IJC
International Journal of Cancer

Antibody targeting of HER2/HER3 signaling overcomes heregulin-induced resistance to PI3K inhibition in prostate cancer

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Dysregulated expression and/or mutations of the various components of the phosphoinositide 3-kinase (PI3K)/Akt pathway occur with high frequency in prostate cancer and are associated with the development and progression of castration resistant tumors. However, small molecule kinase inhibitors that target this signaling pathway have limited efficacy in inhibiting tumor growth, primarily due to compensatory survival signals through receptor tyrosine kinases (RTKs). Although members of the epidermal growth factor receptor (EGFR), or HER, family of RTKs are strongly implicated in the development and progression of prostate cancer, targeting individual members of this family such as EGFR or HER2 has resulted in limited success in clinical trials. Multiple studies indicate a critical role for HER3 in the development of resistance against both HER-targeted therapies and PI3K/Akt pathway inhibitors. In this study, we found that the growth inhibitory effect of GDC-0941, a class I PI3K inhibitor, is markedly reduced in the presence of heregulin. Interestingly, this effect is more pronounced in cells lacking phosphatase and tensin homolog function. Heregulin-mediated resistance to GDC-0941 is associated with reactivation of Akt downstream of HER3 phosphorylation. Importantly, combined blockade of HER2 and HER3 signaling by an anti-HER2/HER3 bispecific antibody or a mixture of anti-HER2 and anti-HER3 antibodies restores sensitivity to GDC-0941 in heregulin-treated androgen-dependent and -independent prostate cancer cells. These studies indicate that the combination of PI3K inhibitors with HER2/HER3 targeting antibodies may constitute a promising therapeutic strategy for prostate cancer.

Prostate cancer represents one of the most common malignancies and the second leading cause of cancer-related death in males.[1,2] The standard of care for metastatic prostate cancer is androgen withdrawal therapy, due to the initial dependence of the tumor cells on androgen receptor (AR) signaling.[3] However, almost all patients develop resistance to this treatment and the tumors recur as hormone refractory prostate cancer. The prognosis of this advanced disease is poor due to a lack of curable treatments.[3] A number of mechanisms including amplification of AR signaling, mutations in the ligand binding domain of AR and induction of AR splice variants have been shown to promote androgen-independent, or castrate-resistant prostate cancer (CRPC) development and progression.[4] In addition, multiple studies indicate an important role for the HER family of receptor tyrosine kinases (RTKs) and their ligands in androgen independent prostate cancer cell survival and progression, suggesting that these pathways might provide useful targets for therapy.[5,6]

Signaling through HER family receptors, which include epidermal growth factor receptor (EGFR), HER2, HER3 and HER4, regulates a variety of cellular functions including cell differentiation, migration, proliferation and survival.[7] With the exception of HER2, specific ligands have been identified for the HER family members which, in the unliganded state, exist in an inactive closed conformation.[8] By contrast, HER2 is present as an open, constitutively active conformer.[9] Ligand binding induces receptor activation, (hetero)dimerization, transphosphorylation within the dimerized receptor pair and propagation of downstream signaling.[10] Aberrant signaling through the HER family of RTKs has been implicated in the pathology of a variety of tumors including prostate cancer.[6,10]

Recently, much interest has focused on targeting HER3 for cancer therapy.[6,10,11] HER3 is distinct from other members of

Key words: engineered antibody, prostate cancer, heregulin, HER2, HER3
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: Cancer Prevention and Research Institute of Texas (CPRIT); **Grant numbers:** RP110069 and RP110070)
DOI: 10.1002/ijc.29378
History: Received 15 Mar 2014; Accepted 18 Nov 2014; Online 4 Dec 2014
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What's new?

Changes in the PI3K/Akt pathway often accompany prostate cancer. But attempts to halt the cancer by targeting this pathway are frustratingly ineffective, as they are thwarted by the HER family of kinases. This study showed that the HER3 ligand, heregulin, allows prostate cancer cells to fend off the PI3K inhibitor, GDC-0941. The authors then went on to demonstrate that they could reverse this effect; targeting HER2 and HER3 with antibodies allowed the inhibitor to again stop the growth of the cancer. Thus, antibodies to HER2 /HER3 may enhance the effectiveness of PI3K inhibitors to treat prostate cancer in patients.

the HER family as it lacks significant kinase activity and requires heterodimerization for phosphorylation and activation. The ligand for HER3, heregulin, is expressed by many tumors and contributes to the development of resistance against HER2/HER3 targeting agents and small molecule kinase inhibitors.[12–14] Although HER3 can form heterodimers with EGFR and HER4, HER2/HER3 heterodimers potentiate the strongest mitogenic signals.[10] The phosphoinositide 3-kinase (PI3K)/Akt and Ras-MEK-MAPK pathways are activated downstream of HER3 phosphorylation.[10] Of the HER family members, HER3 is the most potent stimulator of the PI3K/Akt pathway as it has six binding sites for the p85 regulatory subunit of PI3K, enabling its direct activation.[15]

Genetic alterations in the various components of the PI3K pathway such as activating mutations in the PI3K catalytic gene *PIK3C* and decreased expression of the PI3K regulatory genes *PIK3R1* and *PIK3R3* occur at a high rate in prostate cancer.[16] Further, dysregulated expression and/or mutations of phosphatase and tensin homolog (PTEN) occur in about 40–70% of prostate cancers and are associated with the development of CRPC in both humans and mice.[17–19] PTEN functions as a tumor suppressor by negatively regulating PI3K/Akt signaling. Consequently, alterations in PTEN activity lead to aberrant activation of Akt and its downstream effectors.[16] These results support a critical role for the PI3K/Akt pathway in the pathophysiology of prostate cancer and have led to the investigation of the therapeutic efficacy of small molecule inhibitors that selectively inhibit different nodes of this signaling cascade.[20,21] However, these approaches have had limited success in cancer therapy.

One of the proposed mechanisms for resistance to small molecule inhibitors targeting the PI3K/Akt pathway is the upregulation of HER3 in cancer cells, including prostate tumors.[22,23] Increased HER3 signaling can upregulate and stabilize AR, in addition to activation of the MAPK pathway, leading to decreased sensitivity to androgen deprivation and PI3K/Akt pathway inhibitors.[23] As an alternative to the use of small molecule inhibitors, the targeting of the HER2 signaling axis with the anti-HER2 antibodies, trastuzumab or pertuzumab, as single agents or combined with chemotherapy has also resulted in disappointing responses.[24,25] One possible pathway for tumor escape from HER2 targeting is through the heterodimerization of ligand-activated HER3 with activating receptors such as EGFR or cMet.[10,15] This escape mechanism is dependent on the presence of HER3 ligands, such as heregulin, which is present through autocrine or paracrine production in many tumor types including prostate cancer.[8,12,14,26]

In this study, we demonstrate that although a specific class I PI3K inhibitor, GDC-0941, is effective in blocking the *in vitro* proliferation of prostate cancer cells, the antiproliferative effects are ameliorated by the presence of the HER3 ligand, heregulin. This has motivated an investigation of several different strategies for targeting HER3 in the presence of heregulin using combinations of antibodies and GDC-0941. Specifically, we have analyzed the efficacy of GDC-0941 with antibodies that target HER3 or both HER2 and HER3. The antiproliferative effects of an anti-HER2/HER3 bispecific antibody have been compared with those of mixtures of anti-HER2 and anti-HER3 antibodies. Importantly, we demonstrate that antibody targeting of the HER2/HER3 axis restores sensitivity to GDC-0941 in the presence of heregulin for both androgen-dependent and -independent cell lines. These studies indicate that combination therapy targeting HER2/HER3 signaling with small molecule kinase inhibitors and antibodies has promise for the treatment of prostate cancer.

Material and Methods

Cell lines and reagents

Prostate cancer cell lines LNCaP, 22Rv1, DU145 and MDA PCa 2b were obtained from the American Type Culture Collection (ATCC, catalog nos. CRL-1740, CRL-2505, HTB-81 and CRL-2422, respectively). The C4-2B cell line was a kind gift from Dr. Nima Sharifi, Department of Cancer Biology, Cleveland Clinic, Cleveland, OH. LNCaP, 22Rv1 and C4-2B cells were cultured in RPMI medium (Lonza, Walkersville, MD) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. DU145 cells were cultured in Eagles minimum essential medium (Sigma-Aldrich, St. Louis, MO) with 10% FBS and 1% penicillin and streptomycin. MDA PCa 2b cells were cultured in ATCC formulated F-12K medium (catalog no. 30–2004) following ATCC recommendations. Antibodies specific for phospho-HER3 Y1289 (catalog no. 2842), phospho-Akt (S473; catalog no. 4060), Akt (catalog no. 9272), p-PRAS40 Thr246 (catalog no. 2997), p-P70S6K (catalog no. 9205), phospho-ERK1,2 (Thr202/Tyr204; catalog no. 9101), ERK1,2 (catalog no. 9102), PARP (catalog no. 9542), cleaved caspase-3 (catalog no. 9664) and pBad (Ser136; catalog no. 4366) were obtained from Cell Signaling Technologies (Danvers, MA). Polyclonal anti-HER3 antibody (catalog no. SC-285) and monoclonal anti-c-erbB2 antibody

(catalog no. OP15L) were from Santa Cruz Biotechnology (Dallas, TX) and Millipore (Billerica, MA), respectively. The monoclonal anti-actin antibody (catalog no. 612656) and anti-EEA-1 antibody (catalog no. 610456) were purchased from BD Bioscience (Franklin Lakes, NJ). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (H + L; catalog nos. 111-035-003 and 115-035-003, respectively) were from Jackson Immunoresearch Laboratories (West Grove, PA). The following reagents were also used: Alexa 647-conjugated Cholera toxin subunit B (recombinant; catalog no. C-34778) and charcoal stripped FBS (catalog no. 12676-011), Life Technologies (Grand Island, NY); polyclonal rabbit anti-LAMP-1 antibody (catalog no. AB24170), Abcam (Cambridge, MA); staurosporine (catalog no. 50–230-7494), Fisher BioReagents (Pittsburg, PA); GDC-0941, LC Laboratories (Woburn, MA); recombinant human heregulin-β1 (HRG; catalog no. 100-03), Peprotech (Rockey Hill, NJ). Clinical grade trastuzumab was from the UT Southwestern Pharmacy.

Expression and characterization of recombinant antibodies

Methods for the expression and characterization of the anti-HER3 antibody, Ab6 and the bispecific antibody comprising trastuzumab linked to the Ab6 scFv have been described previously.[27]

Fluorescence microscopy

22Rv1 and C4-2B cells were plated at 50,000 cells per dish, incubated overnight and treated with GDC-0941 (1 μM), heregulin (6.25 nM) or GDC plus heregulin for 24 hr at 37°C. Cells were then fixed with 1.7% (w/v) paraformaldehyde for 10 min at 37°C and stained with Alexa 647 conjugated Cholera toxin subunit B (recombinant). Cells were permeabilized with 0.05% (v/v) saponin for 10 min at room temperature in phosphate-buffered saline (PBS). A preblock with 4% BSA/PBS was carried out prior to staining with polyclonal rabbit anti-LAMP-1 antibody and trastuzumab (50 nM) for 30 min at room temperature. After blocking for 30 min with goat serum (Sigma-Aldrich, catalog no. G6767), primary antibodies were detected by incubation for 30 min at room temperature with the following secondary conjugates: Alexa 555-labeled goat anti-human IgG (H + L; Life Technologies, catalog no. A21433) and Alexa 488-labeled goat anti-rabbit IgG (H + L; Life Technologies, catalog no. A11034). Cells were washed twice with PBS between each incubation step and were stored at 4°C in 1% BSA/PBS prior to imaging.

For analyses of anti-HER3 (Ab6) trafficking, cells were plated as above and incubated with 50 nM Ab6 for 15 min at 37°C and either immediately washed and fixed, or washed and chased in medium for 45 min at 37°C prior to washing and fixation. Fixed cells were permeabilized and stained with anti-EEA-1 or anti-LAMP-1 antibodies, using the same secondary conjugates to detect primary antibodies and Ab6 as above.

Images were acquired using a Zeiss Axiovert 200M inverted fluorescence microscope with a 100X Plan Apochromat objective as described previously.[28] Data was processed using custom written software (MIAtool/LABSoft; http://www.wardoberlab.com/software/miatool/) in MATLAB.

Flow cytometry

22Rv1, LNCaP and C4-2B cells were plated at a density of 200,000 cells per well in 24-well plates and incubated at 37°C overnight. Subsequently, cells were treated with either medium, GDC-0941 (1 μM), heregulin (6.25 nM), GDC plus heregulin or dimethyl sulfoxide (DMSO) for 24 hr. Cells were then incubated with 50 nM anti-HER2 antibody (trastuzumab) for 15 min at 37°C. Following incubation with trastuzumab, cells were washed, trypsinized, fixed with 3.4% (w/v) paraformaldehyde, permeabilized with 0.05% (v/v) saponin and bound trastuzumab detected using Alexa 647-labeled goat anti-human IgG (H + L) antibody (Life Technologies, catalog no. A-21445). Samples were analyzed using a FACS-Calibur and data processed with FLOWJO (Tree Star).

Cell proliferation assay

Cells were plated at 5,000 cells per well in 96-well plates in serum containing medium and incubated at 37°C overnight. The PI3K inhibitor, GDC-0941, was serially diluted (0–1 μM) in culture medium and added to triplicate wells either alone or in the presence of heregulin (HRG, 6.25 nM). Anti-HER2/HER3 targeting antibodies, trastuzumab (anti-HER2), Ab6 (anti-HER3), or the bispecific anti-HER2/HER3 antibody (TA) were used at a concentration of 50 nM. In combination experiments, cells were treated simultaneously with GDC-0941 (500 nM), HRG (6.25 nM) and anti-HER2/HER3 antibodies as indicated in the figure legends. DMSO or PBS were used as vehicle controls for GDC-0941 or HRG/antibodies. Proliferation was assessed following 72 hr of drug treatment using CellTiter 96 Aqueous One Solution Proliferation Assay kit (Promega, catalog no. G3580) according to the manufacturer's instructions.

Cell viability was also assessed by trypan blue exclusion assay. Following 24, 48 and 72 hr of treatment, cells were harvested and stained with trypan blue and counted using a hemocytometer.

Immunoblotting

Immunoblotting was carried out as described previously.[27] In brief, cells grown in 6-well plates ($0.5–1 \times 10^6$ cells per well) were treated as indicated in the figure legends. Cell lysates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, IPVH00010) or nitrocellulose membranes (Bio-Rad, catalog no. 162-0145). Membranes treated with primary antibody overnight at 4°C were washed and incubated with horseradish peroxidase conjugated secondary antibody for 1 hr at room temperature. After washing, bound secondary antibody was visualized using a chemiluminescent detection reagent (Pierce, Rockford, IL or Amersham, Pittsburgh, PA).

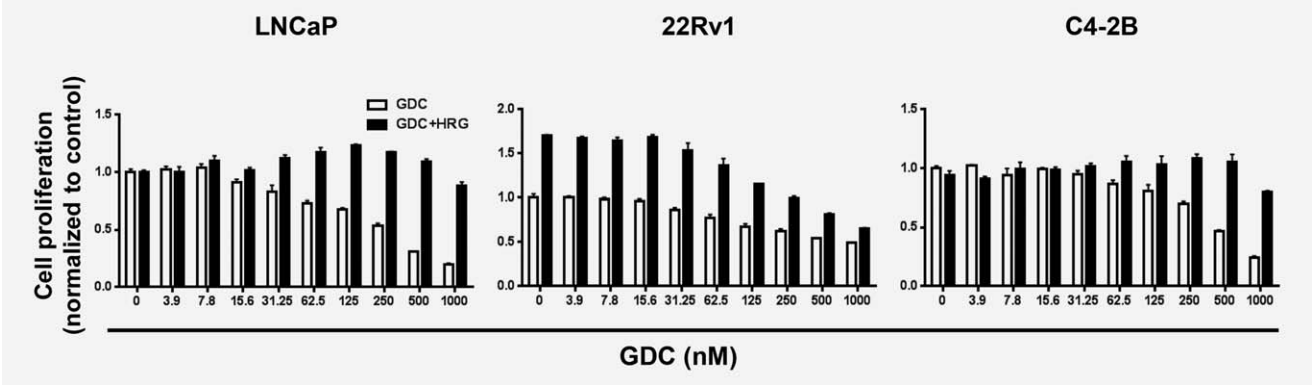

Figure 1. Heregulin reduces the efficacy of GDC-0941 in inhibiting the proliferation of prostate cancer cells. LNCaP, 22Rv1 and C4-2B cells were incubated with different concentrations of GDC-0941 (GDC) in the presence or absence of heregulin (HRG; 6.25 nM) for 3 days. Proliferative responses were normalized against the proliferation of cells incubated in 0 nM GDC. Data shown are means of triplicates ± standard errors of mean (SEMs). Results shown are representative of at least two independent experiments.

Statistical analysis

Statistically significant differences between treatments were determined using Student's *t*-test. Data are presented as mean ± standard error of mean of triplicate values unless otherwise indicated. *p* values of less than 0.05 were considered to be significant.

Results

The efficacy of the PI3K inhibitor, GDC-0941, in inhibiting proliferation and signaling is reduced by heregulin

GDC-0941 is a novel class I PI3K pathway inhibitor that is in clinical development for the treatment of various malignancies.[29] However, the efficacy of this drug in the presence of the HER3 ligand, heregulin, in treating prostate cancer has not been investigated. To address this, we first assessed the responses of androgen dependent (LNCaP) and androgen independent (C4-2B and 22Rv1) prostate cancer cell lines to different doses of GDC-0941 in the presence and absence of 6.25 nM heregulin (Fig. 1). GDC-0941 reduced proliferation of the three cell lines in a dose dependent way, with approximately 80, 50 and 76% inhibition at a concentration of 1 μM in LNCaP, 22Rv1 and C4-2B cells, respectively. However, the presence of heregulin reduced the antiproliferative activity of GDC-0941. The effects of heregulin were particularly marked for the PTEN loss cell lines,[30] LNCaP and C4-2B. Consistent with previous reports,[31] heregulin treatment induced proliferation of 22Rv1 cells, and this ligand-mediated effect was partially blocked by GDC-0941. Interestingly, in the absence of GDC-0941, heregulin did not induce proliferation of LNCaP or C4-2B cells, which is consistent with the previously published differential effects of this growth factor on 22Rv1 versus LNCaP cells.[8,31]

To further investigate the effects of heregulin, analyses of pAkt (S473), pERK1,2 and the downstream targets of Akt and mTOR, pPRAS40 and pP70S6K, respectively, in cells treated with GDC-0941, heregulin or GDC-0941 plus heregulin were carried out (Fig. 2*a*). Consistent with the observations of others,[8,32] heregulin induced phosphorylation of Akt, ERK1,2 and P70S6K. Further, although heregulin-induced pAkt (S473) and pP70S6K levels were reduced by GDC-0941, the inhibitory effects for pAkt (S473) were less marked for LNCaP and C4-2B cells relative to 22Rv1 cells. This is most likely due to PTEN loss and higher levels of constitutive Akt phosphorylation in these cell lines. By contrast with the other signaling molecules in the P13K/Akt pathway, phosphorylation of the downstream target of Akt, PRAS40, remains at basal (medium only) levels in cells in the presence of heregulin and GDC-0941. Further, heregulin-induced phosphorylation of ERK1,2 was not reduced by GDC-0941, which may also account for the decreased antiproliferative effects of this inhibitor in the presence of HER3 ligand. GDC-0941 was also less effective in blocking constitutive PI3K/Akt signaling in the absence of heregulin in LNCaP and C4-2B cells relative to 22Rv1 cells.

Following 24 and 72 hr of treatment, although GDC-0941 reduced pAkt (S473) levels in all of the cell lines tested, these inhibitory effects were greater in 22Rv1 cells than in LNCaP and C4-2B cells (Fig. 2*b*). To further investigate the molecular basis for the reversal of the antiproliferative effects of GDC-0941 in heregulin-treated cells, we analyzed the levels of HER2, HER3 and pHER3 following treatment of cells with GDC-0941, heregulin or GDC-0941 plus heregulin for 24 and 72 hr (Fig. 2*b*). HER3 levels were increased by GDC-0941 treatment, and this was also observed on the cell surface at 24 hr post-treatment (Supporting Information Fig. 1*a*). By contrast, although both total and surface HER2 levels were increased for 22Rv1 cells following GDC-0941 treatment for 24 or 72 hr, HER2 levels were not altered in LNCaP and C4-2B cells. Interestingly, heregulin or heregulin plus GDC-0941 induced a marked reduction in the surface and total HER2 levels in all three cell lines (Figs. 2*b* and 2*c*). Although reductions in surface HER3 levels were observed following 1 hr of heregulin treatment, partial (C4-2B) or complete recovery

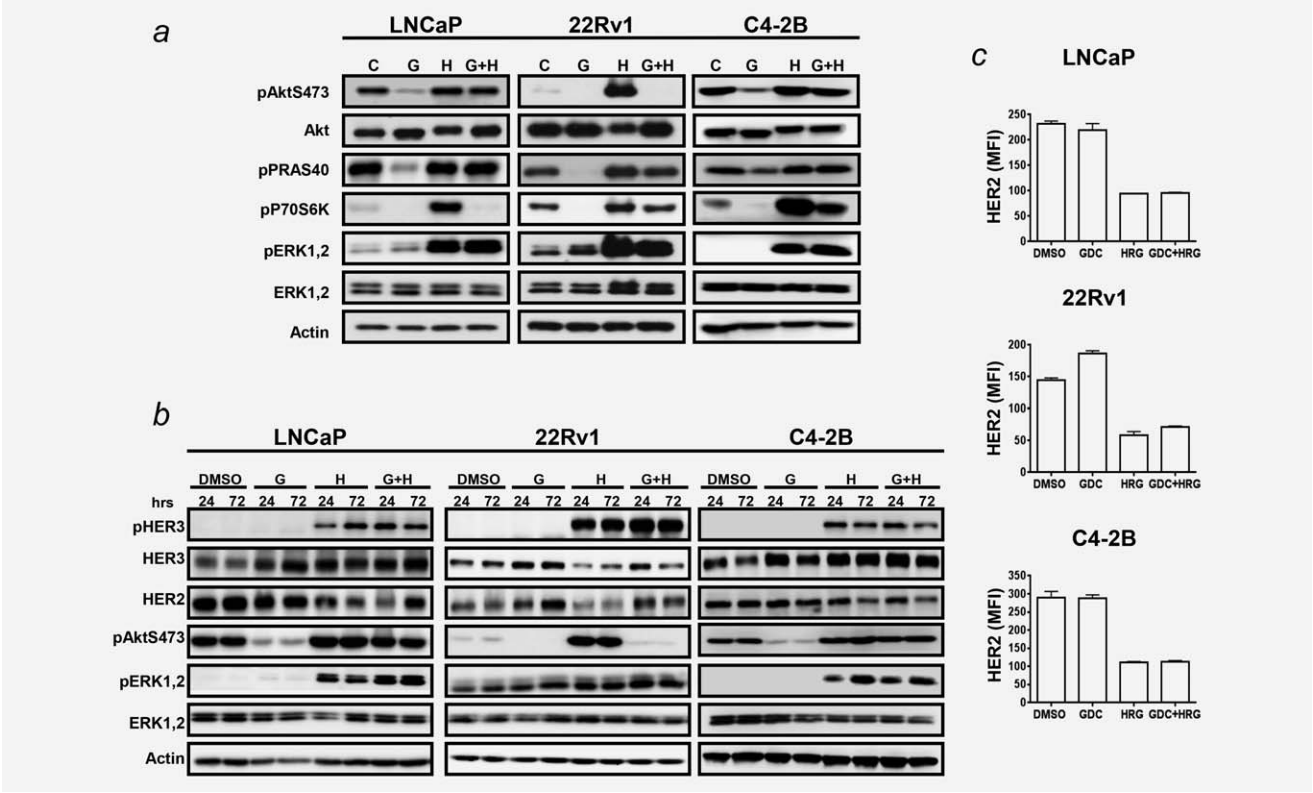

Figure 2. Effects of GDC-0941 on Akt phosphorylation, activation of downstream signaling molecules and total HER2/HER3 expression levels in prostate cancer cells in the presence of heregulin. LNCaP, 22Rv1 and C4-2B cells were incubated with 1 μM GDC-0941 (G) in the presence or absence of 6.25 nM heregulin (H) for 1 hr (*a*) or 24 and 72 hr (*b*). For both (*a*) and (*b*), cell lysates were analyzed by immunoblotting. Data shown are representative of at least two independent experiments. (*c*) Effect of GDC-0941 and heregulin treatment on HER2 levels on the plasma membrane of prostate cancer cells. Cells were treated with 1 μM GDC-0941 (GDC), in the presence or absence of heregulin (HRG; 6.25 nM), for 24 hr. Cell surface HER2 expression was determined by incubation with an anti-HER2 antibody (trastuzumab) followed by Alexa 647-labeled goat anti-human IgG (H + L) antibody. Data shown represent mean fluorescence intensities (MFI) ± SEM of triplicate samples following subtraction of background fluorescence intensities. Results are representative of at least two independent experiments.

(22Rv1) to pretreatment levels occurred within 24 hr (Supporting Information Fig. 1*b*). The heregulin-induced reduction in levels of HER2, which itself has no known ligand, suggests that heregulin-mediated dimerization of HER2 and HER3 may result in lysosomal delivery of HER2. To investigate this possibility, 22Rv1 and C4-2B cells were treated with GDC-0941, heregulin or GDC-0941 and heregulin for 24 hr and stained with an anti-HER2 antibody to determine the intracellular localization of HER2 in the presence of this ligand. Whereas GDC-0941 treatment induced higher levels of surface HER2 in 22Rv1 cells, heregulin exposure resulted in lower HER2 surface levels in both 22Rv1 and C4-2B cells combined with HER2 localization in LAMP-1 positive late endosomal/lysosomal compartments (Fig. 3).

Despite the fluctuations in HER2 and HER3 levels in the presence of heregulin, HER3 phosphoryation was observed at both 24 and 72 hr following heregulin or heregulin/GDC-0941 exposure. Collectively, the data indicate that the inhibitory effects of GDC-0941 are mitigated by activating signals through HER3 in both androgen-dependent and -independent prostate cancer cells.

Combination treatment with antibodies specific for HER2/HER3 and GDC-0941 inhibits prostate cancer cell proliferation

The upregulation of HER3, combined with increased pHER3 levels in GDC-0941-treated cells following heregulin exposure, suggested that the use of antibodies to target the HER2/HER3 axis combined with this PI3K inhibitor might have antiproliferative effects. Consistent with earlier studies in breast cancer cells[33] we observed that the anti-HER2 antibody, trastuzumab, inhibited ligand-independent signaling in C4-2B cells (data not shown). We therefore reasoned that trastuzumab, in combination with an anti-HER3 antibody that blocks heregulin binding to HER3 would be effective in inhibiting both ligand-dependent and -independent signaling. This rationale led to the use of trastuzumab in combination with an anti-HER3 antibody (Ab6), or an in-house engineered anti-HER2/HER3 bispecific comprising trastuzumab and the anti-HER3 antibody, Ab6 (TA).[27] The anti-HER3 antibody, Ab6, inhibits the binding of heregulin to HER3 and was expressed in transfected CHO cells as a biosimilar of the previously described anti-HER3 antibody, MM-121.[11] The

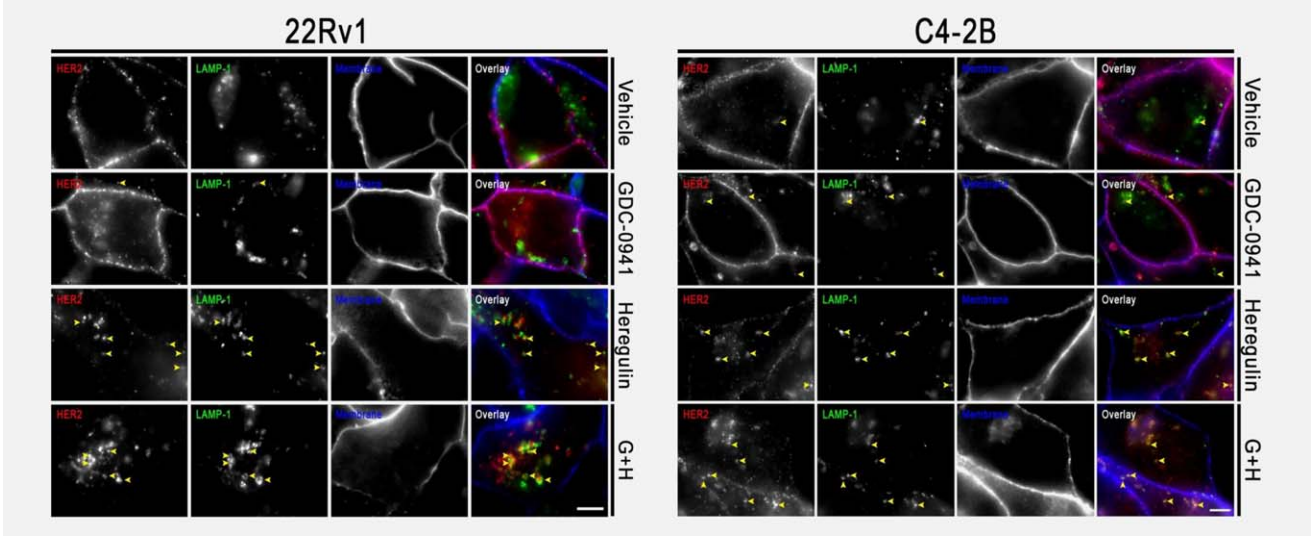

Figure 3. Heregulin treatment results in HER2 trafficking into lysosomes. 22Rv1 and C4-2B cells treated with GDC-0941 (1 μM), heregulin (6.25 nM) or GDC-0941 plus heregulin (G+H) for 24 hr were fixed and stained with Alexa 647-conjugated Cholera toxin B. Cells were then permeabilized and stained with trastuzumab and anti-LAMP-1 antibody for 30 min at room temperature. Primary antibodies were detected by staining with Alexa 555- and Alexa 488-labeled secondary antibodies, respectively. Alexa 488, 555 and 647 are pseudocolored green, red and blue, respectively, in the overlay images. Yellow arrows indicate examples of internalized HER2 in LAMP-1 positive lysosomes. Scale bars = 5 μm.

bispecific anti-HER2/HER3 antibody, TA, comprises trastuzumab with Ab6-derived scFvs connected *via* Gly-Ser-Ser linkers to the CH3 domains of trastuzumab and is therefore tetravalent. This bispecific has been characterized in our earlier studies and has a β-phase half-life of 215 hr in mice.[27] The dissociation constants for trastuzumab and Ab6 for binding to HER2 and HER3, respectively, have been determined previously by others.[11,34] Using surface plasmon resonance, both Ab6 and the bispecific had the expected binding properties for recombinant HER2 and/or HER3 (data not shown), indicating that the trastuzumab and Ab6 (single chain Fv) arms of TA have activities that are similar to those of their parent antibodies. Further, Ab6 and TA migrate on a size exclusion column at the expected molecular weights of 150 kDa and 200 kDa, respectively.[27]

We next analyzed whether antibody targeting of HER2/HER3 signaling restores sensitivity to GDC-0941 in heregulin-treated cells. In addition to LNCaP, 22Rv1 and C4-2B cells, proliferation of two additional cell lines, DU145 and MDA PCa 2b was also assessed. Consistent with the data shown in Figure 1, as a single agent GDC-0941 was ineffective in the presence of heregulin in reducing proliferation of all cell lines tested except DU145 (Fig. 4 and Supporting Information Fig. 2). Although GDC-0941 inhibited the proliferation of DU145 cells, heregulin had no effect on GDC-0941-mediated suppression of DU145 cells (Supporting Information Fig. 2) suggesting that the heregulin-mediated reversal of sensitivity to GDC-0941 is not applicable to all prostate cancer cell lines. For LNCaP, C4-2B and MDA PCa 2b cells, a mixture of trastuzumab and Ab6 was more effective in reducing heregulin-mediated proliferation than the bispecific anti-HER2/HER3 antibody TA or individual antibodies (trastuzumab or Ab6). For 22Rv1 cells, the inhibitory effects of GDC-0941 treatment as a single agent on heregulin-mediated proliferation (Fig. 1) were increased by addition of any of the HER2/HER3 antibodies or combinations, although TA or mixtures of trastuzumab and Ab6 had the most marked effects (Fig. 4). In addition, although 50% inhibition of LNCaP cell proliferation was achieved by ∼50 nM trastuzumab and Ab6 in dose response experiments, similar results were obtained using a twofold higher concentration of the antibodies in C4-2B cells (Supporting Information Fig. 3). This suggests increased sensitivity of androgen-dependent LNCaP cells to combination treatment. In addition, androgen depletion did not alter the response of LNCaP cells to combination treatment (Supporting Information Fig. 4). In the absence of GDC-0941, none of the antibodies inhibited proliferation in the presence of heregulin (Supporting Information Fig. 5). Collectively, the data demonstrate that targeting the HER2/HER3 axis with antibodies in combination with GDC-0941 results in synergistic reductions in cell growth.

We next investigated whether antibody-mediated inhibition of prostate cancer cell proliferation in heregulin and GDC-0941-treated cells is associated with blockade of PI3K/Akt and MAPK/ERK pathway activation. For both LNCaP and 22Rv1 cells following 1 hr of treatment, TA and mixtures of individual antibodies (trastuzumab and Ab6) were the most effective in reducing heregulin-mediated phosphorylation of HER3, Akt, ERK and PRAS40 (Fig. 5). Although pERK1,2 levels were reduced in LNCaP cells by all anti-HER2/HER3 antibodies or combinations by 1 hr, this was not observed for 22Rv1 cells. This difference could be due to

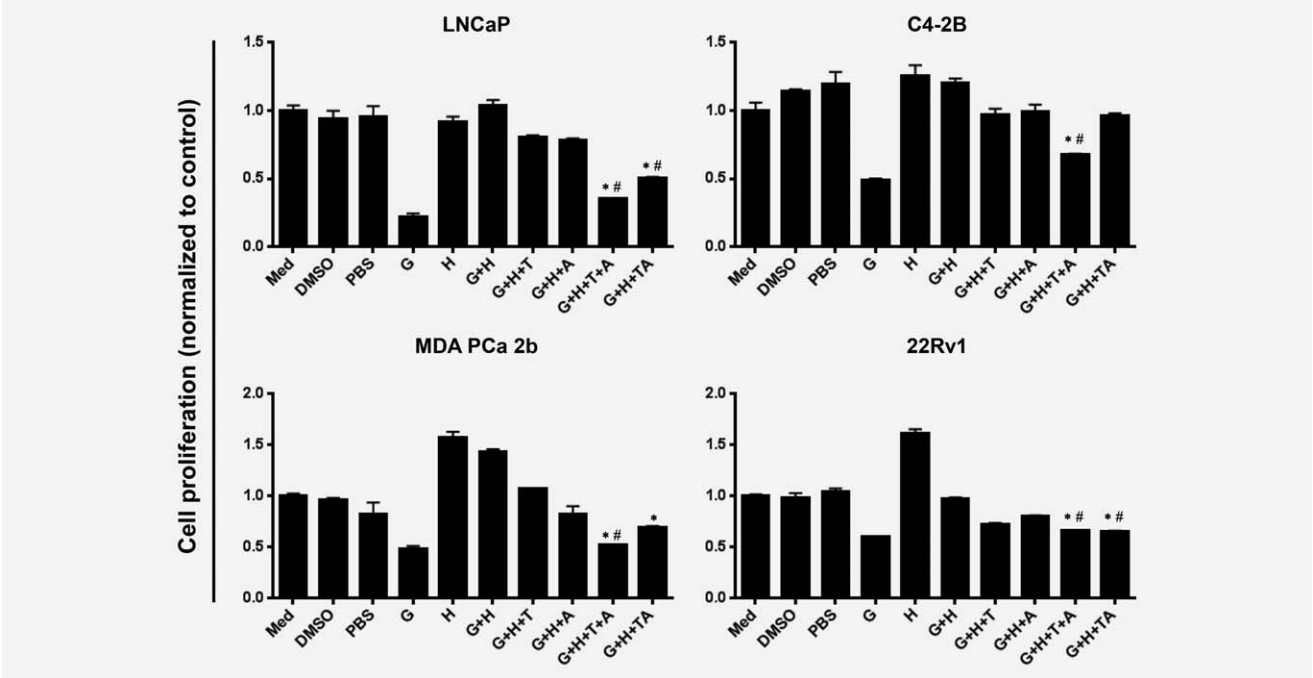

Figure 4. Combined blockade of HER2 and HER3 signals restores sensitivity to GDC-0941 in prostate cancer cells. Cells were incubated with 0.5 μM GDC-0941 (G) in the presence of heregulin (H; 6.25 nM) and treated with 50 nM trastuzumab (T), anti-HER3 Ab6 (A), trastuzumab plus Ab6 (T + A; 50 nM of each) or 50 nM bispecific trastuzumab with anti-HER3 Ab6 scFv (TA) for 3 days. Proliferative responses were normalized against the proliferation of cells incubated in medium (Med) only. Data shown are means of triplicates ± SEM. Statistically significant differences between proliferative responses for cells treated with trastuzumab plus Ab6 (T + A) or bispecific trastuzumab with anti-HER3 Ab6 scFv (TA) *versus* trastuzumab (*) or Ab6 (#) in the presence of GDC-0941 and heregulin are indicated (Student's *t*-test; $p < 0.05$). Results are representative of at least two independent experiments.

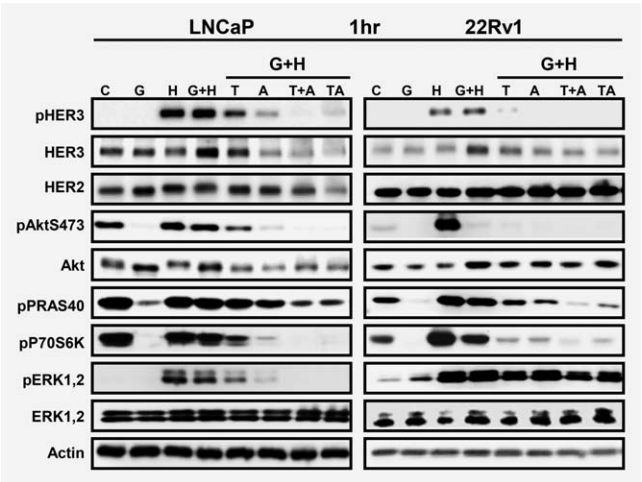

Figure 5. Antibodies specific for HER2 and HER3 synergize with GDC-0941 to prevent heregulin-mediated signaling in prostate cancer cells. LNCaP and 22Rv1 cells were treated with 1 μM GDC-0941 (G), 6.25 nM heregulin (H) and 50 nM trastuzumab (T), 50 nM anti-HER3 Ab6 (A), trastuzumab plus Ab6 (T + A; 50 nM of each), 50 nM bispecific trastuzumab with anti-HER3 Ab6 scFv (TA) as indicated for 1 hr and cell lysates analyzed by immunoblotting. Data shown are representative of at least two independent experiments.

the constitutive activation of ERK in this cell line.[35] Consistent with the proliferative responses (Fig. 4), trastuzumab alone was the least effective of all antibodies/antibody combinations in inhibiting downstream signaling. Significantly, the data indicate that the targeting of HER3 either alone (with Ab6) or in combination with trastuzumab (mixture or bispecific) is more effective than HER2-directed strategies, congruent with the central role of HER3 signaling in tumorigenesis.

We directly compared the effects of the trastuzumab and Ab6 mixture or TA, which had the highest antiproliferative effects (Fig. 4), at 24 and 72 hr on the components of the pHER3 signaling pathway and HER2/HER3 levels. Consistent with their comparable effects on 22Rv1 cell proliferation, TA or a mixture of trastuzumab and Ab6 had similar effects on HER2/HER3 signaling (Fig. 6*a*). However, in LNCaP cells, trastuzumab plus Ab6 treatment was slightly more effective than TA in blocking heregulin-mediated HER3 phosphorylation and downstream signaling. Although total HER3 levels were reduced by treatment with either TA or a mixture of trastuzumab and Ab6 for both LNCaP and 22Rv1 cells, decreases in HER2 levels were more marked for LNCaP relative to 22Rv1 cells. Microscopy analyses of the effect of the anti-HER3 antibody, Ab6, on HER3 trafficking revealed the internalization of Ab6 into early endosomes within 15 min of treatment (Supporting Information Fig. 6). Further, consistent with HER3 degradation in the presence of Ab6, this antibody could be detected within lysosomes within 60 min (Supporting Information Fig. 6).

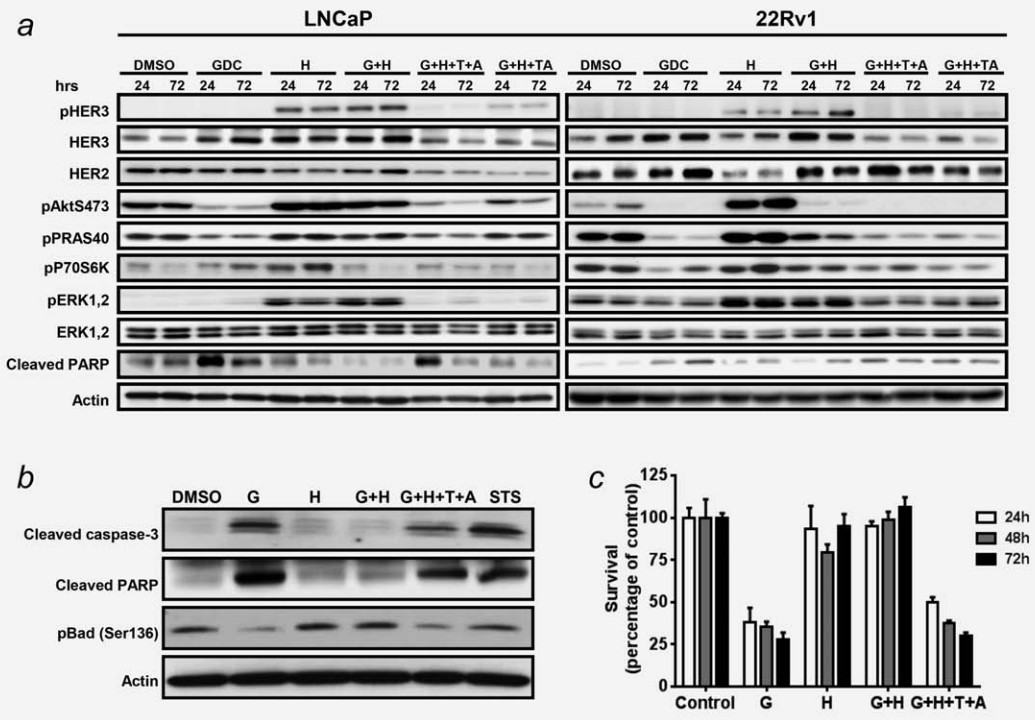

Figure 6. Comparison of the effects of the bispecific anti-HER2/HER3 antibody and a mixture of trastuzumab and Ab6, on heregulin-mediated signaling in GDC-0941-treated cells. (*a*) Cells were treated with GDC-0941 (G; 1 μM), heregulin (H; 6.25 nM), a mixture of trastuzumab and Ab6 (T + A; 50 nM of each) or 50 nM bispecific trastuzumab with anti-HER3 Ab6 scFv (TA) as indicated for 24 and 72 hr and cell lysates analyzed by immunoblotting. (*b*) LNCaP cells were treated with GDC-0941 (G; 1 μM), heregulin (H; 6.25 nM), a mixture of trastuzumab and Ab6 (T + A; 50 nM of each) or staurosporine (STS; 1 μM) as indicated for 3 hr and lysates analyzed by immunoblotting. (*c*) LNCaP cells plated at 5×10^4 cells per well in triplicate were treated with 0.5 μM GDC-0941 (G) in the presence of heregulin (H; 6.25 nM) and trastuzumab plus Ab6 (T + A; 50 nM of each). Cells harvested at 24, 48 and 72 hr after treatment were stained with trypan blue and counted using a hemocytometer. Means of triplicates ± standard deviation are presented. Results shown are representative of two independent experiments.

Treatment of cells with GDC-0941 alone for 24 hr resulted in increased cleaved PARP levels which were reduced by the addition of heregulin. Importantly, trastuzumab plus Ab6 (LNCaP and 22Rv1) or the bispecific, TA (22Rv1) restored cleaved PARP levels to those observed with GDC-0941 treatment (Fig. 6*a*). The levels of cleaved caspase-3 and pBad (Ser136) in LNCaP cells treated with GDC-0941 either alone or in combination with anti-HER2/HER3 antibodies were also analyzed (Fig. 6*b*). Treatment of cells with GDC-0941 for 3 hr induced an increase in cleaved caspase-3 and PARP levels, which were reduced by addition of heregulin. Importantly, trastuzumab plus Ab6 restored the levels of cleaved caspase-3 and PARP to those observed with GDC-0941 treatment. Further, consistent with inhibition of phosphorylation of Akt (S473), trastuzumab plus Ab6 treatment induced suppression of pBad (Ser136) in LNCaP cells treated with GDC-0941 plus heregulin. This indicates a role for apoptotic cell death in the inhibition of cell proliferation by combination treatment. Consistent with data obtained using the MTS assay, direct counting of viable cells confirmed the ability of combined trastuzumab plus Ab6 treatment to recover GDC-mediated cell death in heregulin-treated LNCaP cells (Fig. 6*c*).

Discussion

Hyperactivation of the PI3K/Akt pathway, due to alterations in various signaling components, is critically involved in the progression of prostate cancer.[16,17] Consequently, a number of small molecule kinase inhibitors that target this signaling pathway are in development for the therapy of breast and prostate cancer.[16,21] However, the use of single agents to inhibit the PI3K pathway has met with disappointing results due to a variety of mechanisms. These include compensatory upregulation of cell surface RTKs such as HER3, leading to AR stabilization and transcriptional activity.[22,23] Much data support an important role for members of the HER family members in prostate cancer tumorigenesis.[5,6] The HER2/HER3 signaling axis is one of the most potent stimulators known of the PI3K/Akt pathway, resulting in tumor cell proliferation and compensation for reduced AR activation during androgen deprivation.[10,23] However, by analogy with single agent targeting of the PI3K pathway, monoclonal antibodies or tyrosine kinase inhibitors either alone or in combination

with docetaxel directed toward the HER family RTKs have failed to provide significant clinical benefit in prostate cancer.[24,25] This, combined with the knowledge that the HER3 ligand heregulin is present in autocrine or paracrine fashion in many tumor types including prostate cancer[8,12,26] indicates that combination therapies directed toward both HER2/HER3 and the PI3K pathway could have efficacy in this malignancy.

GDC-0941 is a novel, class I specific PI3K inhibitor that is currently being tested in a variety of tumors.[29,33] Consistent with the previously reported effect of GDC-0941 on PC3 cells,[29] in the absence of heregulin, GDC-0941 was a potent inhibitor of the proliferation of all of the cell lines tested. In the absence of heregulin, the inhibitory effects of GDC-0941 were more pronounced in cells with defective or reduced PTEN function such as LNCaP, C4-2B and MDA PCa 2b.[30,36] This is analogous to the higher sensitivity of breast cancer cell lines that do not express PTEN to PI3K pathway inhibition.[33] However, in the presence of heregulin, the efficacy of GDC-0941 in reducing the proliferation of prostate cancer cells is ameliorated. Reversal of the GDC-0941-mediated growth inhibitory effects on LNCaP and C4-2B cells was also associated with restoration of Akt phosphorylation and phosphorylation of downstream Akt targets. Studies of the effect of heregulin on LNCaP cell proliferation are conflicting, with some reports of a pro-proliferative effect,[37] and others describing an inhibitory effect.[8,38] Although we observed a change in LNCaP cell morphology following heregulin treatment, growth inhibitory effects when cultured in 10% serum containing medium were not significant. Importantly, heregulin rescued LNCaP, C4-2B and MDA PCa 2b cells from the growth inhibitory effects of GDC-0941. However, in the presence of GDC-0941, heregulin was only partially effective or ineffective in restoring proliferation in the PTEN-sufficient cell lines, 22Rv1 and DU145, respectively.[36]

The intracellular fate of HER3 following ligand binding has not been investigated in prostate cancer cells. Although the E3 ubiquitin ligase Nrdp1 mediates HER3 ubiquitination and proteasomal degradation in a ligand-dependent manner in MCF-7 breast cancer cells,[39] it is not clear whether HER3 expression is regulated similarly in prostate cancer cells. Inefficient delivery of HER3 into lysosomal compartments following ligand binding has also been reported to occur due to receptor–ligand dissociation, leading to recycling of HER3 back to the cell membrane of HER3-transfected CHO cells.[40] Although we observe an initial decrease in surface HER3 levels in prostate cancer cells (C4-2B and 22Rv1) following heregulin exposure, the molecular details as to how the levels recover within 24 hr warrant further investigation.

Our observation that GDC-0941 has reduced efficacy in the presence of heregulin prompted us to investigate whether this PI3K inhibitor in combination with anti-HER2 or HER3 antibodies, or bispecifics/mixtures specific for both receptors, decreases PI3K/Akt activation and downstream signaling. Importantly, we demonstrate that targeting HER2 and HER3 with antibodies restores sensitivity to GDC-0941-mediated antiproliferative effects in prostate cancer cells. The trastuzumab plus Ab6 combination or TA induced extensive HER3 degradation, consistent with the propensity of anti-HER3 antibodies to internalize HER3.[41] Significantly, individual anti-HER2 or anti-HER3 antibodies are less effective than dual antibody targeting of HER2 and HER3. In addition, our results support a model in which the inhibition of Akt phosphorylation and concomitant activation of Bad initiate downstream signaling and induction of the apoptotic pathway involving caspase-3 and PARP.

Antibody targeting of individual members of the HER family tyrosine kinases, particularly HER2, have limited therapeutic effects in prostate cancer.[24,25] This has motivated the development of several alternative antibody-based approaches for the treatment of this malignancy. For example, a bispecific T cell engaging (BiTE) antibody that recognizes both CD3ε of the T cell receptor complex and prostate specific membrane antigen (PSMA) is under preclinical evaluation.[42] In addition, an anti-PSMA monoclonal antibody conjugated to a cytotoxic drug (PSMA-ADC) is currently in clinical trials.[43] The recent observation that the presence of heregulin can reduce the cytotoxic effect of a HER2-directed antibody drug conjugate, trastuzumab emtansine (T-DM1), in HER2-overexpressing breast cancer cells provides support for strategies to target HER3.[44]

Development of resistance to inhibition of the PI3K/Akt pathway in HER2-overexpressing breast cancer cells is associated with enhanced HER3 expression and phosphorylation and is in part dependent on FOXO transcription factors.[22] However, an Akt specific compensatory mechanism has also been described.[45] Upregulation of HER3 expression occurs in prostate cancer cells following treatment with BEZ235, a dual PI3K/mTOR inhibitor.[23] Consistent with these findings, we observe upregulation of HER3 in both LNCaP and 22Rv1 cells. However, by contrast with HER2-overexpressing breast cancer cells, phosphorylation of HER3 and re-activation of the downstream PI3K/Akt pathway was not observed in prostate cancer cells in the absence of heregulin. A likely reason for this difference in behavior is the higher level of HER2 expression in the former cell type.[9]

Of direct relevance to the current study, the findings concerning heregulin expression in prostate cancer are mixed with some groups detecting heregulin in the majority of prostate cancers[26] and others reporting undetectable levels.[46] However, heregulin was shown to be expressed by prostate stroma and other cell types[46] and therefore may function in a paracrine fashion to induce HER3 signaling. In addition, increased expression of HER3 has been demonstrated in prostate tumors compared with normal prostate[47] and elevated HER2/HER3 levels in association with PTEN loss correlate with poor prognosis in prostate cancer.[48]

In an earlier study, combined targeting of both HER3 dimerization partners, EGFR and HER2, was required to

block the growth of androgen-dependent prostate cancer cells undergoing androgen withdrawal treatment.[32] This treatment strategy was ineffective in CRPC cells or in the presence of androgens, suggesting that the timing of HER-targeting is critical in the context of androgen withdrawal therapy. Following progression to the castration resistant phenotype, it may not be possible to effectively inhibit tumor growth using this strategy due to enhanced Akt activation[49] and increased growth factor receptor stimulation.[19] By contrast, we demonstrate that antibody targeting of both HER2 and HER3 combined with PI3K/Akt inhibitors reduces signaling and proliferation in both androgen dependent and CRPC cell lines that are deficient in PTEN expression. Significantly, this combination overcomes the resistance to PI3K inhibition induced by the HER3 ligand, heregulin, suggesting that this approach could have promise for the treatment of prostate cancer.

Acknowledgements

We thank Dr. Sripad Ram for expert advice and assistance with microscopy experiments, Dr. Pankaj Bansal, Dr. Siva C. Devanaboyina and J. Bregg Reedy for help with expression plasmid generation and CHO cell transfections and Ramraj Velmurugan for computational assistance. E. Sally Ward, Raimund Ober, Jeffrey Kang and Jayakumar Poovassery are inventors on a patent (owned by UT Southwestern Medical Center) related to the use of bispecific and multivalent antibodies that target HER2 and HER3 in cancerous cells.

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