

Sustained Inhibition of HER3 and EGFR Is Necessary to Induce Regression of *HER2*-Amplified Gastrointestinal Carcinomas

Simonetta M. Leto^{1,2}, Francesco Sassi², Irene Catalano^{1,2}, Valter Torri³, Giorgia Migliardi^{1,2}, Eugenia R. Zanella^{1,2}, Mark Throsby⁴, Andrea Bertotti^{1,2,5}, and Livio Trusolino^{1,2}

Abstract

Purpose: Preclinical studies in *HER2*-amplified gastrointestinal cancer models have shown that cotargeting *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* (*HER* receptors), 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. *Clin Cancer Res*; 21(24): 5519–31. ©2015 AACR.

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 and pertuzumab) and reversible ATP-mimetics (lapatinib; ref. 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 are less satisfactory (7–9). Although trastuzumab has been approved for gastric cancer 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

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

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A. Bertotti and L. Trusolino contributed equally to this article and share senior authorship.

Corresponding Authors: Andrea Bertotti, Laboratory of Translational Cancer Medicine, Candiolo Cancer Institute, Strada Provinciale 142, 10060 Candiolo, Torino, Italy. Phone: 39-011-993-3202; Fax: 39-011-993-3225; E-mail: andrea.bertotti@unito.it; and Livio Trusolino, E-mail: livio.trusolino@unito.it

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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 coextinction targets in *HER2*-amplified GI tumors and propose monotherapy with irreversible *HER2* inhibitors as a viable alternative to dual *HER2* blockade.

of a subset (5%–10%) of metastatic colorectal carcinomas (mCRC) refractory to *EGFR* blockade (15–17). Preclinical findings suggest that anti-*HER2* monotherapy may also be poorly effective 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 biologic 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 the 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 nontargeting control vector, were purchased from Sigma (target sequences: shRNA_1-pLKO.1-puro and shRNA_1-pLKO.1-neo, CCGGAGGTTAGGAGTAGATATTGACTCGAGTCAATATCTACTCCTAACCTCTTTTGTG; shRNA_2-pLKO.1-puro, CCGGCTTCGTCATGTTGAACTATAAATCGAGTATAGTTC AACATGACGAAGTTTTTGTG; shRNA_2-pLKO.1-neo, CCGGAATTCTCTACTCTACCATTGCTCGAGCAATGGTAGAGTAGAGAATTCTTTTGTG). 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 of cetuximab to avoid the potential emergence of *HER2*-negative subclones.

Biologic 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 (2×10^4 cells/well), cultured in the absence or presence of 20 µg/mL of 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 2 weeks after seeding and quantitated using ImageJ. Lapatinib and trastuzumab were given at the indicated concentrations once weekly.

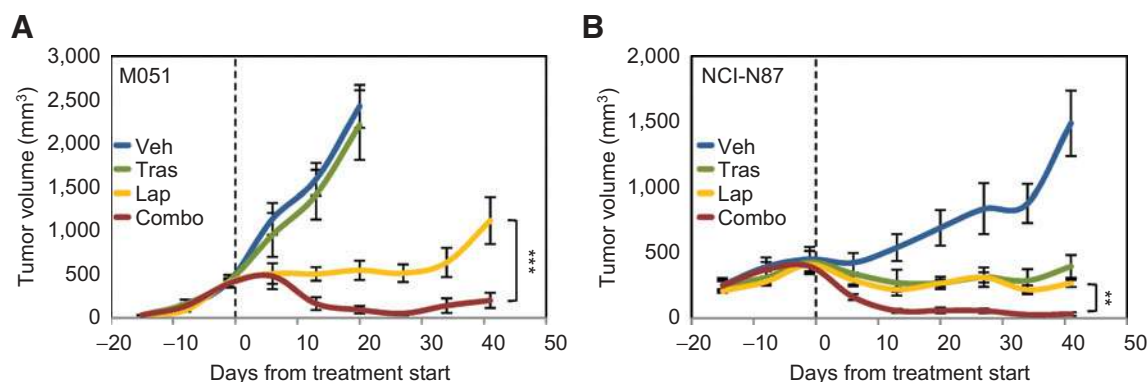


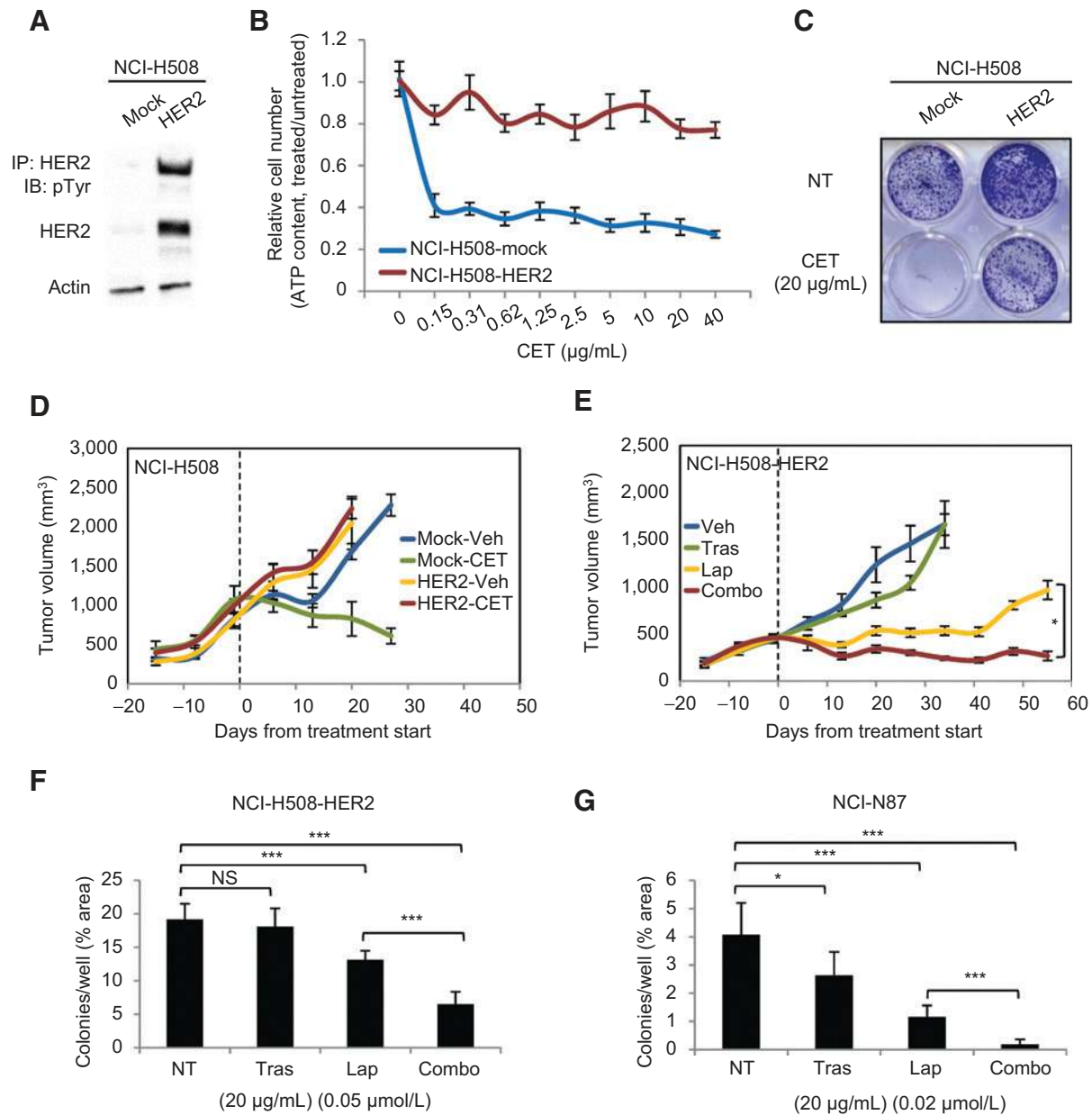
Figure 1.

Effect of anti-*HER2* therapies in *HER2*-amplified patient-derived colorectal cancer tumorgrafts and gastric cancer cell-line xenografts. A and 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, SEM. Veh, vehicle (saline); Tras, trastuzumab; Lap, lapatinib; Combo, trastuzumab plus lapatinib. ***, $P < 0.0001$; **, $P < 0.003$ by repeated measures ANOVA.

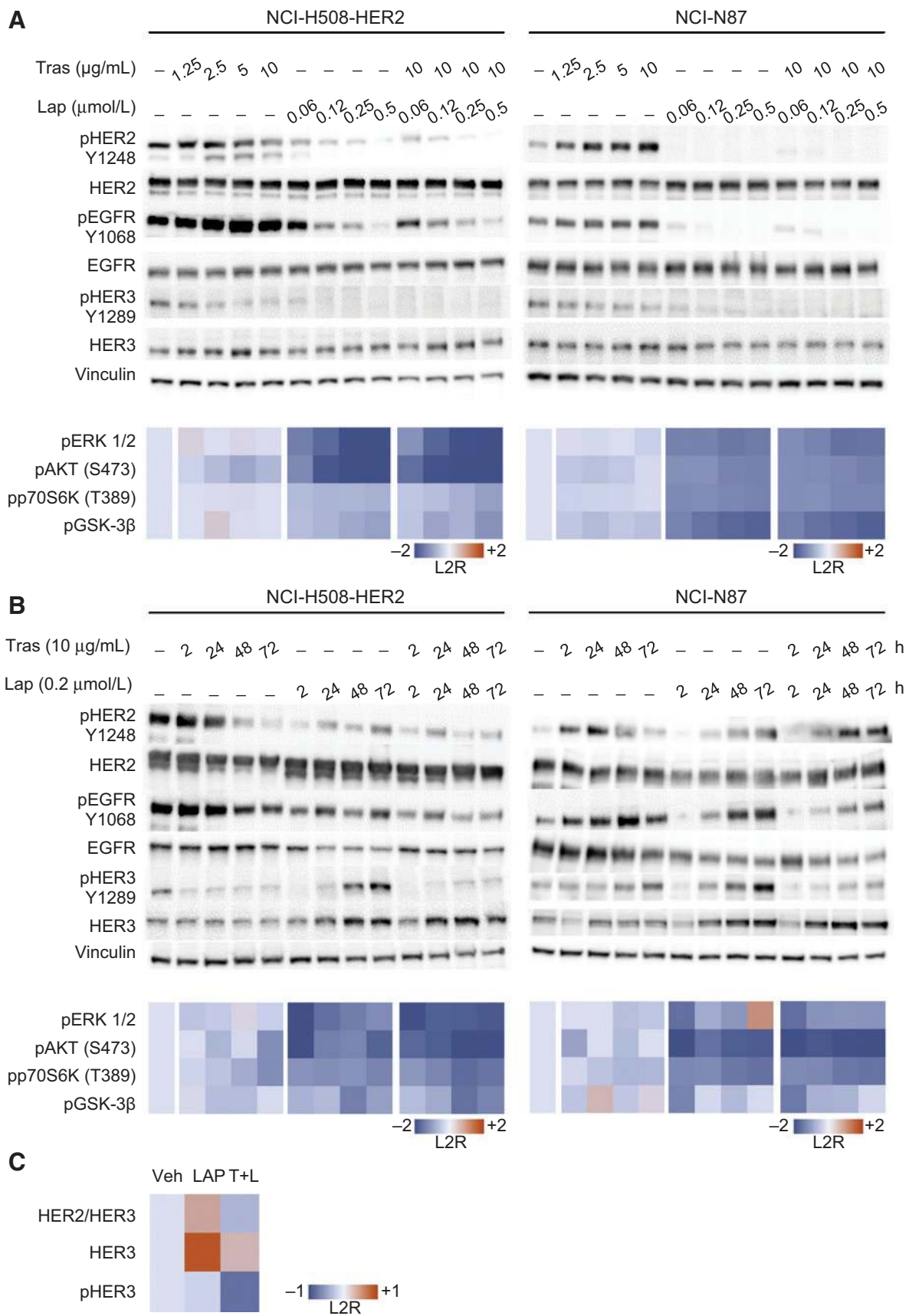
Immunoprecipitation and immunoblot analysis

Proteins were extracted with cold EB buffer (50 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 5 mmol/L EDTA, 5 mmol/L 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

**Figure 2.**

Biologic 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 \pm SEM of five independent experiments, each performed in three biologic replicates. C, effect of long-term treatment with cetuximab in mock-transduced and HER2-overexpressing NCI-H508 cells. D and 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 and G, anchorage-independent growth of NCI-H508-HER2 (F) and NCI-N87 (G) treated with the indicated modalities. Results are the means \pm SD of two independent experiments, each performed in three biologic replicates. NS, not significant; *, $P < 0.05$; ***, $P < 0.001$ by the two-tailed Student *t* test. NT, not treated; Veh, vehicle (saline); CET, cetuximab; Tras, trastuzumab; Lap, lapatinib; Combo, trastuzumab plus lapatinib.



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-3 β (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 tumorgrafts using a fluorescent antibody-based proximity assay (VeraTag; Monogram Biosciences; refs. 20–22).

Patient-derived tumorgrafts 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 or 3.5×10^6 NCI-H508 cells in 30% Matrigel were injected subcutaneously into the right flank of 6-week-old NOD/SCID mice. Established tumors (average volume 400 mm³) 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 $4/3\pi \cdot (d/2)^2 \cdot D/2$, 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 a two-tailed Student *t* test or two-way ANOVA. For *in vivo* assays in xenografts or patient-derived tumorgrafts, statistical analyses were performed by repeated measures ANOVA. The 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 time point along treatment was used. A generalized linear model procedure accounting for repeated measures was then used to estimate the coefficient and relative standard error of treatment time and the interaction between therapies and time. The estimates of the effect were then 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

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 (top) or analyzed for pAKT/pp70S6K/pGSK-3 β and pERK1/2 by MSD multiarrays (bottom). 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 (top) or to MSD multiarrays (bottom). Relative phosphoprotein modulations for downstream transducers were calculated as described in A. Two-way ANOVA statistics for MSD results: lapatinib versus 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 β , not significant. Lapatinib versus 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 antiphosphoprotein detection but on different gels. All Western blots are representative of two experiments on independent biologic 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 6 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) biologic replicates. Veh, vehicle (saline); Tras, trastuzumab; Lap, lapatinib; T+L, trastuzumab plus lapatinib.

trastuzumab, a dual treatment modality that is used investigational 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, whereas 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 vs. 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 colorectal carcinoma cell lines are resistant to cetuximab and sensitive to the combination of trastuzumab and lapatinib

None of 151 immortalized colorectal carcinoma 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 colorectal carcinoma cell lines (NCI-H508, DiFi, and HDC-142) reported to be sensitive to anti-EGFR antibodies (refs. 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; 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

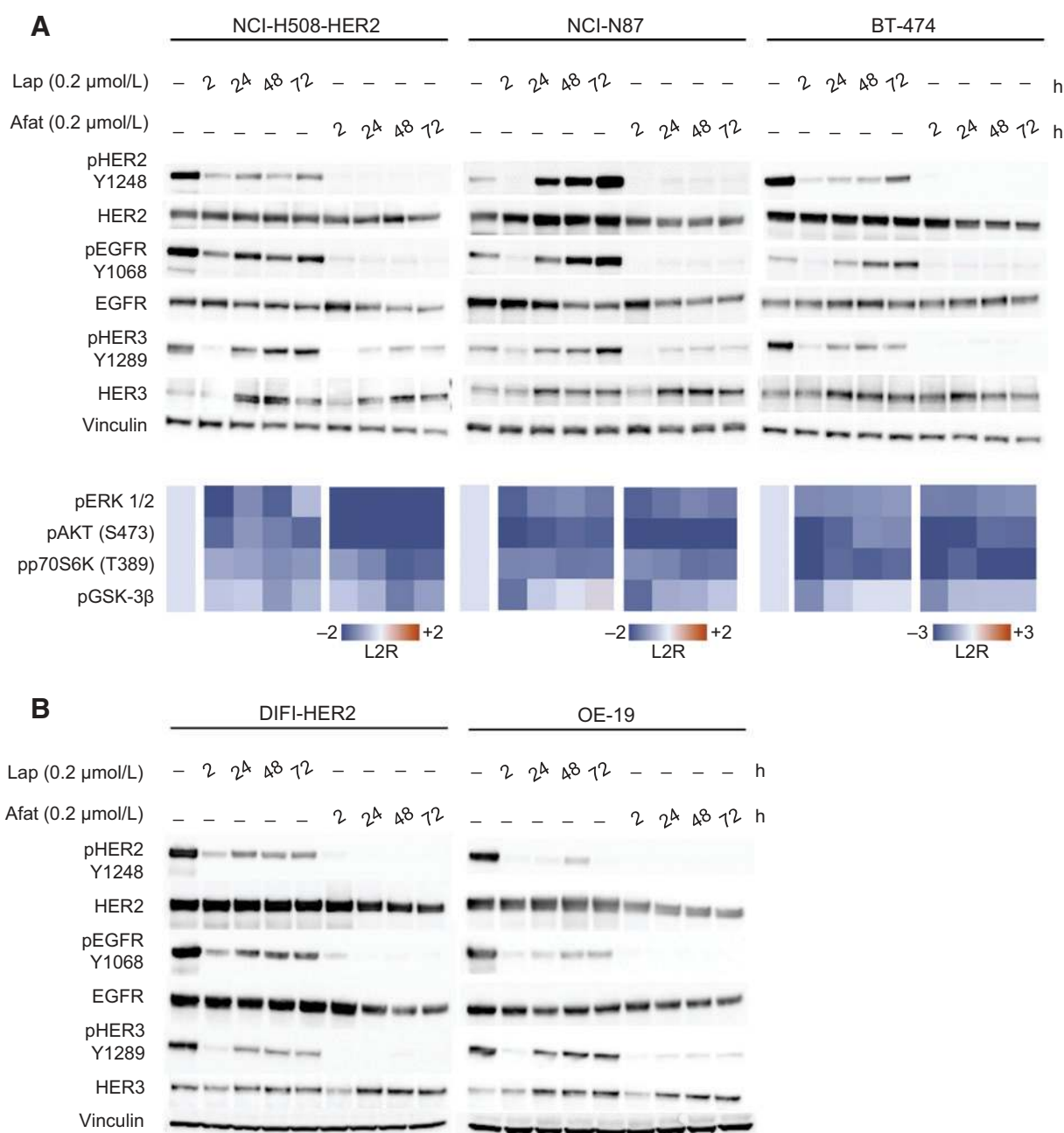
trastuzumab and lapatinib; trastuzumab monotherapy was ineffective; and lapatinib alone induced initial disease stabilization followed by resumption of tumor growth (Fig. 2E). These *in vivo* results were recapitulated in soft-agar assays, with higher growth-inhibitory activity exerted by the combination 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 alone or in combination with trastuzumab potently inhibited phosphorylation of all HER receptors (Fig. 3A; Supplementary Fig. S3A and 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-3 β* (as readouts of *PI3K*-dependent signals) using the 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-3 β* (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 the one hand, trastuzumab specifically impaired *HER3* phosphorylation, with no activity against *EGFR*, paradoxical hyperphosphorylation of *HER2*, and negligible repercussions on downstream signals; on the other hand, lapatinib and combo similarly depressed all HER receptors and transducers (Fig. 3A; Supplementary Fig. S3A and 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).

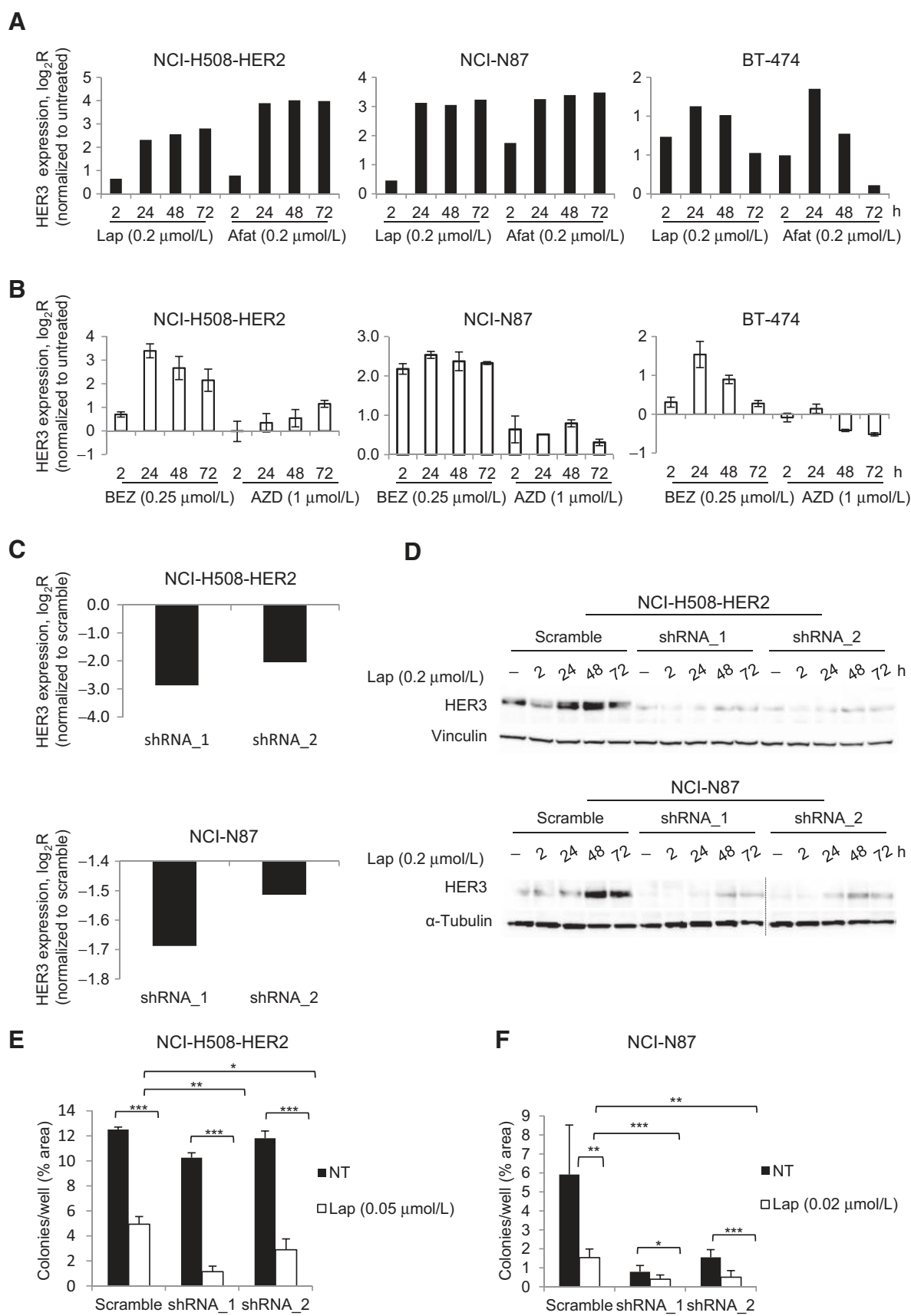
Dose–response assays after 2 hours of treatment did not reveal marked differences in *HER*-based signaling pathways that could account for the stronger effect of the combination therapy. We therefore carried out time-course experiments in NCI-H508-*HER2* cells to assess potential variations in signal 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 and Supplementary Table S1). Of note, treatment with lapatinib acutely

**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 (top) or to MSD multiarrays (bottom). Representative Western blots for ERK and AKT are shown in Supplementary Fig. S6. Two-way ANOVA statistics for MSD results: lapatinib versus afatinib in NCI-H508-HER2: phospho-AKT, $P < 0.001$; phospho-p70S6K, $P < 0.001$; phospho-ERK, $P < 0.01$; phospho-GSK-3 β , not significant. Lapatinib versus afatinib in NCI-N87: phospho-GSK-3 β , $P < 0.05$; other signals, not significant. Lapatinib versus afatinib in BT-474: phospho-AKT, $P < 0.01$; phospho-p70S6K, $P < 0.01$; phospho-GSK-3 β , $P < 0.01$; 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 antiphosphoprotein detection but on different gels. All Western blots are representative of two experiments on independent biologic replicates (Supplementary Fig. S5). Lap, lapatinib; Afat, afatinib.

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 and 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 and 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 potentially depressed HER3 phosphorylation (Fig. 3C; Supplementary Table S1).

At the level of downstream signals, evaluated by both MSD-based phosphoproteomics (Fig. 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 and 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 reactivation of ERK and AKT also in NCI-N87 (Fig. 3B; Supplementary Figs. S3B and S4B; Supplementary Table S1). In accordance with results in NCI-H508-HER2, cotreatment 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 Figs. S3B and 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 reactivation 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 Figs. S5A and 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 Figs. S5A and 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 colorectal carcinoma 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

Figure 5.

Effects of PI3K or MEK blockade on HER3 overexpression and consequences of HER3 knockdown on sensitivity to lapatinib. A and 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 \pm range of two independent experiments, each performed in three technical replicates (B). C, expression of HER3 transcript in NCI-H508-HER2 (top) and NCI-N87 (bottom) 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, nontargeting shRNA (scramble). D, expression of HER3 protein under basal conditions and following lapatinib treatment for the indicated times in NCI-H508-HER2 (top) and NCI-N87 (bottom) transduced with control (scramble), HER3 shRNA_1, or HER3 shRNA_2. E and 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 \pm SD of 1 (NCI-H508-HER2) or two (NCI-N87) experiments, each performed in three biologic replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by the two-tailed Student *t* test. NT, not treated; Lap, lapatinib; Afat, afatinib; BEZ, BEZ235; AZD, AZD6244.

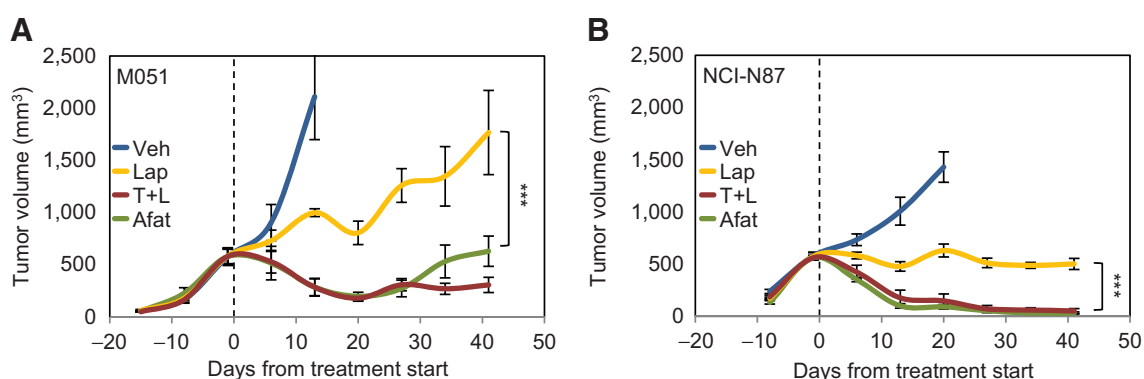


Figure 6.

Antitumor activity of afatinib monotherapy in *HER2*-amplified patient-derived colorectal cancer tumorgrafts and gastric cancer cell-line xenografts. A and 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.

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 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 potentially 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 colorectal carcinoma 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 gastric cancer 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 colorectal carcinoma, 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; ref. 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 biologic 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 colorectal carcinoma 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 $\mu\text{mol/L}$ of 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 anti-tumor activity of HER2 inhibition, afatinib alone prompted regression of HER2-amplified GI carcinomas, similar to the trastuzumab-lapatinib combination. It should be noted that, while lapatinib is equipotent toward HER2 and EGFR (40), afatinib is more active against EGFR (41). Therefore, afatinib higher therapeutic efficacy is also likely to rely on more drastic neutralization of EGFR-driven transphosphorylation of HER2 and HER3. This preferential activity toward 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 pretreated 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 gastric cancer. Finally, HER3-neutralizing antibodies have demonstrated to synergize with trastuzumab and lapatinib in HER2-amplified breast tumors (21) and are now being tested in phase I first-in-human studies. Again, it will be interesting to explore the value of HER3-targeted therapies in HER2-amplified tumors of different origin.

Disclosure of Potential Conflicts of Interest

L. Trusolino reports receiving a commercial research grant from Merus B.V. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S.M. Leto, A. Bertotti, L. Trusolino
Development of methodology: S.M. Leto, F. Sassi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Leto, F. Sassi, I. Catalano, G. Migliardi, E.R. Zanella, M. Throsby
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.M. Leto, F. Sassi, I. Catalano, V. Torri, E.R. Zanella, M. Throsby, A. Bertotti, L. Trusolino
Writing, review, and/or revision of the manuscript: S.M. Leto, F. Sassi, A. Bertotti, L. Trusolino
Study supervision: A. Bertotti, L. Trusolino

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