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Molecular Cancer Therapeutics

ErbB3 Inhibitory Surrobodies Inhibit Tumor Cell Proliferation *In Vitro* and *In Vivo*

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Abstract

ErbB3 is an important regulator of tumorigenesis and is implicated in development of resistance to several currently used oncology drugs. We have identified ErbB3 inhibitors based on a novel biologic scaffold termed a surrobody. Two of these inhibitors appear to work by a previously unrecognized mechanism of action. As a consequence, they not only inhibited cell proliferation and intracellular signaling driven by stimulation with the ErbB3 ligand neuregulin (NRG), but also inhibited signaling and proliferation that was driven by overexpression of ErbB2 in the absence of ligand stimulation. In addition, the surrobodies inhibited tumor growth *in vivo* in both ErbB2-overexpressing and nonoverexpressing cells. In ErbB2-overexpressing cells, both of the anti-ErbB3 surrobodies significantly augmented the activities of trastuzumab, lapatinib, and GDC-0941, agents that inhibit cell proliferation by different mechanisms. Moreover, although NRG diminished the efficacy of these agents, when they were combined with anti-ErbB3 surrobodies the affect of NRG was abrogated. In this capacity, the anti-ErbB3 surrobodies were more effective than the ErbB2/ErbB3 dimerization inhibitory antibody pertuzumab. Despite the fact that these surrobodies appear to engage ErbB3 differently than previously described anti-ErbB3 antibodies, they retain all of the beneficial characteristics of this class of agents, including the ability to augment drugs that inhibit EGF receptor. These anti-ErbB3 agents, therefore, show substantial promise for development as single agents or in combination with other ErbB-directed antibodies or small molecules and may provide for a broader range of therapeutic indications than previously described anti-ErbB3 antibodies. *Mol Cancer Ther; 11(7); 1–10. ©2012 AACR.*

Introduction

Inappropriate signaling through the EGF receptor (EGFR, ErbB) family of receptors can promote tumorigenesis and many epithelial cancers are marked by functional activation of ErbB family members. The ErbB family consists of 4 related tyrosine kinase receptors EGFR, ErbB2 (HER2/NEU), ErbB3, and ErbB4. ErbB3 is unique among these because it is deficient in tyrosine kinase activity. However, it is readily phosphorylated upon heterodimerization with ligand-activated EGFR or with ErbB2. In its fully phosphorylated state, it contains 6 binding sites for phosphoinositide 3-kinases (PI3K) and therefore constitutes one of the most potent activators of the AKT signaling pathway. Because the AKT pathway is intimately involved in coordinating cell proliferation, survival, metabolism, size, and angiogenesis, ErbB3 represents a pivotal node in regulating these pathways.

Several U.S. Food and Drug Administration–approved cancer therapeutics and a number of molecules currently in development target the ErbB family of receptors. Among these are therapeutic antibodies including cetuximab and panitumumab targeting EGFR, and trastuzumab and pertuzumab targeting ErbB2. In addition, targeted small molecules are in clinical use including erlotinib and gefitinib targeting EGFR, and lapatinib, which inhibits both EGFR and ErbB2 tyrosine kinase activities. However, despite the fact that EGFR and ErbB2 are key oncogenic drivers, only a subset of patients derive clinical benefit from these drugs and the development of resistance is common.

The mechanisms by which tumors are rendered refractory to, or develop resistance to, treatment with these agents are not fully elucidated. However, activation of ErbB3 likely plays an important role (1–5). Inhibition of ErbB3 activity is, therefore, a promising approach to addressing some of the limitations posed by drugs that are currently in use.

We have used a novel biologic scaffold termed a surrobody (6) to identify 2 ErbB3 inhibitors, SL-175 and SL-176. Surrobodies are derived from the pre-B-cell receptor complex that, in nature, is the universal precursor to

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the B-cell receptor. They are composed of a diversified immunoglobulin heavy chain complexed with an invariant surrogate light chain (Supplementary Fig. S1) that together confer specific high-affinity binding to their targets.

In this article, we show that SL-175 and SL-176 are potent inhibitors of ErbB3 both *in vitro* and *in vivo*. In addition, we show that these molecules enhance the antiproliferative activities of other ErbB family-targeted agents, highlighting the potential for direct ErbB3 inhibition in a combination setting. Moreover, in contrast to previously described anti-ErbB3 antibodies, SL-175 and SL-176 inhibit both neuregulin (NRG)-driven and ligand-independent AKT signaling and proliferation in cells that overexpress ErbB2. The results suggest that both of these surrobodies act by a novel mechanism.

Materials and Methods

Cell culture and proliferation assays

All cell lines were obtained from American Type Culture Collection and propagated as recommended. No independent authentication of cell lines was carried out. For proliferation assays in non-ErbB2–overexpressing cell lines, cells were plated in serum-free medium, treated for 1 hour with test article, and then treated with 1.25 nmol/L NRG1β EGF domain (R&D Systems, hereafter referred to as NRG). Cells were then grown for 96 hours and relative cell number was determined using CellTiter-Glo (Promega). For ErbB2-overexpressing lines (SKBR3, BT-474, and N87), cells were treated for 1 hour with test article before stimulation with 0.125, 0.375, or 1.25 nmol/L NRG, respectively, and then grown for 3 days (SKBR3, N87) or 6 days (BT-474) in complete medium. For ligand-independent growth, test article was added to complete medium and cells were grown in the absence of NRG for 6 days. Lapatinib, GDC-0941, and erlotinib (Fig. 7) were obtained from LC Laboratories.

Expression and purification of surrobodies and antibodies

Trastuzumab, pertuzumab, and cetuximab were either purchased and/or expressed in HEK293F cells using the humanized sequences of 4D5 (7), 2C4 (8), or C225 (9), respectively. Surroglobulins (SgGs) and IgGs were purified via protein A chromatography.

Affinity determinations

Affinities of the surrobodies were determined by binding to immobilized ErbB3-Fc (R&D Systems) by ELISA or by binding to cells by flow cytometry. Affinity values reported represent the concentration at which half maximal binding was achieved as calculated by GraphPad Prism using a 4 parameter fit.

Phosphorylation assays

Cells were seeded in a 12-well plate in serum-free medium and starved overnight. They were then incubated with Surrobody for 1 hour followed by stimulation with 1.25 nmol/L NRG for 12 minutes. For testing ligand-independent signaling in ErbB2-overexpressing cells, the cells were seeded in 12-well plates in complete medium and incubated with surrobody for 1 hour. Cells were lysed and pErbB3, p-AKT, and p-ERK were determined in triplicate using PathScan ELISA kits (Cell Signaling Technology). Total protein was determined in the lysates using a BCA Protein Assay Kit (Pierce).

In vivo studies

All animal studies were conducted at Piedmont Research Center, Charles River Discovery and Imaging Services, in compliance with the recommendations of the Guide for Care and Use of Laboratory Animals.

A total of 5×10^6 BxPC-3 cells were injected subcutaneously as a Matrigel suspension into nude mice and allowed to grow to an average of 175 mm^3. A 25 mg/kg loading dose of the indicated compound was administered, followed by maintenance doses of 12.5 mg/kg twice weekly for the duration of the study.

A total of 1×10^7 NCI-N87 cells were injected subcutaneously as a Matrigel suspension into C.B-17 SCID mice. When tumors reached 120 to 170 mm^3, they were dosed as indicated above.

For both studies, 10 mice were evaluated per treatment group. A Student *t* test was used to assess statistical significance using GraphPad Prism.

Results

Identification of anti-ErbB3 surrobodies

We have previously shown that surrobodies can be expressed in *E. coli* and are amenable to phage display (6). In addition, surrobodies with high affinity and selectivity to a therapeutically significant target were identified by panning a diverse population of phage-displayed surrobodies.

We constructed a 2.95×10^{10} member fully human phage displayed surrobody library (Xu and colleagues, unpublished data) and panned it against an ErbB3-Fc fusion protein. Sixty-nine unique clones were identified encoding surrobodies that bind to ErbB3-Fc as determined by ELISA. Monovalent surrobodies (Supplementary Fig. S1) were prepared from *E. coli* extracts and evaluated for their ability to inhibit NRG-induced dimerization of ErbB2 and ErbB3 using the U2OS PathHunter Functional Assay (DiscoveRx). Eight surrobodies were identified that showed inhibitory activity. Two, SL-175 and SL-176, were chosen for further characterization. They were expressed as bivalent surrobodies (Supplementary Fig. S1) using full-length heavy chains in HEK293 cells as described (6). The resulting surrobodies, termed SgGs (for surroglobulins), were expressed at levels yielding between 89 and 154 mg/L of culture and formed the basis of all subsequent analysis.

The SgGs were evaluated by ELISA for their ability to bind ErbB3-Fc. Both SL-175 and SL-176 bound human

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ErbB3-Fc with affinities less than 50 pmol/L and showed strong cross-reactivity with murine and cynomolgus ErbB3. These 2 clones likely bind to the same or overlapping epitopes as they compete with one another for binding. SL-175 and SL-176 were also evaluated by flow cytometry for their ability to bind ErbB3 on human pancreatic BxPC-3 cells. On these cells the affinity was 40 pmol/L for SL-176 and 138 pmol/L for SL-175 (Supplementary Table S1). No binding was observed to EGFR or ErbB2 by ELISA (Supplementary Fig. S2). In further support for the specificity of these surrobodies to ErbB3, cell surface staining with these agents was not impacted in cell lines that vary dramatically in cell surface expression of EGFR and ErbB2.

Surrobodies suppress NRG-stimulated cell signaling and proliferation

Both SL-175 and SL-176 were further evaluated for their ability to inhibit ErbB3 function in *in vitro* cell proliferation assays. The anti-ErbB3 surrobodies suppressed NRG-driven cell growth in 8 different human cell lines derived from a broad range of tumor types. Maximum suppression ranged between 40% to 80% and potencies were approximately 1 to 100 nmol/L (Fig. 1). Overall suppression was superior to that observed using the ErbB2/ErbB3 dimerization inhibitor pertuzumab (10) in both ErbB2-overexpressing (Fig. 1F–H) and nonoverexpressing (Fig. 1D) cells. Notably, these anti-ErbB3 surrobodies reduced proliferation of A549 cells that harbor a *K-ras* gene mutation (Fig. 1E) and Colo205 cells that harbor a *BRAF* mutation (Fig. 1B).

Because ErbB3 is a pivotal regulator of AKT activation, the effect of treating cells with anti-ErbB3 surrobodies on NRG-stimulated intracellular signaling was examined. BxPC-3 cells (Fig. 2A–C) or SKBR3 cells (which overexpress ErbB2; Fig 2D–F), were treated with SL-175 or SL-176 followed by NRG stimulation and examined for their levels of phospho-AKT, phospho-ErbB3, and phospho-ERK1/2. The anti-ErbB3 surrobodies reduced the levels of all 3 active phosphorylated species to the level of unstimulated cells, or lower, in both cell lines (Fig. 2A–F). Concentrations as low as 1 nmol/L were sufficient to inhibit phosphorylation. The ability of the surrobodies to inhibit ErbB3 phosphorylation and both the AKT and ERK downstream pathways thus corresponds to their ability to suppress cellular proliferation.

Surrobodies suppress *in vivo* tumor growth

To determine whether suppression of proliferation *in vitro* correlates with reduction in tumor growth, SL-175 and SL-176 were individually used to treat established BxPC-3 tumors in nude mice. As a comparator, an

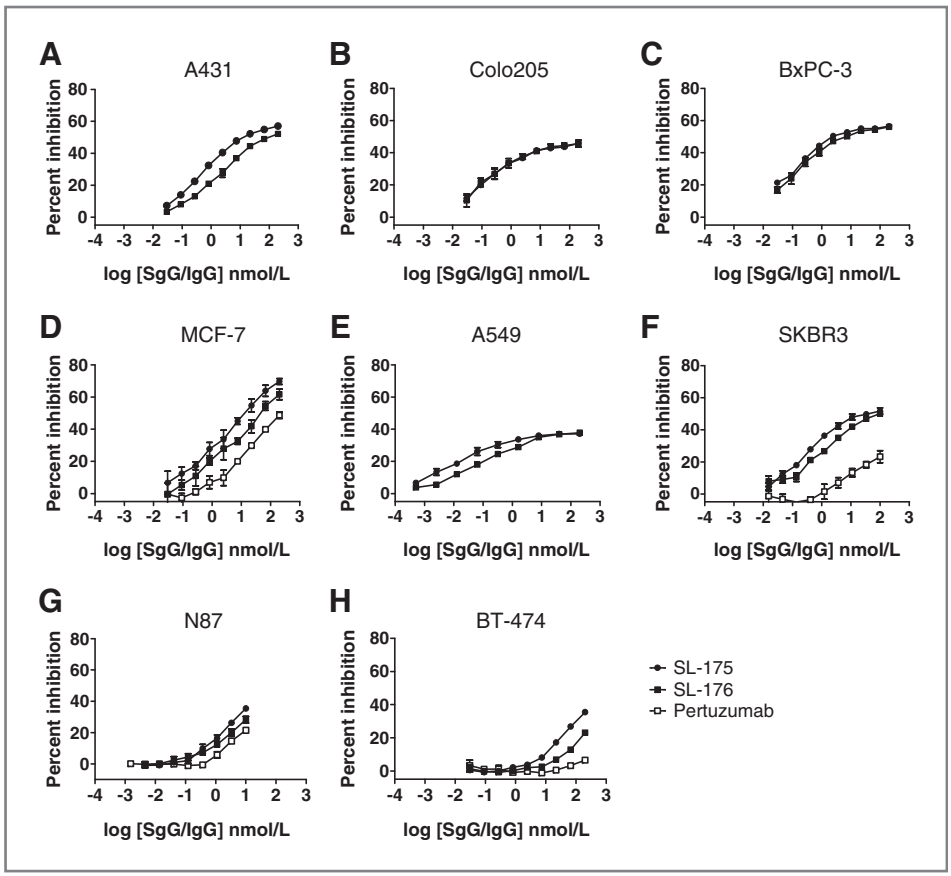

Figure 1. SL-175 and SL-176 inhibit NRG-dependent proliferation of a variety of cell lines. The designated cell lines were incubated with indicated amounts of surrobody (SgG) or antibody (IgG) for 1 hour before stimulation with NRG. Percent inhibition was determined relative to untreated cells. Error bars indicate SD.

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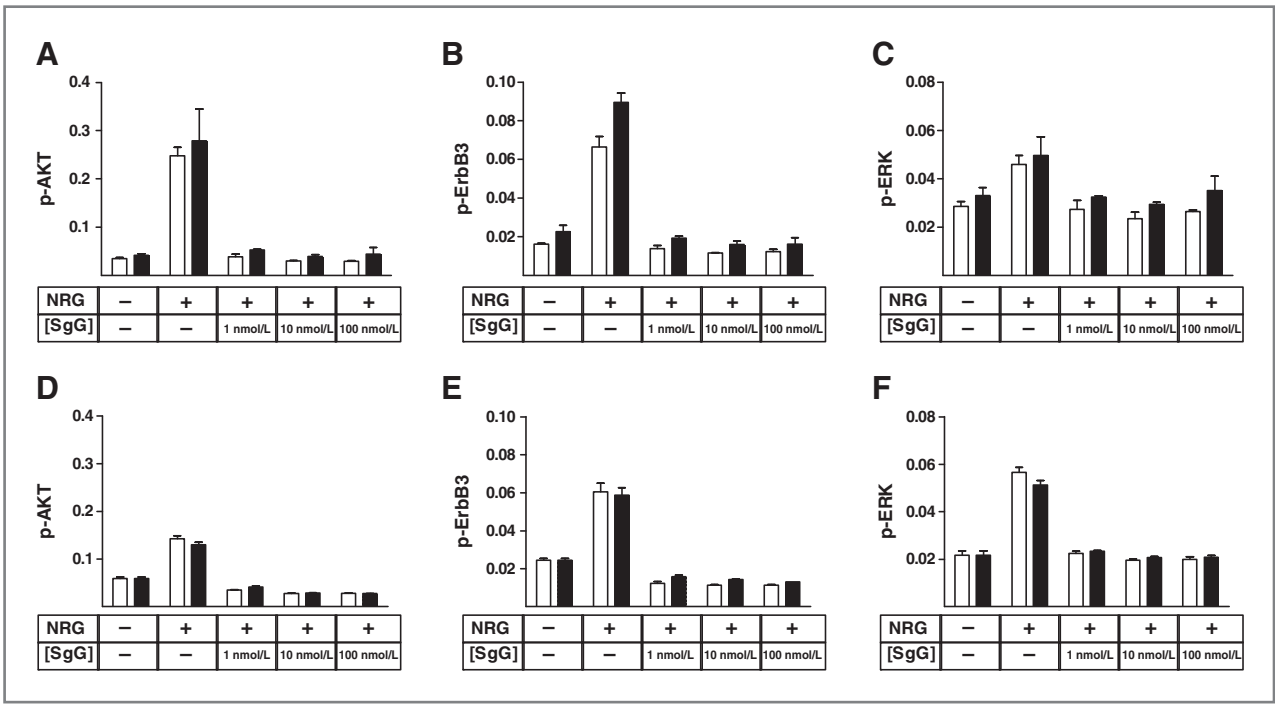

Figure 2. Inhibition of NRG-stimulated signaling by SL-175 and SL-176. BxPC-3 cells (A–C) or SKBR3 cells (D–F) were treated with either SL-175 (open bars) or SL-176 (filled bars), then stimulated with NRG. Phospho-AKT (Ser473; A and D), phospho-ErbB3 (panTyr; B and E), and phospho-ERK1/2 (Thr202/Tyr204; C and F) were determined in cell lysates by ELISA. *Y*-axis units are A_{450}/μg protein. Error bars indicate SD.

additional group was treated with cetuximab. Both Surrobodies significantly reduced tumor growth and conferred a marked survival advantage compared with control PBS-treated animals (Fig. 3). Specifically, tumor growth delay for SL-176 was 40.4 days ($P < 0.05$) and tumor growth delay for SL-175 was 19.5 days ($P < 0.05$). In addition, the SL-176 group showed a 20% incidence (2 of 10) of complete tumor remissions at study termination (day 106). Four of the remaining mice treated with SL-176 exhibited partial tumor regressions. The mean tumor size in animals treated with SL-176 was also significantly reduced compared with animals treated with cetuximab ($P = 0.006$ at day 18). In the SL-175 group there was a single mouse that exhibited a complete remission.

Both surrobodies were well tolerated. No treatment-related deaths, unusual clinical signs, or post-study necropsy findings were noted.

Activity in ErbB2-overexpressing tumor cell lines

ErbB2 has no known ligand and its overexpression is sufficient to drive cell proliferation in a ligand-independent manner. In tumors driven by ErbB2 overexpression, ErbB3 has been shown to be an obligate dimerization partner for ErbB2 (11, 12). The anti-ErbB2 antibody trastuzumab predominantly inhibits ligand-independent proliferation, which in part is attributed to binding to domain IV of ErbB2 and thereby inhibiting interaction of ErbB2 with unstimulated ErbB3 (13). In

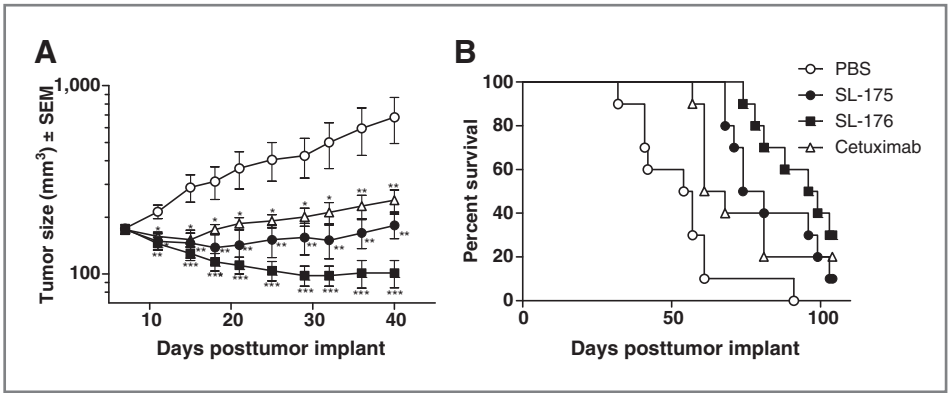

Figure 3. SL-175 and SL-176 inhibit BxPC-3 tumor growth *in vivo*. Animals received a 25 mg/kg loading dose of the indicated test article followed by twice weekly injections at 12.5 mg/kg. A, mean tumor volume ± SEM. Student *t* test was used to assess statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). B, Kaplan–Meier plot indicating percent survival.

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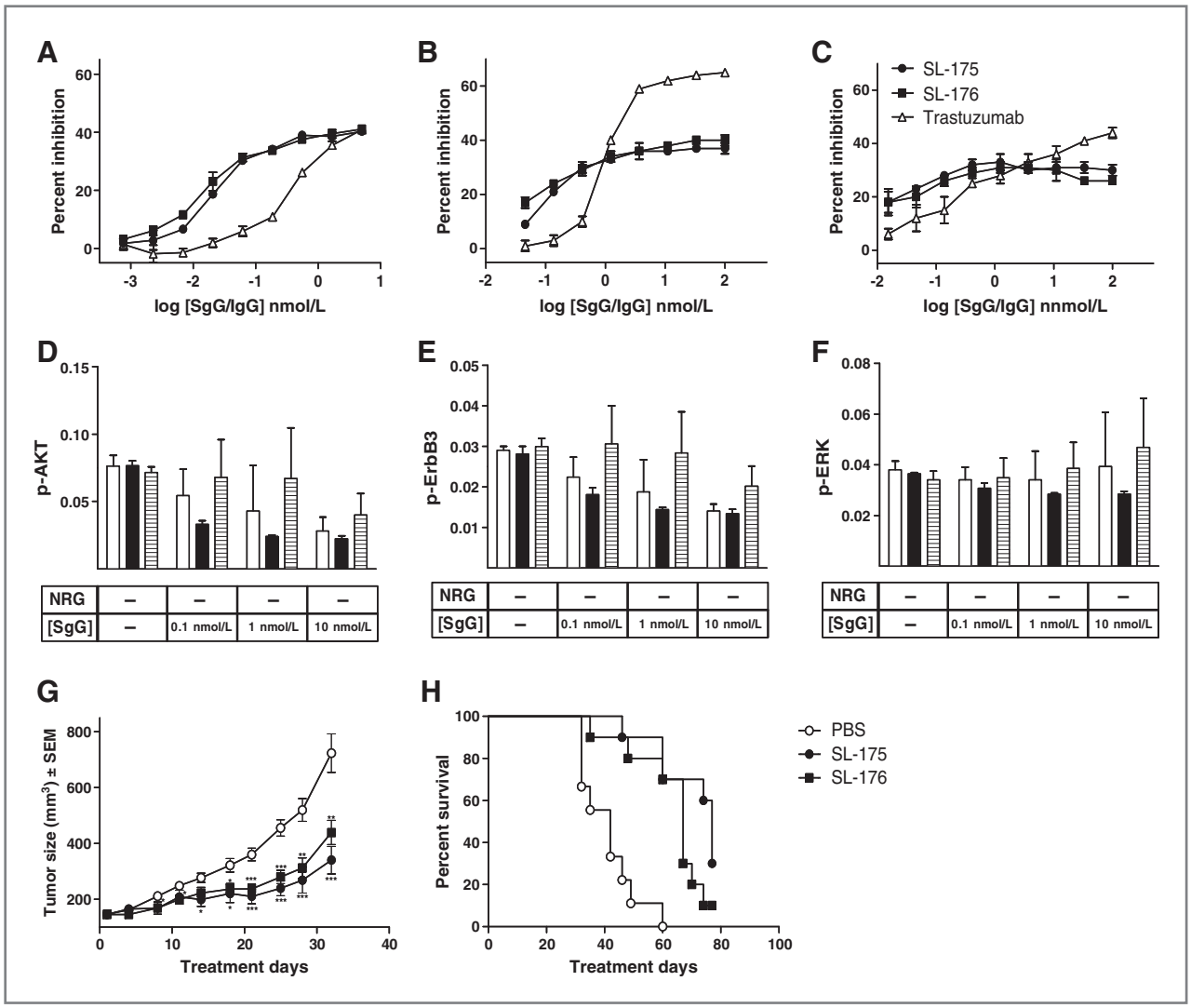

Figure 4. SL-175 and SL-176 reduce AKT signaling and proliferation in ErbB2-overexpressing cells *in vitro* and *in vivo*. SKBR3 cells (A), BT-474 cells (B), or NCI-N87 (C) cells were treated for 6 days with the indicated surrobody (SgG) or antibody (IgG). Percent growth inhibition was determined relative to untreated cells. D–F, SKBR3 cells were treated for 1 hour with the indicated amounts of SL-175 (open bars), SL-176 (filled bars), or trastuzumab (hatched bars). Phospho-AKT (Ser473; D), phospho-ErbB3 (panTyr; E), and phospho-ERK1/2 (Thr202/Tyr204; F) were determined by ELISA. *Y*-axis units are A_{450}/μg protein. Error bars indicate SD. G, NCI-N87 tumor growth in C.B-17 SCID mice. Animals received a 25 mg/kg loading dose of the indicated test article followed by twice weekly injections of 12.5 mg/kg. Values represent mean tumor volume ± SEM. Student *t* test was used to assess statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). H, Kaplan–Meier plot indicating percent survival.

contrast, the anti-ErbB2 antibody pertuzumab primarily inhibits ligand-driven proliferation by inhibiting dimerization via the canonical dimerization domain (domain II; refs. 10, 14).

The activities of previously described anti-ErbB3 antibodies are limited to inhibition of ligand-driven signaling (15). To test whether the activities of the anti-ErbB3 surrobodies were similarly restricted to ligand-dependent growth, we examined their activity on unstimulated ErbB2-overexpressing cells. Two breast cancer cell lines (SKBR3 and BT-474) and one gastric cancer cell line (NCI-N87) were chosen. We (data not shown) and others (13, 15) have determined that these cells do not secrete appreciable levels of ErbB3 ligand. Remarkably, SL-175 and SL-176 reduced proliferation in all 3 cell lines even in the absence of NRG. Furthermore, in SKBR3 cells the surrobodies were as effective as trastuzumab, with markedly superior potency (Fig. 4A). Similarly in BT-474 cells and NCI-N87 cells, the Surrobodies exhibited superior potency, though trastuzumab generated a higher overall percent inhibition (Fig. 4B and C).

To investigate how the anti-ErbB3 surrobodies affect proliferative signaling pathways in unstimulated cells, we examined the levels of phospho-ErbB3, phospho-AKT, and phospho-ERK1/2 upon exposure to several

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concentrations of SL-175 and SL-176. Both phospho-ErbB3 and phospho-AKT were reduced in a dose-dependent manner (Fig. 4D and E). Consistent with their superior potency in proliferation assays, the anti-ErbB3 surrobodies were more effective than trastuzumab in reducing ErbB3 and AKT phosphorylation at low doses. Neither the anti-ErbB3 surrobodies nor trastuzumab significantly reduced phospho-ERK1/2 levels (Fig. 4F). Similar results were obtained with BT-474 cells (data not shown).

To determine whether the novel ability to suppress ErbB2-driven proliferation is also manifest *in vivo*, SL-175 and SL-176 were individually used to treat established N87 tumors in C.B-17 SCID mice. The surrobodies significantly delayed tumor growth by 84% and 62%, respectively, and conferred a considerable survival advantage (Fig. 4G and H).

Taken together, these results show that the anti-ErbB3 surrobodies reduce cell proliferation both when it is driven by NRG and when it is driven by ErbB2 overexpression. We therefore surmise that the anti-ErbB3 surrobodies act by a mechanism distinct from previously described anti-ErbB3 antibodies that purportedly exclusively inhibit NRG-stimulated growth (15, 16)

Anti-ErbB3 surrobodies surpass pertuzumab in their ability to augment the activity of trastuzumab in ErbB2-overexpressing cells

Combination treatment of ErbB2-overexpressing tumors with trastuzumab and pertuzumab provides improved efficacy over individual agent treatment regimens in animal models and in the clinic (17–20). Because ErbB2-overexpressing tumors are likely to be heterogeneous with respect to the amount of NRG in the tumor microenvironment, the superior efficacy of combination treatment may result from the differential abilities of pertuzumab and trastuzumab to inhibit NRG-driven and ligand-independent signaling, respectively.

We investigated whether the anti-ErbB3 surrobodies provide a benefit analogous to pertuzumab in combination with trastuzumab. SL-175 or pertuzumab were tested as single agents or in combination with trastuzumab in ErbB2-overexpressing cells (Fig. 5). Consistent with previous observations (13), pertuzumab, as a single agent, had little activity in the absence of NRG in SKBR3 cells. Moreover, pertuzumab displayed little, if any, enhancement of trastuzumab activity when the 2 were combined (Fig. 5A). In contrast, the combination of SL-175 and trastuzumab was considerably more effective at suppressing proliferation than either SL-175 alone or trastuzumab

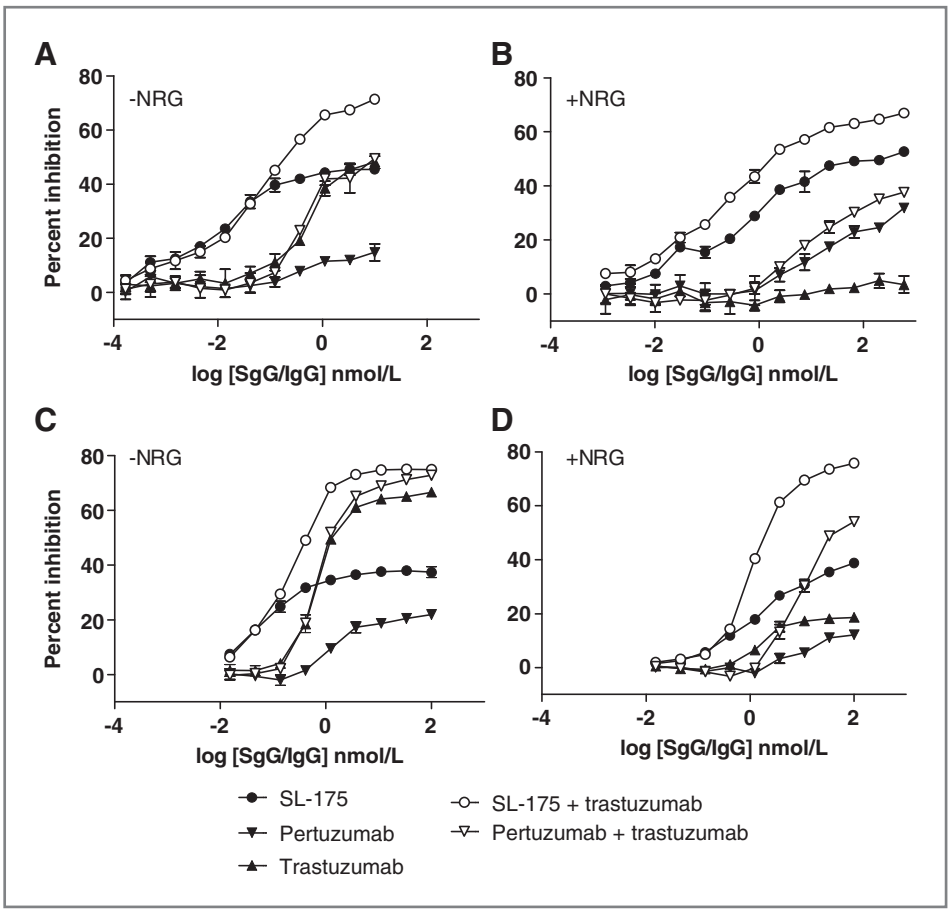

Figure 5. Combining SL-175 with trastuzumab augments inhibition of proliferation. SKBR3 cells (A and B) or BT-474 cells (C and D) were treated with trastuzumab or a combination of trastuzumab and either SL-175 or pertuzumab, each at the indicated concentration. A and C, cells were grown in the absence of ligand. B and D, cells were treated with NRG. Error bars indicate SD.

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alone. Similar results were seen in BT-474 cells (Fig. 5C), though the advantage of the SL-175/trastuzumab combination over trastuzumab or the pertuzumab/trastuzumab combination was manifest primarily as an increase in potency. Analogous experiments comparing SL-176 in combination with trastuzumab to the pertuzumab/trastuzumab combination showed similar beneficial results (Supplementary Fig. S3).

Combinations of anti-ErbB3 surrobodies with trastuzumab were also tested in the same cell lines stimulated with NRG. In accordance with previous findings (21), trastuzumab had very limited activity as a single agent under these conditions. Remarkably, however, the SL-175/trastuzumab combination was substantially more effective than SL-175 alone (Figs. 5B and D). Furthermore, the SL-175/trastuzumab combination was far more potent and effective at reducing proliferation than the pertuzumab/trastuzumab combination. Again similar results were obtained when SL-176 was combined with trastuzumab, showing superiority to the trastuzumab/pertuzumab combination (Supplementary Fig. S3). Preliminary analysis suggests the ErbB3 surrobodies synergize with trastuzumab with confidence interval (CI) values ranging from approximately 0.1 to 0.8 depending on the cell line and whether NRG is present or absent.

Anti-ErbB3 surrobodies augment the activity of targeted kinase inhibitors in ErbB2-overexpressing cells

Trastuzumab acts synergistically with the dual EGFR/ErbB2 kinase inhibitor lapatinib in ErbB2-overexpressing cells *in vitro* and *in vivo* (22). Because the anti-ErbB3 surrobodies displayed antiproliferative activity on ErbB2-overexpressing cells, it was of interest to determine whether they similarly augment lapatinib. We carried out an experiment in which a single concentration (10 nmol/L) of antibody or surrobody was combined with varying concentrations of lapatinib. This concentration for the biologic agents was chosen because it is physiologically achievable and represents the approximate plateau of inhibitory activity for SL-175, SL-176, and trastuzumab in these cells in the absence of NRG (Fig. 4A). As expected, trastuzumab dramatically enhanced the antiproliferative activity of lapatinib in SKBR3 cells (Fig. 6A). Remarkably, the anti-ErbB3 surrobodies enhanced activity to a similar extent as trastuzumab, whereas pertuzumab showed a more modest benefit under these conditions.

The same combinations were tested on cells grown in the presence of NRG. In accordance with previously published results (23), we found that NRG dramatically

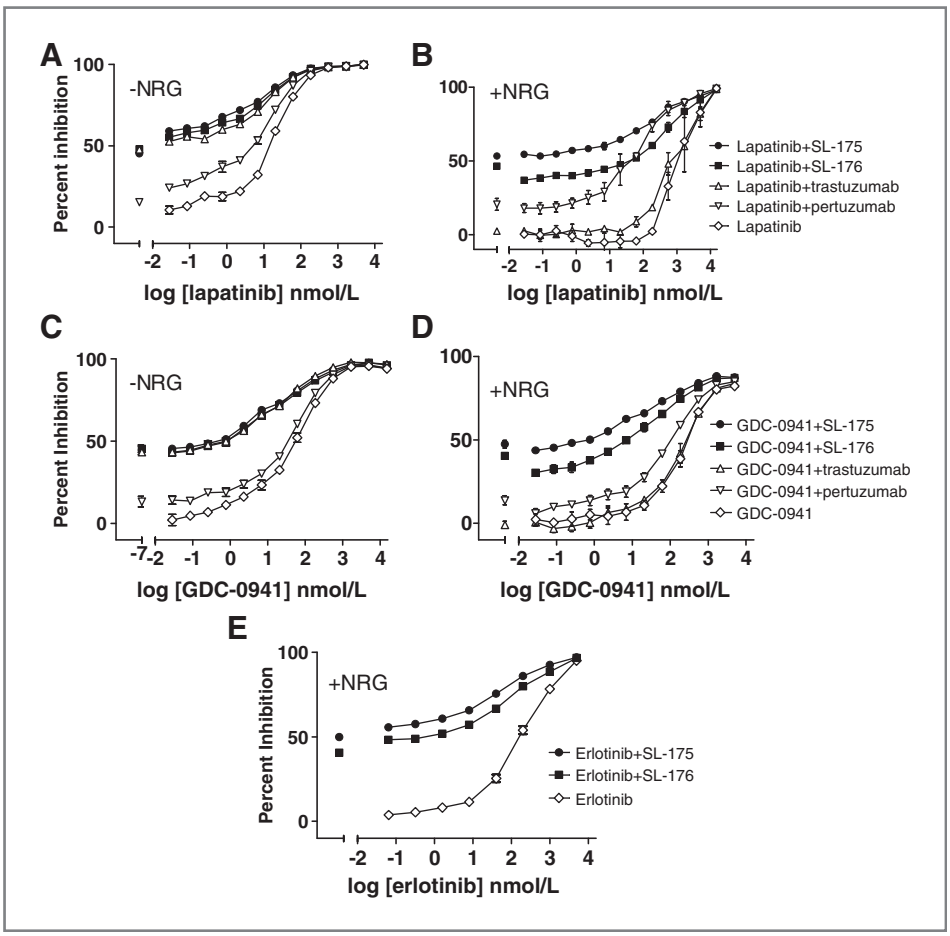

Figure 6. Combining SL-175 and SL-176 with targeted kinase inhibitors augments inhibition of proliferation. A, SKBR3 cells were treated with the indicated concentrations of lapatinib alone, or in combination with 10 nmol/L SL-175, SL-176, pertuzumab, or trastuzumab. B, SKBR3 cells were treated as in A, except that they were stimulated with NRG. C, SKBR3 cells were treated with the indicated the concentrations of GDC-0941 alone or in combination with 10 nmol/L SL-175, SL-176, pertuzumab, or trastuzumab. D, SKBR3 cells were treated as in C, except that they were stimulated with NRG. E, A431 cells were treated with the indicated concentrations of erlotinib alone, or in combination with 10 nmol/L SL-175, SL-176. A431 cells were stimulated with 1.25 nmol/L NRG. In all panels, the values to the left of the break in the *x*-axis correspond to the level of inhibition achieved with the indicated SgG or IgG as a single agent at 10 nmol/L. Error bars indicate SD.

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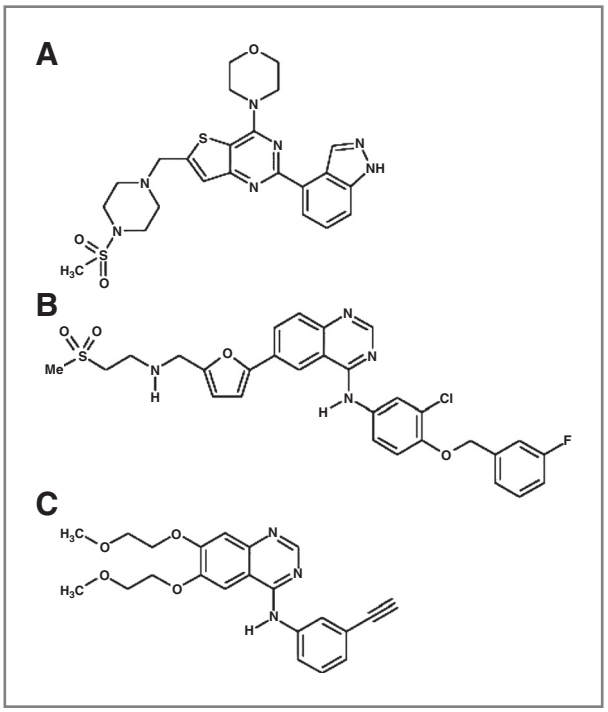

Figure 7. Chemical structures. The chemical structures of GDC-0941 (A), lapatinib (B), and erlotinib (C) are indicated.

decreased the potency of lapatinib from an IC_{50} of 17 nmol/L to an IC_{50} of approximately 1 μmol/L, supporting the notion that that ErbB3 activation may limit its efficacy (Fig. 6B). We found that in NRG-stimulated cells, the antiproliferative activity of the trastuzumab/lapatinib combination was not appreciably different from that of lapatinib alone. In contrast, combining lapatinib with SL-175 substantially improved the overall potency of inhibition. SL-176 also maintained its ability to augment lapatinib in the presence of NRG, though to a lesser extent than SL-175. By comparison, pertuzumab enhanced the inhibitory activity of lapatinib, though not to the extent of either of the anti-ErbB3 surrobodies.

Direct inhibition of ErbB3 via anti-ErbB3 surrobodies thus appears to enhance the activity of lapatinib more effectively than inhibiting ErbB2/ErbB3 dimerization inhibition via pertuzumab. Moreover, in contrast to trastuzumab, the anti-ErbB3 surrobodies maintain their ability to augment lapatinib in the presence or in the absence of ligand.

GDC-0941 is another targeted kinase inhibitor in development for treatment of ErbB2-overexpressing tumors. GDC-0941 is a potent and selective inhibitor of PI3K (24) whose activity is markedly influenced by phosphorylation of ErbB3. Trastuzumab and GDC-0941 synergistically inhibit the PI3K/AKT pathway, cell proliferation, and *in vivo* tumor growth (13). Inhibition of the PI3K/AKT pathway leads to compensatory increases in phosphorylated ErbB3, which is linked to AKT-mediated negative feedback signaling (3, 13). It was therefore of interest to determine whether surrobody-mediated inhibition of ErbB3 impacts the potency of GDC-0941 in proliferation assays.

In agreement with previously published data, trastuzumab dramatically enhanced the ability of GDC-0941 to inhibit ligand-independent proliferation (Fig. 6C). Interestingly, SL-175 and SL-176 enhanced its activity to a similar degree as trastuzumab. As was observed for lapatinib, stimulating with NRG decreased the potency of GDC-0941. However, the decrease in potency and resulting shift in IC_{50} was far smaller than that observed for lapatinib (from 50–176 nmol/L; Fig. 6D). As in combinations with lapatinib, the anti-ErbB3 surrobodies maintained their ability to potentiate GDC-0941 whereas trastuzumab did not. Pertuzumab augmented GDC-0941 in the presence of NRG but to a lesser extent than the anti-ErbB3 surrobodies.

Anti-ErbB3 surrobodies augment the activity of EGFR inhibitors

EGFR is an oncogenic driver in many tumor types. Combined treatment with EGFR inhibitors and an anti-ErbB3 antibody has been shown to be beneficial for inhibiting tumor growth preclinically (25) and is currently being tested in the clinic.

Because the anti-ErbB3 surrobodies appear to work through a distinct mechanism of action, we queried whether they, like previously described anti-ErbB3 antibodies, augment EGFR inhibitors. The anti-proliferative activities of SL-175 or SL-176 combined with the kinase inhibitor erlotinib were determined in A431 cells (Fig. 6E). Combination treatments were more effective at inhibiting proliferation than erlotinib or anti-ErbB3 surrobodies alone. In corresponding experiments, the anti-ErbB3 Surrobodies were examined for their ability to augment the activity of cetuximab. In these experiments, the addition of either anti-ErbB3 surrobody substantially increased the antiproliferative effects of cetuximab (Supplementary Fig. S4). The surrobodies, therefore, have retained the ability to impact the EGFR signaling axis in addition to impacting the ErbB2 signaling axis in a novel manner.

Discussion

Here, we show the *in vitro* and *in vivo* antitumor capacities of 2 surrobody molecules directed against ErbB3. These results further show the capabilities of this novel scaffold to specifically and potently address therapeutic targets in a manner that is comparable with antibody-based therapeutics.

Both of the described anti-ErbB3 surrobodies appear to engage ErbB3 in a manner that confers additional activities beyond those previously described for ErbB3 antagonists. Specifically, in addition to inhibiting NRG-dependent cell activation, the surrobodies reduce proliferation and AKT signaling that is driven by overexpression of ErbB2. At this time it is unclear whether these unique

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activities are enabled by the surrobody structure. This activity may allow for broader therapeutic use compared with previously described ErbB3 inhibitory antibodies that inhibit only NRG-driven signaling.

In ErbB2-overexpressing tumors, the ErbB2/ErbB3 dimer is a particularly important oncogenic driver. Trastuzumab is commonly used to treat these tumors and one mechanism by which trastuzumab inhibits tumor growth appears to be through inhibition of the ligand-independent dimerization of ErbB2 with ErbB3 (13). However, trastuzumab, as a single agent, is unable to inhibit NRG-dependent dimerization, and elaboration of NRG is a possible mechanism by which some tumors acquire trastuzumab resistance. The ErbB2-binding antibody pertuzumab inhibits ligand-driven formation of ErbB2/ErbB3 heterodimers. Coadministration of trastuzumab and pertuzumab strongly enhances growth inhibition *in vitro* and *in vivo* (17). Accordingly, pertuzumab has shown great promise in combination with trastuzumab in the clinic (20).

However, as shown in Fig. 5, the ability of pertuzumab to augment trastuzumab *in vitro* is primarily manifest when cells are stimulated with NRG. In contrast, the anti-ErbB3 surrobodies augmented the activity of trastuzumab regardless of whether NRG was present or absent. Moreover, the surrobody/trastuzumab combinations were more potent and more effective than the pertuzumab/trastuzumab combinations under both conditions. Given the likely heterogeneity in the tumor microenvironment with respect to the presence of NRG, this breadth of activities provides a compelling case that both of the anti-ErbB3 surrobodies described here hold significant promise for combination treatment.

Our studies showed that the anti-ErbB3 surrobodies were also able to augment the activity of targeted kinase inhibitors. A number of ErbB-targeted kinase inhibitors are in clinical use or in development, but their inhibition of ErbB signaling can result in compensatory alterations, such as upregulation of ErbB3 expression (3, 26–29). In addition, NRG dramatically diminishes the effectiveness of these agents *in vitro* (23). In experiments with ErbB2-overexpressing cells we found trastuzumab augmented lapatinib (as has been previously shown; refs. 22, 23), but only in the absence of ErbB3 ligand stimulation. In contrast, pertuzumab partially augmented lapatinib only in the presence of NRG. By comparison, each of the anti-ErbB3 surrobodies augmented lapatinib both in NRG-stimulated and in unstimulated cells, and abrogated the ability of NRG to diminish lapatinib effectiveness. Analogously, trastuzumab and pertuzumab only conditionally improved the antiproliferative activity of the PI3K inhibitor, GDC-0941. Again, in sharp contrast to trastuzumab and pertuzumab, both of the anti-ErbB3 surrobodies augmented GDC-0941 activity regardless of the presence or absence of NRG. Taken together, these results suggest that combining an anti-ErbB3 surrobody with these respective kinase inhibitors might decrease resistance and improve their therapeutic index compared with combination treatments using the anti-ErbB2 antibodies. However, because anti-ErbB2 antibodies can confer antibody-dependent cell-mediated cytotoxicity toward ErbB2-overexpressing tumors *in vivo*, the relative role of ErbB3 inhibition in this context remains to be seen.

It is likely that the anti-ErbB3 surrobodies inhibit cell signaling and proliferation as a composite of multiple distinct ErbB3-based mechanisms. Information is provided in Supplementary Fig. S5 that the anti-ErbB3 surrobodies decreased ErbB3 cell surface expression. However, induction of receptor internalization has also been reported for anti-ErbB3 antibodies that do not inhibit ligand-independent signaling driven by ErbB2 overexpression (30). Junttila and colleagues (13) and others have raised the possibility that when ErbB2 is overexpressed in the absence of ligand, the ErbB2/ErbB3 interaction is fundamentally different from the canonical ligand-induced, receptor-mediated dimerization model proposed by structural studies. Trastuzumab, which binds to domain IV of ErbB2, appears to disrupt this interaction and may also influence the composition or existence of higher order oligomers of ErbBs (31). SL-175 and SL-176 may analogously bind to a domain that similarly disrupts ErbB2/ErbB3 interactions. The fact that trastuzumab combined with either of the anti-ErbB3 surrobodies is more effective than the single agents may indicate that neither type of agent completely disrupts these interactions and/or that disruption with the 2 different agents may be occurring through different means.

We, and others, have also shown that combining ErbB3 inhibition and EGFR inhibition is beneficial. It is noteworthy that even though the anti-ErbB3 surrobodies apparently uniquely engage ErbB3 they maintain the important ability to augment inhibition of the EGFR axis.

These results in total suggest that the anti-ErbB3 Surrobodies encompass the breadth of beneficial activities previously described for anti-ErbB3 antibodies currently in development. These anti-ErbB3 agents therefore show substantial promise for therapeutic use, either singly or in combination with other agents, and may afford greater therapeutic potential than previously described anti-ErbB3 antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P.K. Foreman, M. Gore, P.A. Kobel, R.R. Bhatt
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.K. Foreman, M. Gore, P.A. Kobel, C. Hannum, S.M. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.K. Foreman, M. Gore, P.A. Kobel, C. Hannum, R.R. Bhatt
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