Function-Blocking ERBB3 Antibody Inhibits the Adaptive Response to RAF Inhibitor

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Abstract

ERBB3/HER3 expression and signaling are upregulated in mutant BRAF melanoma as an adaptive, prosurvival response to FDA-approved RAF inhibitors. Because compensatory ERBB3 signaling counteracts the effects of RAF inhibitors, cotargeting ERBB3 may increase the efficacy of RAF inhibitors in mutant BRAF models of melanoma. Here, we corroborate this concept by showing that the ERBB3 function-blocking monoclonal antibody huHER3-8 can inhibit neuregulin-1 activation of ERBB3 and downstream signaling in RAF-inhibited melanoma cells. Targeting mutant BRAF in combination with huHER3-8 decreased cell proliferation and increased cell death in vitro, and decreased tumor burden in vivo, compared with targeting either mutant BRAF or ERBB3 alone. Furthermore, the likelihood of a durable tumor response in vivo was increased when huHER3-8 was combined with RAF inhibitor PLX4720. Together, these results offer a preclinical proof of concept for the application of ERBB3-neutralizing antibodies to enhance the efficacy of RAF inhibitors in melanoma to delay or prevent tumor regrowth. As ERBB3 is often upregulated in response to other kinase-targeted therapeutics, these findings may have implications for other cancers as well. Cancer Res; 74(15); 4122–32. ©2014 AACR.

Introduction

BRAF (v-raf murine sarcoma viral oncogene homolog B1) is a serine/threonine kinase that is mutated in approximately 50% of melanomas. The prevailing BRAF mutation is a valine to glutamic acid missense mutation at amino acid 600 (V600E) that results in a constitutively active kinase (1). BRAFV600E signals in a deregulated manner through the MEK–ERK1/2 pathway, which promotes cell-cycle progression, survival, and invasion. Vemurafenib and dabrafenib are small-molecule inhibitors with selectivity toward mutant BRAF versus wild-type BRAF in melanoma cells (2, 3) and are FDA approved for patients with metastatic BRAFV600E melanoma. In phase III clinical trials, patients with BRAFV600E melanoma receiving vemurafenib or dabrafenib had RECIST-criteria response rates of 48% and 50%, and median progression-free survival (PFS) of 5.3 months and 5.1 months, respectively. These effects were a significant improvement compared with the 5% to 7% response rate and 1.6 to 2.7 months PFS for patients on dacarbazine, the previous standard of care. Unfortunately, approximately 15% of patients fail to achieve any tumor shrinkage with vemurafenib or dabrafenib treatment, and almost all responding patients develop resistance within 1 year (3, 4). Mechanisms of acquired resistance that develop following continued exposure of tumor to vemurafenib frequently include expression of mutant RAS and aberrant BRAF splice variants (5). In addition, the combination of the mutant BRAF inhibitor dabrafenib and the MEK inhibitor trametinib has recently received accelerated FDA approval for the treatment of patients with BRAFV600E melanoma. This was based on phase I/II clinical trial data showing a RECIST-criteria response rate of 76% and a median survival of 9.4 months (6). Although the approval of the dabrafenib/trametinib combination therapy marks an improvement in the treatment of mutant BRAF melanomas, the issue of relapse and the need to improve efficacy remain.

One approach to improve the clinical benefit of RAF inhibitors is to identify strategies that produce more effective induction of tumor cell killing. In contrast to acquired resistance, much less is known about the adaptive mechanisms that are rapidly switched on in the presence of RAF inhibitor and promote cell survival. Initial preclinical models indicate a resetting of the ERK1/2 pathway (7) and upregulation and enhanced neuregulin-1 (NRG1) activation of ERBB3/HER3 in BRAFV600E melanoma cells (8). Enhanced ERBB3 signals in concert with ERBB2 leading to activation of the PI3K–AKT (v-akt murine thymoma viral oncogene) pathway, which has been implicated in the survival of melanoma cells in response to vemurafenib (9–11). Although there is much effort to develop PI3K–AKT inhibitors to use in combination with RAF inhibitors, their use clinically has been limited by toxicities (12). Furthermore, experience from other tumor types indicates that the efficacy of PI3K–AKT inhibitors will be
hampered by upregulation of ERBB3 signaling as a compensatory response (13). On the basis of these findings, we have tested the signaling and antitumor effects of directly targeting ERBB3 in combination with vemurafenib in BRAF-mutant melanoma cells. These experiments showed that the anti-ERBB3 antibody, huHER3-8, inhibits the enhanced NRG1 signaling that is observed in RAF-inhibited melanoma cells, with concomitant decreased proliferation and survival of cells in vitro. The combination of RAF inhibition with huHER3-8 was also more efficacious than either treatment alone at inhibiting tumor growth and promoting durable responses in vivo.

Materials and Methods

Inhibitors

Vemurafenib (PLX4032), PLX4720, and trametinib (GSK1120212) were purchased from Selleck Chemicals LLC. huHER3-8 is a humanized antagonistic ERBB3 antibody (14).

Cell culture

WM115, WM793, 1205Lu, and SK-MEL-28 cells were cultured in MCDB 153 with 2% FBS, 20% Leibovitz L-15 medium, 5 μg/mL insulin. A375 cells were cultured in DMEM with 10% FBS. M238 cells were cultured in RPMI with 10% FBS and 2 mmol/L L-glutamine. All media contained 1% penicillin/streptomycin. All cells were cultured at 37°C and 5% CO2 in a humidified chamber.

Western blotting

Lysates were separated by SDS-PAGE and proteins transferred to polyvinylidene difluoride membrane. Immunoreactivity was detected using horseradish protein conjugate secondary antibodies (CalBioTech) and chemiluminescence substrate (ThermoScientific) on the Versadoc Imaging System (BioRAD). BRF (sc-5284), ERK2 (sc-1647), and p-ERBB3 (Tyr1328, sc-135654) antibodies were purchased from Santa Cruz Biotechnology Inc. ERBB3 (#4754), p-ERBB3 (Tyr1197, #4561), p-ERBB3 (Tyr1289, #4791), ERBB2 (#4290), p-ERBB2 (Tyr1196, #6942), p-ERBB2 (Tyr1221/1222, #2243), AKT (#9272), p-AKT (Ser473, #6942), p-AKT (Thr380, #9265), p-ERK1/2 (Thr202/Tyr204, #9101), and GAPDH (#2118) antibodies were purchased from Cell Signaling Technology. Actin (A2066) antibody was purchased from Sigma-Aldrich Co.

Reverse phase protein array analysis

A375 and 1205Lu cells were plated in 6-welled dishes at 2 × 10⁵ cells per well. Cells were treated with DMSO control or 1 μmol/L vemurafenib for 24 hours. huHER3-8 (10 μg/mL) was then added for 45 minutes followed by 10 ng/mL NRG1 for an additional 15 minutes or 24 hours. Cells were lysed in reverse phase protein array (RPPA) lysis buffer, and proteins prepared and analyzed by RPPA, as previously described (15). Lysates were run against 172 validated antibodies, and analyses were performed using normalized data.

Pathway analysis

Pathway analysis was performed using triplicate normalized RPPA data that were transformed for use with the Gene Set Enrichment Analysis (GSEA) software (16). To generate a single value for each gene, RPPA values of phosphorylated proteins were averaged and normalized to their unphosphorylated counterpart when possible. These values were uploaded to GSEA and analyzed comparing NRG1-treated versus NRG1 and huHER3-8–treated samples in the presence of vemurafenib using Gene Ontology gene sets available from MSigDB. Cytoscape interactomes were generated using significantly (t test metric ranking) normalized enrichment score ≥ 1.49; nominal P value ≤ 0.01; gene sets ≥ 5 genes) enriched pathways (17). Values used for GSEA analysis were then averaged and normalized to vemurafenib alone–treated samples to generate color labeling of proteins associated with enriched pathways.

Annexin V staining

Cells were treated with 1 μmol/L vemurafenib for 24 hours. Medium containing vemurafenib was then replenished and cells treated with 10 μg/mL of huHER3-8 antibody for 45 minutes before stimulation with 10 ng/mL NRG1. After 72 hours, floating and adherent cells were washed and incubated with 5 μL Annexin V-APC (BD Biosciences) in 100 μL 1× binding buffer (0.1 mol/L HEPES, 1.4 mol/L NaCl, 25 mmol/L CaCl₂), and 0.2 mg/mL propidium iodide for 15 minutes at room temperature. Cells were diluted in binding buffer and analyzed on the FACS Calibur Flow Cytometer. Experiments were performed in triplicate, and statistical analysis was done using a two-tailed t test assuming unequal variance with error bars representing SD.

EdU incorporation assays

A375 cells were treated with vemurafenib and huHER3-8, and incubated with 10 μmol/L EdU (5-ethyl-2’-deoxyuridine). After 72 hours, cells were prepared according to the Alexa Fluor 647 Flow Cytometry Assay Kit (Invitrogen) protocol, and analyzed using the FACS Calibur Flow Cytometer. Experiments were performed in triplicate, and statistical analysis was done as in Annexin V protocol.

Crystal violet

A375 (3 × 10⁵) cells were plated into 33-mm diameter dishes and treated with vemurafenib for 24 hours before the addition of huHER3-8 and NRG1. After 14 days, cells were washed, fixed, and stained with crystal violet. Pictures were taken at ×40 magnification on the Nikon ECLIPSE Ti (Nikon Instruments Inc.). ImageJ was used to quantify percent plate coverage.

In vivo assays

Female athymic mice (NU/J: Jackson) were injected intradermally with human melanoma cells (~1.0 × 10⁶), and cells allowed up to 2 weeks to reach appropriate tumor volume. huHER3-8 (100 μL of 1 mg/mL) was injected intraperitoneally every 3 days of the experiment. For shRNA experiments, mice were given doxycycline (2 mg/ml) in the drinking water 3 days before huHER3-8 treatment that was replenished every 3 days for the duration of the experiment. For PLX4720 chow experiments, PLX4720 was formulated into rodent chow at 90 mg/kg (Research Diets Inc.). Tumors were
measured using digital calipers, and volume was calculated using the formula $V = \frac{L \times W^2}{C}$. Some animals were euthanized due to the development of skin necrosis that prevented them from reaching the maximum allowed tumor volume (1,000 mm$^3$). At the conclusion of each experiment, tumors that were larger than 1.00 (progression), less than 1.00 (regression), or equal to 0.00 (complete regression) were recorded. Animal experiments were performed at Thomas Jefferson University (Association for Assessment and Accreditation of Laboratory Animal Care-accredited) and approved by the Institutional Animal Care and Use Committee. Statistical analysis was performed using a mixed effect model where error bars represent SE.

Results

**NRG1–ERBB3 signaling in vemurafenib-treated mutant BRAF melanoma cells is inhibited by huHER3-8**

We tested the ability of the humanized anti-ERBB3 monoclonal antibody, huHER3-8, to inhibit ERBB3 phosphorylation in BRAF$^{V600E,D}$ melanoma cells. huHER3-8 binds within residues 20 and 342 of ERBB3 with an affinity of 0.17 nmol/L toward human ERBB3 in FACS assay using SKBR3 human breast adenocarcinoma cells (14) and outcompetes NRG1 binding and prevents ERBB3 dimerization with ERBB2. A 10 μg/mL dose of huHER3-8 was used for experiments based on dose-dependent inhibition of NRG1-mediated ERBB3 phosphorylation (Fig. 1A). In the mutant BRAF cell lines, 1205Lu, M238, and A375, basal levels of phosphorylated ERBB3 were low (Fig. 1A and B). Consistent with our previous findings, NRG1 stimulates phosphorylation of ERBB3, an effect that was dramatically enhanced by overnight pretreatment with vemurafenib (8). Pretreatment with huHER3-8 efficiently inhibited NRG1-induced phosphorylation of ERBB3 in both untreated and vemurafenib-treated cells (Fig. 1B).

To better understand the effects of ERBB3 on mutant BRAF melanoma cells, we performed an RPPA analysis on 1205Lu and A375 cells treated with vemurafenib and NRG1 in the absence/presence of huHER3-8 (15). PI3K/AKT pathway signaling was most affected by NRG1 treatment in both cell lines (Supplementary Tables S1 and S2). Importantly, pretreatment with huHER3-8 prevented the phosphorylation of AKT induced by NRG1 (Fig. 2A and B). Analysis of the RPPA data using Gene Ontology gene sets was performed to determine the pathways affected by NRG1 and huHER3-8 treatment. In 1205Lu cells treated with vemurafenib and NRG1, there was a significant enrichment of cellular pathways involving phosphorylation and receptor signaling (Fig. 2C). By contrast, huHER3-8 pretreatment effectively inhibited the activation of NRG1-dependent signaling and significantly enriched pathways involved in the regulation of cell death and apoptosis (Fig. 2D). A375 cells treated with vemurafenib and NRG1 exhibited a significant enrichment of pathways involved in PI3K/AKT signaling, as well as other cellular pathways (Supplementary Fig. S1). Pretreatment with huHER3-8 in these cells prevented the enrichment of these pathways, but did not result in a significant enrichment of cell death and apoptosis pathways (Supplementary Tables S1 and S2 for full data set). Taken together, these data...
Figure 2. NRG1-dependent activation of ERBB3 results in increased AKT signaling and is inhibited by huHER3-8. A, 1205Lu cells were treated with 1 μmol/L vemurafenib overnight. Cells were then treated with or without 10 μg/mL huHER3-8 for 45 minutes followed by treatment with 10 ng/mL NRG1 for 15 minutes or 24 hours, as indicated. Cell lysates were prepared for RPPA analysis. Linear RPPA scores were averaged and normalized to vemurafenib alone samples. Proteins that were regulated 1.5-fold or greater are shown. B, A375 cells were treated as in A. Proteins regulated 1.5-fold or greater are shown. C, RPPA data generated from vemurafenib-treated 1205Lu cell samples were transformed and analyzed using GSEA. Significantly enriched pathways in the presence of NRG1 were visualized in Cytoscape where pathway node sizes are indicative of normalized enrichment scores relative to each other, as indicated. Data from samples treated with NRG1 for 15 minutes and 24 hours were averaged together and normalized to vemurafenib alone samples to generate the color labeling of protein nodes shown. D, significantly enriched pathways in 1205Lu samples treated with vemurafenib, NRG1, and huHER3-8. Pathway analysis was performed as in C.
suggest that the PI3K/AKT signaling pathway is activated by NRG1 and inhibited by anti-ERBB3 antibodies in RAF-inhibited mutant BRAF melanoma cells.

**huHER3-8 prevents NRG1/ERBB3-dependent long-term activation of the PI3K/AKT pathway in mutant BRAFV600E/D melanoma cell lines**

Western blotting confirmed RPPA data that NRG1 stimulated ERBB2 and AKT phosphorylation in RAF-inhibited 1205Lu, M238, and A375 cells and that these effects were ablated by huHER3-8 treatment (Fig. 3A). huHER3-8 also inhibited NRG1-stimulated phosphorylation of ERK1/2 in vemurafenib-treated cells. These results were dose-dependent and observed in several other mutant BRAF melanoma cell lines (Supplementary Fig. S2A and S2B). As the above experiments used a short-term (15 minutes) stimulation with NRG1, we performed a time course to support the RPPA data that huHER3-8 elicits persistent effects. In six different BRAFV600E/D melanoma cell lines, huHER3-8 inhibited phosphorylation of ERBB3 and AKT over a 24-hour time course (Fig. 3B). Notably, huHER3-8 caused a downregulation of ERBB3 in all cell lines (Figs. 1A and B, 3B, and Supplementary Fig. S2A and S2C). A small degree of reactivation of signaling was noted in some cell lines (e.g., WM793 cells) after 24 hours (Fig. 3B). We also observed an increase in ERK1/2 activation following NRG1 treatment of vemurafenib-treated cells (Fig. 3A and B). This effect was independent of PTEN status, because overexpression of PTEN in PTEN-deficient 1205Lu cells had no effect on NRG1 stimulation of phospho-ERK1/2 (Supplementary Fig. S3). These results confirm the RPPA data that huHER3-8 inhibits NRG1-stimulated phosphorylation of ERBB3 and AKT in RAF inhibitor–treated mutant BRAFV600E/D melanoma cells.

**huHER3-8 cooperates with vemurafenib to increase cytotoxicity and decrease proliferation in BRAFV600E/D melanoma cell lines in vitro**

Next, we investigated the effects of huHER3-8 in combination with vemurafenib on cell death and proliferation. As measured by Annexin V and propidium iodide staining, vemurafenib induced cell death in five of the six tested BRAF-mutant human cell lines (Fig. 4A). In 1205Lu and other mutant BRAF melanoma cells, NRG1 treatment reduced the levels of vemurafenib-induced cell death. Consistent with the pathway analysis, huHER3-8 significantly enhanced cell death in vemurafenib-treated, NRG1-stimulated cells to levels similar to those observed in cells treated with vemurafenib alone. Furthermore, huHER3-8 treatment had no effect on cell death in DMSO-treated (Fig. 4B) or unstimulated cells, suggesting that the low basal activation of ERBB3 does not play a role in cell survival. Because A375 cells displayed intrinsic resistance to cell death in this assay, consistent with previous data (10), we analyzed effects on S phase entry in this cell line. NRG1 increased EdU incorporation in vemurafenib-treated A375 cells by 5-fold (Fig. 4C). Notably, huHER3-8 significantly reduced NRG1-induced increase in EdU incorporation. The effects of huHER3-8 were long lasting as huHER3-8 reversed NRG1-mediated stimulation of A375 growth in 2-week colony growth assays (Fig. 4D). These results suggest that targeting ERBB3 can reverse the protective effects of NRG1 in BRAF-inhibited melanoma cells in vitro.

**huHER3-8 combines with BRAF targeting to delay tumor regrowth in vivo**

Next, we generated 1205Lu and A375 tetracycline responsive cells expressing an inducible shRNA targeted against *BRAF*. Upon treatment with doxycycline, the reduced BRAF expression led to inhibition of phospho-ERK1/2 and upregulation of ERBB3 and NRG1-dependent phosphorylated ERBB3, similar to vemurafenib treatment (Fig. 5A and Supplementary Fig. S4A). We used these cells to form xenografts in immune-compromised mice and tested the effects of huHER3-8 in combination with targeting BRAF in vivo. In the absence of BRAF depletion in vivo, huHER3-8 had no significant effect on tumor growth in 1205Lu xenografts (Fig. 5B). However, the combination of BRAF targeting by shRNA and huHER3-8 treatment resulted in a significant reduction of xenograft growth (Fig. 5B). We also noted that 2 of 9 mice harboring 1205Lu xenografts in the *BRAF* shRNA plus huHER3-8 treatment combination had complete regressions compared with 0 of 8 mice in the BRAF-depleted alone 1205Lu xenografts (Fig. 5C). These effects were durable as indicated by their lack of regrowth following doxycycline and huHER3-8 removal.

By contrast, effects of BRAF depletion alone and BRAF depletion plus huHER3-8 treatment were comparable on the growth of A375 xenografts, probably due to the strong initial effect of BRAF knockdown in these cells (Supplementary Fig. S4B and S4C). Thus, we examined the effect of huHER3-8 in combination with pharmacologic inhibition of BRAFV600E/D using 90 mg/kg PLX4720 chow, which produces plasma concentrations close to those detected in patients treated with vemurafenib (G. Bollag, personal communication). Notably, 4 of 8 mice treated with PLX4720 in combination with huHER3-8 achieved complete (no detectable tumor) or near-complete (94% reduction in tumor size) regression compared with only 1 of 8 mice treated with PLX4720 alone (Fig. 6C and D). To determine whether the regressions were durable, these mice were removed from treatment on day 27 and monitored for an additional 30 days. No tumor regrowth was observed 30 days after treatment in all mice that underwent complete regression; however, one xenograft in the combination group that underwent 94% regression did regrow once removed from treatment (Fig. 6D). These data suggest that the combination of huHER3-8 treatment with BRAF inhibition in vivo may increase the likelihood of a durable response. Together, these results indicate that targeting ERBB3 may enhance the effects of RAF inhibition in mutant BRAFV600E/D xenografts *in vivo*.

**Discussion**

There is strong interest in targeting ERBB3 in distinct tumor types (18). Although ERBB3 is known to play an important function in the melanocytic lineage during development (19), its role in melanomas is poorly characterized. Here, we provide evidence that supports the use of neutralizing ERBB3 antibodies in mutant BRAF melanoma as a part of combinatorial approach to counteract adaptive resistance to RAF inhibitors.
Previous studies showed that ERBB3 signaling is upregulated in BRAF<sup>V600E</sup> melanoma cells treated with RAF inhibitors such as vemurafenib (7, 8). Although we have previously used lapatinib to target ERBB3–ERBB2 complexes in combination with vemurafenib/PLX4720 (7, 8), there are toxicity concerns about the use of this small-molecule ERBB2/EGFR inhibitor in combinatorial approaches. Furthermore, upregulation of ERBB3 is a common compensatory mechanism in response to lapatinib and PI3K/AKT pathway inhibitors (13, 20), arguing for direct targeting of ERBB3. Initially, we
show that the neutralizing ERBB3 antibody, huHER3-8 (14) inhibits NRG1-dependent ERBB3 activation in BRAFV600E/D melanoma cells in vitro. RPPA and Western blot analyses showed that AKT signaling is potently activated by NRG1 and is inhibited by ERBB3 targeting. These data are consistent with ERBB3 potently binding PI3K (21) and our findings that constitutively active AKT provides strong protection against vemurafenib/PLX4720-mediated effects on melanoma cell growth and survival (8, 10). We show that huHER3-8 inhibits AKT signaling in mutant BRAF melanoma and enhances the antitumor effects of vemurafenib (Fig. 7). An acute increase in ERK1/2 phosphorylation was observed following 15 minutes of NRG1 treatment in RAF-inhibited BRAFV600E/D cells; however, this phosphorylation subsided within 3 hours. Because sustained ERK1/2 activation has been linked to proliferation (22, 23), the acute effects observed here are unlikely to contribute to the beneficial growth provided by NRG1. ERK1/2 activation at 24 hours observed in some cell lines seemed to be NRG1-independent and is more likely due to loss of negative feedback inhibition on the ERK1/2 pathway (24). NRG1 provides strong...
protection against vemurafenib/PLX4720-mediated effects on melanoma cell growth and survival (8, 10), and, importantly, this protection was inhibited by huHER3-8. Although effects of cell death were observed in most cell lines, targeting ERBB3 in A375 cells did not promote cell death but instead inhibited NRG1-mediated S phase entry. Consistent with this point, the antiapoptotic protein MCL-1 was upregulated in RAF-inhibited 1205Lu cells following 24 hours of NRG1 treatment, and

![Figure 5](image-url)

**Figure 5.** 1205LuTR BRAF shRNA-expressing cells mimic the effects of vemurafenib and combine with huHER3-8 to reduce tumor growth in vivo. A, 1205LuTR cells expressing an inducible BRAF shRNA were grown in the absence/presence of 100 ng/mL doxycycline for 96 hours. 1205Lu parental cells were grown in the presence of 1 μmol/L vemurafenib for 24 hours. Cells were then treated/untreated with 10 μg/mL huHER3-8 for 45 minutes followed by stimulation with 10 ng/mL NRG1 for 15 minutes. Cells were then lysed and analyzed via Western blot. B, parental 1205Lu or 1205LuTR cells harboring an inducible BRAF shRNA were injected intradermally into the backs of immune-deficient mice. Tumors were allowed to form for approximately 2 weeks. Doxycycline was added, where appropriate, to the drinking water 3 days before the start of the experiment. As indicated, mice were treated with huHER3-8 (parental, n = 6; BRAFsh, n = 9) or vehicle (parental and BRAFsh both n = 8) via i.p. injection every 3 days. The graph indicates the mean tumor volumes following huHER3-8 treatment. Statistically significant comparisons are indicated by asterisks, where *, P ≤ 0.05 and **, P ≤ 0.01. Bars, SE. C, fold change of tumor volumes relative to day 0 of individual xenografts from B. Mice that underwent regression were removed from treatment on day 27 and monitored for an additional 30 days.
this upregulation was partially reduced by huHER3-8 (Supplementary Fig. S5A). A375 cells had intrinsically high levels of MCL-1, which was unaffected by treatment with huHER3-8 (Supplementary Fig. S5B). Despite evidence of NRG1–ERBB3 regulation of p38/MAP kinase and JNK signaling in other cancers (25, 26), we did not observe regulation of these pathways in BRAFV600E melanoma cells by RPPA analysis.

Enhanced effects of the anti-ERBB3 antibody/RAF inhibitor combinations versus either alone were also observed in vivo. huHER3-8 combined with BRAF knockdown was more effective than BRAF knockdown alone at inhibiting the growth of mutant BRAF 1205LuTR cell xenografts. In A375 cells, huHER3-8 gave no additive effect to BRAF targeting when analyzing inhibition of xenograft growth, but did increase the number of xenografts that underwent complete and long-lasting regression. These data indicate that ERBB3-neutralizing agents may have varied effects in mutant BRAF melanomas. This is perhaps unsurprising given the knowledge that the phosphorylation of multiple receptor tyrosine kinases can be upregulated in response to RAF inhibitors (13, 20). These data emphasize the need to identify biomarkers that will predict the response to huHER3-8 and other ERBB3 targeting agents.

Although other ERBB3-neutralizing antibodies such as MM-111 (Merrimack) and LJM716 (Novartis) are currently being tested in early-stage clinical trials (19), dual specific ERBB3-targeting antibodies are also being developed. For example, MM-111 is a bispecific antibody with affinity for both ERBB3 and ERBB2 (27), and MEHD7945A is a dual ERBB3/EGFR targeting human IgG that is more efficacious than monospecific ERBB3 and EGFR antibodies in xenograft models (28).

Recent therapies designed to stimulate the immune response to melanoma have resulted in durable responses in patients. In addition to the antitumor effects of ERBB3 inhibition, the Fc regions on monospecific and dual targeting antibodies interact with specific receptors on natural killer cells and activate antibody-dependent cell-mediated cytotoxicity (ADCC; ref. 32). For example, ADCC contributes to the...
importantly, our studies extend beyond melanoma and possibly increase the number of long-term responses seen in patients. The recent approval of the dabrafenib and trametinib combination has provided a clinical benefit to patients harboring BRAFV600E melanoma. However, the response is heterogeneous and the majority of patients (59%) still progress within 1 year while on treatment (6), indicating that adaptive responses still contribute to tumor survival. Upregulated ERBB3 signaling following RAF/MEK inhibition was also inhibited by huHER3-8 (Supplementary Fig. SSC). Therefore, as the treatments for melanoma. Vemurafenib treatment relieves a negative feedback on ERBB3 transcription, resulting in an increase of ERBB3 at the membrane. In the presence of its ligand (NRG1), ERBB3 heterodimerizes with ERBB2 to become active. Active ERBB3 recruits PI3K, which signals to the AKT pathway promoting survival of mutant BRAF melanoma cells treated with vemurafenib. Treatment with the anti-ERBB3 monoclonal antibody huHER3-8 outcompetes NRG1 and prevents the activation of ERBB3 and AKT signaling and enhances the efficacy of vemurafenib treatment.

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In summary, the data presented here show for the first time in melanoma that a clinically designed anti-ERBB3 antibody combines with vemurafenib to increase cell death and decrease cell proliferation in vitro, while reducing tumor growth and increasing long-term response to RAF inhibition in vivo. Our work highlights the potential benefits of using ERBB3-targeting antibodies in combination with ERK1/2 pathway inhibition for the treatment of patients with mutant BRAFV600E melanoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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The Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretation, or conclusion.

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