HER2 and HER3 signaling by actively triggering their transphosphorylation (27-30). We reasoned that long-lasting blockade of HER2 and EGFR by irreversible inhibitors might prevent HER3 reactivation. We therefore used Western blots and MSD to analyze the consequences of afatinib (an irreversible inhibitor of HER2 and EGFR, with stronger activity on EGFR) versus lapatinib (which reversibly inactivates both receptors at equimolar concentrations) in time-course experiments. As expected, in both NCI-H508-HER2 and NCI-N87, lapatinib was unable to maintain durable inhibition of HER receptors and downstream signals (Fig. 4A, Supplementary Fig. S5A, Supplementary Fig. S6, Supplementary Table S1). Conversely, afatinib induced protracted receptor dephosphorylation and more sustained inhibition of downstream transducers in the face of higher HER3 levels (Fig. 4A, Supplementary Fig. S5A, Supplementary Fig. S6, Supplementary Table S1).

As a proof of concept for generalizing these observations, we extended the comparative evaluation of lapatinib versus afatinib in BT-474, a prototypical breast cancer cell line with HER2 amplification. Also in this setting, afatinib frustrated the deferred rephosphorylation of EGFR and HER3 observed in the presence of lapatinib (Fig. 4A, Supplementary Fig. S5A, Supplementary
Table S1). MSD and Western blot analysis revealed that, similar to the trastuzumab-lapatinib combination, afatinib monotherapy proved to be more efficient than lapatinib alone in achieving durable inhibition of downstream effectors (Fig. 4A, Supplementary Fig. S6, Supplementary Table S1). Prolonged neutralization of HER2, HER3 and EGFR by afatinib was also observed in another HER2-overexpressing CRC cell line (DiFi-HER2) (Fig. 4B, Supplementary Fig. S5B, Supplementary Table S1) and in HER2-positive OE-19 gastric cancer cells (Fig. 4B, Supplementary Fig. S5B, Supplementary Table S1).

**HER3 overexpression is recapitulated by PI3K blockade, and HER3 knockdown exacerbates the inhibitory effects of lapatinib on cancer cell growth**

Both lapatinib and afatinib caused increased expression of HER3 in all cell lines tested and both compounds were particularly effective in abrogating (transiently for lapatinib and durably for afatinib) downstream activation of AKT and ERK. In HER2-positive breast cancer, lapatinib-induced inhibition of AKT is known to relieve feedback suppression of HER3 expression by FOXO-mediated transactivation of the HER3 promoter (31). In colorectal cancer, inhibition of the MEK-ERK axis impedes transcriptional repression of HER3 by MYC (32). To dissect the contribution of either pathway to the regulation of HER3 expression in our models, we treated NCI-H508-HER2, NCI-N87 and BT-474 cells with lapatinib, afatinib, the PI3K-mTOR inhibitor BEZ235, or the MEK inhibitor AZD6244 and analyzed HER3 transcript level by RT-qPCR in time-course experiments. As expected, treatment with lapatinib or afatinib upregulated HER3 mRNA levels in all cell lines (Fig. 5A). HER3 upregulation was also observed in the presence of BEZ235, whereas the effect of AZD6244 was negligible (in NCI-H508-HER2 and NCI-N87) or absent (in BT-474) (Fig. 5B). This indicates that modulation of HER3 expression by lapatinib and afatinib mainly depends on the PI3K/AKT pathway in both GI and breast tumors.

Rephosphorylation of HER3 and EGFR as a consequence of lapatinib treatment was likely due to increased HER3 expression, which is expected to facilitate formation of HER heterodimers and EGFR transphosphorylation. As an additional means to achieve permanent HER3 neutralization, we silenced HER3 expression by two different short hairpin RNA (shRNA) vectors (Fig. 5C and D) and tested HER3-deficient cells in soft agar assays in the absence or presence of lapatinib. In agreement with the assumption that selective blockade of HER3 signaling is expected to increase the efficacy of lapatinib, silencing of HER3 in NCI-H508-HER2 sensitized to lapatinib treatment (Fig. 5E). In NCI-N87, HER3 knockdown potently impaired anchorage-independent growth under basal conditions and enhanced the inhibitory activity of lapatinib (Fig. 5F). Collectively, these results indicate that HER3 activity causally attenuates responsiveness to lapatinib in colorectal cancer and has a more general role in sustaining the transformed phenotype of gastric tumors.
Afatinib monotherapy induces tumor regression in vivo

If durable abrogation of HER3 and EGFR phosphorylation is the mechanistic basis for the enhanced therapeutic activity of the trastuzumab-lapatinib association, then afatinib monotherapy – which also leads to persistent inhibition of HER receptors – is expected to induce regression of HER2-amplified tumors. We therefore treated the M051 CRC tumorgraft model and the NCI-N87 cell-line xenografts with lapatinib alone, the trastuzumab-lapatinib combination, and afatinib alone. In accordance with initial results (see Fig. 1), lapatinib inhibited tumor growth and trastuzumab plus lapatinib prompted tumor shrinkage (Fig. 6A and B). Notably, tumors underwent regression also when treated with afatinib monotherapy (Fig. 6A and B).

In conclusion, all these findings indicate that: i) the poor response to trastuzumab alone is likely due to inefficient inactivation of HER2 and EGFR, with consequent negligible output on downstream signaling; ii) the modest antitumor effects of lapatinib monotherapy can be ascribed to regained HER3 and EGFR activation/phosphorylation over time; iii) sustained targeting of HER3 and EGFR by irreversible or dual blockade of HER2/EGFR is required to achieve overt tumor regression.
DISCUSSION

Preclinical results in HER2-positive GC cell-line xenografts, confirmed in the present study, indicate that trastuzumab and lapatinib have higher antitumor activity than either treatment alone (14). In the case of CRC, we have previously found that regression of HER2-amplified patient-derived tumorgrafts occurs only when lapatinib is administered together with antibodies against EGFR (cetuximab) or the EGFR/HER2 heterodimer (pertuzumab) (15). Using HER2-amplified patient-derived tumorgrafts, we show here that a similar effect on tumor shrinkage could be also obtained by combining lapatinib and trastuzumab. On the basis of these in vivo data, we thoroughly investigated the signaling consequences of HER2 blockade by trastuzumab and/or lapatinib to identify limitations in their mechanism of action as single agents and to analyze cooperative modalities that might explain the higher therapeutic efficacy of the two inhibitors when given together.

We found that trastuzumab monotherapy durably blunted the phosphorylation levels of HER3, with negligible effects on the activation of HER2. On the other hand, lapatinib prompted immediate and potent inhibition of HER2 but also upregulation and rephosphorylation of HER3 after initial transient blockade. Hence, each of the two agents could rectify the inadequacy of the other. Lapatinib-driven upregulation of HER3 was mimicked by blockade of the PI3K/AKT pathway, indicating that HER3 expression in GI tumors is negatively regulated by active signaling downstream from HER2. Analogous results have been observed in HER2-positive breast cancer cell lines treated with lapatinib (30) and in other cancer cell lines treated with PI3K or AKT inhibitors (33, 34).

The ability of trastuzumab to prevent lapatinib-driven reactivation of HER3 is conceivably due to trastuzumab-mediated perturbation of HER receptor clustering at the cell surface, given the established mechanism of action of trastuzumab through disruption of ligand-independent HER heterodimers (35). Further pointing to the biological and clinical relevance of these findings, we observed heightened expression of HER3, higher representation of HER2/HER3 heterodimers, and lack of HER3 inhibition in patient-derived CRC tumorgrafts after lapatinib treatment; conversely, the trastuzumab-lapatinib combination reduced the ratio of HER2/HER3 heterodimers and suppressed HER3 phosphorylation. This mechanism is expected to complement an independent mode of therapeutic cooperation that relies on the ability of lapatinib to induce accumulation of HER2 at the cell surface, which enhances immune-mediated trastuzumab-dependent cytotoxicity (36, 37).

The inhibitory effect of lapatinib on HER2 phosphorylation tended to dissipate after prolonged treatment. Therefore, the fact that lapatinib prompted not only HER3 protein upregulation but also its rephosphorylation can be explained by compound exhaustion over time and the ensuing restoration of residual HER2 kinase function, which reverberates on overexpressed HER3. Indeed,
it has been demonstrated that a suprapharmacological dose of 5 μM lapatinib (which likely limits long-term drug consumption by granting higher inhibitor availability) abrogates recovery of phosphorylated HER3 in HER2-positive breast cancer cell lines (31, 38). Inhibitor consumption, together with greater density of HER heterodimers favored by HER3 overexpression, is likely to also account for EGFR rephosphorylation following lapatinib treatment, although this rescue did not appear to be associated with increased protein expression.

EGFR/HER2 irreversible inhibitors, such as afatinib and neratinib, have been demonstrated to be more potent and to protract target inhibition compared with lapatinib (39). We therefore reasoned that the use of irreversible EGFR/HER2 inhibitors would avoid HER3 and EGFR rephosphorylation produced by lapatinib. Indeed, a new notable finding of our study is that irreversible HER2 inhibition by single-agent afatinib could substitute for dual blockade by trastuzumab and lapatinib for induction of sustained inhibition of HER3 and EGFR. In keeping with the notion that such persistent blockade is instrumental to enhancing the antitumor activity of HER2 inhibition, afatinib alone prompted regression of HER2-amplified gastrointestinal carcinomas, similar to the trastuzumab-lapatinib combination. It should be noted that, while lapatinib is equipotent towards HER2 and EGFR (40), afatinib is more active against EGFR (41). Therefore, afatinib higher therapeutic efficacy is likely to also rely on more drastic neutralization of EGFR-driven transphosphorylation of HER2 and HER3. This preferential activity towards EGFR might be particularly important in colorectal cancers, in which EGFR signaling plays a major proliferative role in the absence of mutations along the RAS pathway (42).

Our observation that afatinib was more effective than lapatinib in inducing shrinkage of HER2-amplified GI tumors has some clinical correlates. Monotherapy with the irreversible HER2 inhibitor neratinib in HER2-positive breast cancer showed considerable clinical activity, with 56% objective response rates in trastuzumab-naïve patients (43). Similarly, afatinib could overcome trastuzumab resistance in heavily pre-treated HER2-amplified mammary tumors (44). According to information available on the web, a clinical trial with afatinib and trastuzumab in trastuzumab-resistant, HER2-positive gastroesophageal tumors is currently recruiting participants (NCT01522768). It will be interesting to analyze whether the good efficacy of irreversible HER2 inhibition in mammary tumors will be confirmed clinically in GC. Finally, HER3-neutralizing antibodies have demonstrated to synergize with trastuzumab and lapatinib in HER2-amplified breast tumors (45) and are now being tested in phase I first-in-man studies. Again, it will be interesting to explore the value of HER3-targeted therapies in HER2-amplified tumors of different origin.
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LEGENDS TO FIGURES

Figure 1.
Effect of anti-HER2 therapies in HER2-amplified patient-derived colorectal cancer tumorgrafts and gastric cancer cell-line xenografts. A, B, Growth curves of M051 patient-derived mCRC tumorgrafts (A) and NCI-N87 gastric cancer cell-line xenografts (B) treated with the indicated modalities. n = 6 (M051) or 5 (NCI-N87) for each treatment arm. Error bars indicate SEM. VEH, vehicle (saline); TRAS, trastuzumab; LAP, lapatinib; COMBO, trastuzumab plus lapatinib. *** P < 0.0001; ** P < 0.003 by repeated measures ANOVA.

Figure 2.
Biological characterization of HER2-overexpressing NCI-H508 and NCI-N87 cells. A, Ectopic HER2 overexpression and constitutive phosphorylation in NCI-H508. Cells were stably transduced with a HER2-encoding lentiviral vector or the empty vector (mock). Lysates were subjected to anti-HER2 immunoprecipitation (IP) followed by Western blot using anti-phosphotyrosine (P-Tyr) or anti-total HER2 antibody. Actin was used as a loading control. B, Short-term response of mock-transduced and HER2-overexpressing NCI-H508 cells to increasing doses of cetuximab. Viable cells were measured after 96 hours of treatment using ATP content as a proxy of cell numbers. Data were plotted relative to untreated controls. Results are the means ± SEM of five independent experiments, each performed in three biological replicates. C, Effect of long-term treatment with cetuximab in mock-transduced and HER2-overexpressing NCI-H508 cells. D, E, Tumor growth curves of mock-transduced and HER2-overexpressing NCI-H508 xenografts treated with cetuximab (D) or the indicated anti-HER2 agents (E). n = 6 for each treatment arm. Error bars indicate SEM. *, P = 0.0224 by repeated measures ANOVA. F, G, Anchorage-independent growth of NCI-H508-HER2 (F) and NCI-N87 (G) treated with the indicated modalities. Results are the means ± SD of two independent experiments, each performed in three biological replicates. NS, not significant; * P < 0.05; *** P < 0.001 by two-tailed Student’s t-test. NT, not treated; VEH, vehicle (saline); CET, cetuximab; TRAS, trastuzumab; LAP, lapatinib; COMBO, trastuzumab plus lapatinib.

Figure 3.
Signaling consequences of treatment with trastuzumab, lapatinib and their combination in HER2-positive colorectal and gastric cancer. A, Activation status/phosphorylation of HER receptors and downstream transducers in NCI-H508-HER2 and NCI-N87 cells in dose-response experiments. Cells were treated with the indicated concentrations of trastuzumab, lapatinib or the combination of both for 2 hours. Cell extracts were immunoblotted with the indicated antibodies (upper panels) or analyzed for pAKT/pp70S6K/pGSK-3β and pERK1/2 by MSD multi-arrays (lower panels).
Phosphoprotein response for downstream transducers was reported as a heatmap. The color scale represents relative protein phosphorylation changes calculated as Log2 ratio (treated/untreated) of the median of three independent experiments. Each cell position in the rows corresponds to the experimental conditions of the above blots. Two-way ANOVA statistics for MSD results indicate that the effects of lapatinib versus those of trastuzumab plus lapatinib were not significantly different in either NCI-H508 or NCI-N87. B, Activation status/phosphorylation of HER receptors and downstream transducers in NCI-H508-HER2 and NCI-N87 cells in time-course experiments. Trastuzumab, lapatinib or the combination of both were added to cells at the beginning of the experiment at the indicated concentrations. Cells were incubated in the presence of the inhibitor(s) for the indicated times and lysates subjected to Western blot analysis (upper panels) or to MSD multi-arrays (lower panels). Relative phosphoprotein modulations for downstream transducers were calculated as described in (A). Two-way ANOVA statistics for MSD results: lapatinib vs. trastuzumab plus lapatinib in NCI-H508-HER2: phospho-AKT, \( P < 0.05 \); phospho-p70S6K, \( P < 0.01 \); phospho-ERK, \( P < 0.05 \); phospho-GSK-3\( \beta \), not significant. Lapatinib vs. trastuzumab plus lapatinib in NCI-N87: phospho-AKT, \( P < 0.05 \); other signals, not significant. In (A) and (B) Western blot experiments, vinculin was used as a loading control. Western blots for total proteins were run with the same lysates as those used for anti-phosphoprotein detection but on different gels. All Western blots are representative of two experiments on independent biological replicates (Supplementary Fig. S3). Representative Western blots for ERK and AKT are shown in Supplementary Fig. S4. C, VeraTag assessment of HER2/HER3 heterodimers and phospho/total HER3 quantitation in patient-derived mCRC tumorgrafts (M051) after six weeks of treatment with lapatinib or trastuzumab plus lapatinib. Formalin-fixed paraffin-embedded tumor sections were analyzed by VeraTag assays. Results were reported as Log2 ratio (treated/untreated) of the median of three (vehicle and lapatinib) or two (trastuzumab plus lapatinib) biological replicates. VEH, vehicle (saline); TRAS, trastuzumab; LAP, lapatinib; T+L, trastuzumab plus lapatinib.

Figure 4.

Signaling consequences of afatinib monotherapy and comparison with lapatinib. A, Activation status/phosphorylation of HER receptors and downstream transducers in NCI-H508-HER2, NCI-N87 and BT-474 treated with lapatinib or afatinib in time-course experiments. The compounds were added to cells at the beginning of the experiment at the indicated concentrations. Cells were incubated in the presence of the inhibitor for the indicated times and lysates subjected to Western blot analysis (upper panels) or to MSD multi-arrays (lower panels). Representative Western blots for ERK and AKT are shown in Supplementary Fig. S6. Two-way ANOVA statistics for MSD results: lapatinib vs. afatinib in NCI-H508-HER2: phospho-AKT; \( P < 0.001 \); phospho-p70S6K, \( P < 0.001 \); phospho-ERK, \( P < 0.01 \); phospho-GSK-3\( \beta \), not significant. Lapatinib vs. afatinib in NCI-N87:
phospho-GSK-3β, $P < 0.05$; other signals, not significant. Lapatinib vs. afatinib in BT-474: phospho-AKT, $P < 0.01$; phospho-p70S6K, $P < 0.01$; phospho-GSK-3β, $P < 0.01$; phospho-ERK, $P < 0.05$. B, Activation status/phosphorylation of HER receptors in DiFi-HER2 colorectal cancer cells and OE-19 gastric cancer cells treated with lapatinib or afatinib in time-course experiments. For Western blot experiments, vinculin was used as a loading control. Western blots for total proteins were run with the same lysates as those used for anti-phosphoprotein detection but on different gels. All Western blots are representative of two experiments on independent biological replicates (Supplementary Fig. S5). LAP, lapatinib; AFAT, afatinib.

Figure 5.
Effects of PI3K or MEK blockade on HER3 overexpression and consequences of HER3 knockdown on sensitivity to lapatinib. A, B, RT-qPCR to evaluate the transcript expression of HER3 in NCI-H508-HER2, NCI-N87 and BT-474 cells treated with lapatinib (A), afatinib (A), the dual PI3K/mTOR inhibitor BEZ235 (B) or the MEK inhibitor AZD6244 (B) in time course experiments. Results are the means of one experiment performed in three technical replicates (A) or the means ± range of two independent experiments, each performed in three technical replicates (B). C, Expression of HER3 transcript in NCI-H508-HER2 (upper panel) and NCI-N87 (lower panel) transduced with two different shRNA lentiviral vectors targeting HER3 (shRNA_1 and shRNA_2). Results are the means of one experiment performed in three technical replicates, relative to HER3 transcript expression in cells transduced with a control, non-targeting shRNA (scramble). D, Expression of HER3 protein under basal conditions and following lapatinib treatment for the indicated times in NCI-H508-HER2 (upper panel) and NCI-N87 (lower panel) transduced with control (scramble), HER3 shRNA_1, or HER3 shRNA_2. E, F, Anchorage-independent growth of NCI-H508-HER2 (E) and NCI-N87 (F) transduced with control (scramble), HER3 shRNA_1, or HER3 shRNA_2, treated with the indicated modalities. Results are the means ± SD of one (NCI-H508-HER2) or two (NCI-N87) experiments, each performed in three biological replicates. * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by two-tailed Student’s t-test. NT, not treated; LAP, lapatinib; AFAT, afatinib; BEZ, BEZ235; AZD, AZD6244.

Figure 6.
Antitumor activity of afatinib monotherapy in HER2-amplified patient-derived colorectal cancer tumorgrafts and gastric cancer cell-line xenografts. A, B, Growth curves of M051 patient-derived mCRC tumorgrafts (A) and NCI-N87 gastric cancer cell-line xenografts (B) treated with the indicated modalities. $n = 6$ for each treatment arm. Error bars indicate SEM. VEH, vehicle (saline); LAP, lapatinib; T+L, trastuzumab plus lapatinib; AFAT, afatinib. *** $P < 0.0001$ (M051) and $P = 0.0003$ (NCI-N87, AFAT vs. LAP) by repeated measures ANOVA.
Leto et al., Figure 1

A

**M051**

Days from treatment start

B

**NCI-N87**

Days from treatment start

<table>
<thead>
<tr>
<th>Tumor volume (mm$^3$)</th>
<th>VEH</th>
<th>TRAS</th>
<th>LAP</th>
<th>COMBO</th>
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Research.
A

B

M051 and NCI-N87 cell lines. The graphs show the change in tumor volume (mm$^3$) over time from treatment start. The x-axis represents the number of days from the start of treatment, and the y-axis represents the tumor volume in mm$^3$. The treatments compared are VEH (vehicle), LAP (Lapatinib), AFAT (Afatinib), and T+L (a combination of T and L).

- **M051**
  - VEH: Red line with data points
  - LAP: Blue line with data points
  - T+L: Yellow line with data points
  - AFAT: Green line with data points

- **NCI-N87**
  - VEH: Red line with data points
  - LAP: Blue line with data points
  - T+L: Yellow line with data points
  - AFAT: Green line with data points

Significance levels are indicated by asterisks: *** indicates a significant difference.

Leto et al., Figure 6
Sustained inhibition of HER3 and EGFR is necessary to induce regression of HER2-amplified gastrointestinal carcinomas

Simonetta M Leto, Francesco Sassi, Irene Catalano, et al.

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