Pan-HER, an Antibody Mixture Simultaneously Targeting EGFR, HER2, and HER3, Effectively Overcomes Tumor Heterogeneity and Plasticity

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Abstract

Purpose: Accumulating evidence indicates a high degree of plasticity and compensatory signaling within the human epidermal growth factor receptor (HER) family, leading to resistance upon therapeutic intervention with HER family members.

Experimental Design/Results: We have generated Pan-HER, a mixture of six antibodies targeting each of the HER family members EGFR, HER2, and HER3 with synergistic pairs of antibodies, which simultaneously remove all three targets, thereby preventing compensatory tumor promoting mechanisms within the HER family. Pan-HER induces potent growth inhibition in a range of cancer cell lines and xenograft models, including cell lines with acquired resistance to therapeutic antibodies. Pan-HER is also highly efficacious in the presence of HER family ligands, indicating that it is capable of overcoming acquired resistance due to increased ligand production. All three target specificities contribute to the enhanced efficacy, demonstrating a distinct benefit of combined HER family targeting when compared with single-receptor targeting.

Conclusions: Our data show that simultaneous targeting of three receptors provides broader efficacy than targeting a single receptor or any combination of two receptors in the HER family, especially in the presence of HER family ligands. Pan-HER represents a novel strategy to deal with primary and acquired resistance due to tumor heterogeneity and plasticity in terms of HER family dependency and as such may be a viable alternative in the clinic.

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Introduction

The human epidermal growth factor receptor (HER) family consists of four members: EGFR/HER1, ErbB-2/HER2, ErbB-3/HER3, and ErbB-4/HER4. These receptors play an important role in normal cell growth, metabolism, proliferation, survival, and differentiation. However, deregulation through mutation, overexpression, or gene amplification of the HER family is commonly associated with development, progression, or acquired resistance of many human cancers (1). Homo- or hetero-dimerization induced by binding of ligands within the EGF family of growth factors results in cross-phosphorylation of the dimerization partners, ultimately triggering intracellular signaling (2, 3).

EGFR and HER2 are clinically validated targets in squamous cell carcinoma of the head and neck, colorectal, breast, gastric, and non–small cell lung cancers, and growing evidence suggests that HER3 will prove to be a clinically relevant target as well (2, 4–6).

In contrast, the role of HER4 in cancer pathogenesis is less clear. Both small-molecule tyrosine kinase inhibitors (TKI), targeting individual or multiple members of the HER family, and therapeutic monoclonal antibodies have been approved for treatment of various cancers. However, currently approved antibody therapeutics only address individual receptors (e.g., cetuximab and panitumumab against EGFR and trastuzumab and pertuzumab against HER2), although pertuzumab was selected to prevent HER2 from dimerizing with other HER family members (7). Bispecific antibodies and combinations of antibodies that target two members of the HER family are also in clinical development (8, 9).

Accumulating evidence shows that the HER family displays a high degree of plasticity and provides compensatory signaling leading to acquired resistance in response to therapeutic intervention (10, 11). Examples include HER family TKI (gefitinib and erlotinib) resistance in HER2-driven breast cancer due to increased HER3 expression (12), anti-HER2 trastuzumab resistance in breast cancer due to overexpression of EGFR and EGFR ligands (13) or increased EGFR and/or HER3 expression (14), EGFR/HER2 TKI lapatinib resistance in breast cancer due to HER3 upregulation (15), and anti-EGFR cetuximab resistance in colorectal cancer mediated by upregulation of HER2 or HER3 ligand neuregulin (heregulin; ref. 16). Simultaneous targeting of more than one HER family receptor is frequently able to reverse the resistance to the initial drug and is often more efficacious than single-receptor targeting alone (15–17). Inhibiting more than one HER family member may thus be critical to limit acquired resistance and more effectively treat cancer.
Translational Relevance

Human epidermal growth factor receptor (HER) family expression heterogeneity and plasticity in response to therapeutic intervention are important drivers of primary and acquired drug resistance and pose challenges to effective cancer treatment in the clinic. To address these challenges, we have generated Pan-HER, a mixture of six antibodies targeting each of EGFR, HER2, and HER3 with a pair of synergistic antibodies. We show here that by targeting three receptors of the HER family, a range of cancers of various tissue origin and genetic background are effectively inhibited. Importantly, by downmodulating all three targets simultaneously, Pan-HER prevents compensatory receptor upregulation and also renders the receptors unavailable for ligand binding and activation. The results presented here show that Pan-HER overcomes acquired resistance due to HER family expression heterogeneity and plasticity and thus support clinical evaluation of Pan-HER in patients with HER family–dependent tumors.

In addition, the cells within a tumor may display extensive heterogeneity and therefore do not respond equally to therapy. This intratumor heterogeneity comprises not only clinically important traits such as ability to metastasize and resist therapy, but also the expression of biomarkers or potential therapeutic targets, including the members of the HER family (18, 19). Heterogeneous expression patterns have been described for EGFR in glioblastoma (20, 21) and for HER2 in breast cancer (22, 23), gastric cancer (24), and salivary duct carcinoma (20). In addition, biomarker studies in primary breast cancer have revealed a highly heterogeneous expression pattern for the whole HER family (25). Consequently, biomarker assessment and subsequent selection of an optimal targeted therapy remains challenging even when multiple biopsies are available.

In light of these findings, we hypothesized that simultaneous targeting and downmodulation of EGFR, HER2, and HER3 would provide efficacious tumor growth inhibition across a broad range of cancers as well as overcome acquired resistance due to receptor plasticity or increased ligand expression. As shown previously (26–28), certain combinations of two antibodies targeting non-overlapping epitopes on EGFR or HER2 act synergistically and are superior to individual antibodies in terms of target elimination and cancer cell growth inhibition. This receptor elimination also results in superior activity versus individual antibodies (e.g., cetuximab) in the presence of EGFR ligands (27). Here we have taken this concept one step further by the generation of Pan-HER—an antibody mixture comprised of synergistic pairs of antibodies targeting EGFR, HER2, and HER3.

Materials and Methods

Cell lines

Cell lines were obtained from cell line repositories or kindly provided by the Department of Radiation Biology, National University Hospital, Denmark. All cell lines were mycoplasma free, cultured according to supplier recommendations, continuously evaluated for visual changes, and either used within 6 months of purchase or STR-profiled using the ATCC Cell Authentication Testing Service. Source, origin, subtype, and growth medium for each cell line are summarized in Supplementary Table S1. Amplification and mutation status for the cell lines was extracted from the Cancer Cell Line Encyclopedia (http://www.broadinstitute.org/ccle/home).

Resistant HN5, OE19, and MDA-MB-175-VII cells were established by exposure to increasing concentrations of cetuximab, trastuzumab, or pertuzumab, respectively, for up to 12 months. The level of antibody resistance was tested every month in a WST-1 viability assay (see below) until a pool of resistant cells was established. Single-cell clones were subsequently generated by limited dilution.

Antibodies against EGFR, HER2, and HER3

To generate antibodies against EGFR, HER2, and HER3, mice were immunized with soluble, recombinant antigens and/or human cancer cells overexpressing the targets as described elsewhere (28, 29). The resulting antibody repertoires were cloned from single splenic B cells by the Symplex technology (30, 31) and expressed as full-length mouse-human chimeric or humanized IgG1 in mammalian cells. The anti-EGFR antibodies 1277 and 1565, anti-HER2 antibodies 4384 and 4517, and the anti-HER3 antibodies 5038 and 5082 were identified by screening for binders to soluble, recombinant antigens as well as human cancer cells as described before (29). Relative binding specificities were determined as described previously (28, 29).

Anti-EGFR antibody cetuximab (Erbitux) and anti-HER2 antibody trastuzumab (Herceptin) were obtained from Merck KGaA and Roche, respectively. Analogues of anti-HER3 antibody MM-121 (32) and anti-HER2 antibody pertuzumab (33) were generated by cloning and expressing the variable region sequences in human IgG1 format in mammalian cells as described above.

Cell proliferation and growth assays

A WST-1 (Roche Diagnostics) viability assay was used to measure growth inhibition following antibody treatment for 96 hours as described previously (27). Antibody mixtures were prepared immediately before performing experiments by mixing equal amounts of the constituent antibodies and each treatment was titrated in duplicate from 50 μg/mL. Antibody mixture concentration refers to total antibody concentration. For ligand stimulation, human recombinant epidermal growth factor (EGF; 1 nmol/L final concentration) or neuregulin (5 nmol/L final concentration), both obtained from R&D Systems, was added to the cells immediately before antibody exposure.

Analysis of receptor modulation and downstream signaling

Cell lines were plated in serum-starved growth medium and exposed to antibody treatments (40 μg/mL total antibody concentration) for 48 hours before harvest. To study the effect of ligand on receptor phosphorylation and downstream signaling, the cells were treated with antibodies for 48 hours followed by exposure to 1 nmol/L EGF or 5 nmol/L neuregulin for 15 minutes immediately before harvest. Cell lysates were prepared as described elsewhere (27).

Size-based Simple Western and traditional Western blot analysis

Samples for Simple Western analysis of total receptor levels were diluted to a final protein concentration of 1 to 3 μg/mL in a master mix containing internal fluorescent standards and DTT,
and processed under standard conditions in a Sally Simple Western instrument (ProteinSimple). Rabbit primary antibodies against EGFR (D38B1), HER2 (28D8), HER3 (D22C5) from Cell Signaling Technology (all diluted 1:50) were used.

Traditional Western blot analysis was performed using the NuPAGE SDS-PAGE Gel System (Life Technologies). The membranes were developed using an Odyssey infrared imaging (LI-COR Biosciences) with Image Studio software version 2.1. The following antibodies were used (clone number, phosphorylation site, and stock dilution shown in brackets): Rabbit primary antibodies against EGFR, HER2, and HER3 (same as above diluted 1:1,000), pEGFR (Y1068, 1:1,000), pH2R2 (6B12, Y1221/1222, 1:1,000), pHER3 (21D3, Y1289, 1:2,000), AKT (1:1,000), pAKT (S473, 1:1,000), ERK1/2 (1:1,000), pERK1/2 (D13.14.4E, T202/Y204, 1:1,000), and PARP (1:500) were obtained from Cell Signaling Technology. Donkey anti-rabbit IRDye 800 CW secondary antibodies were used (clone number, phosphorylation site, and dilution shown in brackets).

Results

Identification of Pan-HER, a potent mixture of antibodies targeting EGFR, HER2, and HER3

Pairs of antibodies against EGFR, HER2, and HER3 for inclusion in the Pan-HER antibody mixture were identified by screening panels of approximately 50 antibodies against each receptor for synergistic in vitro inhibition of cancer cell growth and induction of target downmodulation in multiple cancer cell lines (28, 29 and data not shown). As shown in Fig. 1A, the identified antibody pairs recognize nonoverlapping epitopes on their target receptors. The two antibodies against EGFR (1277 and 1565) bind nonoverlapping epitopes on domain III and IV of the receptor, respectively, and the two antibodies against HER3 (5038 and 5082) bind nonoverlapping epitopes on domain I of the target receptor. The antibodies bind to both human and cynomolgus receptors, but do not cross-react with the murine counterparts (data not shown).

Targeting each receptor with an antibody pair demonstrated superior in vitro growth inhibitory activity compared with targeting with either of the monoclonal antibodies alone (Fig. 1B). Each antibody pair also demonstrated strong binding synergy, i.e., the combination displayed greater growth inhibitory activity than either antibody on its own at the same total antibody concentration (Fig. 1B and Supplementary Fig. S1). In addition, more pronounced receptor downmodulation was observed in cell lines with dependency on each of the three targets upon treatment with the antibody pair (Fig. 1C).

Pan-HER broadly inhibits a large and diverse panel of cancer cell lines with different EGFR, HER2, and HER3 expression levels and dependencies

The Pan-HER antibody mixture was tested in vitro against an extensive panel of more than 100 cancer cell lines originating from 13 clinical indications (Supplementary Table S1) to address the effect of simultaneous intervention with EGFR, HER2, and HER3. To assess the contribution of each target specificity in the Pan-HER antibody mixture, the antibody pairs comprising the mixture (individually or combined), and the reference antibodies cetuximab, trastuzumab, and MM-121, were included for comparison. Results from this large screen demonstrated that Pan-HER inhibited all included cell lines with a dependency on EGFR, HER2, or HER3 signaling (Fig. 2A and B). In most cases, Pan-HER effectively inhibited cancer cell growth and outperformed single-receptor targeting antibodies. Highly varying responses to the various dual- or triple-receptor targeting treatments were observed in different cell lines and the precise HER family dependency was difficult to predict based on molecular signatures. Pan-HER was superior to single-receptor and HER2+HER3 targeting antibody mixtures across the cell line panel (P < 0.001; Fig. 2B) and also to the EGFR+HER2 and EGFR+HER3 targeting mixtures in a number of cell lines, three of which are shown in Fig. 2C. Pan-HER was also superior to the combination of cetuximab, trastuzumab, and MM-121 in a limited number of cell lines where this was tested (Supplementary Fig. S2). Importantly, Pan-HER was broadly efficacious in cell lines carrying mutations in a range of clinically relevant oncogenes, including TP53 and KRAS and amplified or mutated EGFR and ERBB2 genes (Fig. 2A). However, the strong efficacy was not restricted to cell lines with HER gene amplification and/or distinct mutational profiles. The fact that not all cell lines were inhibited to the same degree demonstrates that the activity is not due to a general cytotoxic effect.

Pan-HER retains efficacy in the presence of ligands and in cancer cells with acquired resistance to HER family targeting antibodies

Due to the well-established role of HER family ligands in inducing resistance to HER-targeted treatments (13, 16), the efficacy of Pan-HER treatment was also tested in the full cell line panel in the presence of EGF (EGFR ligand) or neuregulin (HER3 ligand). The results demonstrate that the broad and potent inhibitory effect of Pan-HER is maintained in the presence of ligands (Fig. 3A–C). Upon EGF stimulation, Pan-HER...
demonstrated broader efficacy than all the single-receptor targeting treatments, including EGFR ($P < 0.05$), and the HER2+HER3 targeting mixture ($P < 0.001$; Fig. 3B). The ligand-induced neutralization of single and dual HER–targeting treatments was particularly striking upon neuregulin stimulation, where Pan-HER displayed significantly broader activity across the cell line panel than all the other treatments ($P < 0.01$; Fig. 3B).

As outlined above, development of acquired resistance is frequently observed in response to treatment with antibodies targeting a single HER family member. To investigate the efficacy of Pan-HER in the event that acquired resistance ensues, cetuximab-, trastuzumab-, or pertuzumab-resistant cells were generated and tested for sensitivity to Pan-HER treatment. As shown in Fig. 3D, trastuzumab-resistant cells retained partial sensitivity and cetuximab- and pertuzumab-resistant cells retained full sensitivity to Pan-HER. Interestingly, the combination of trastuzumab and pertuzumab was unable to reverse resistance to either of the single antibodies in trastuzumab and pertuzumab resistant HER2- and HER3- dependent cell lines (Fig. 3D), emphasizing the need for targeting more than one HER family member to overcome acquired resistance.

Pan-HER effectively downmodulates target receptors and inhibits compensatory receptor upregulation, HER phosphorylation, and downstream signaling

To investigate how simultaneous targeting of EGFR, HER2, and HER3 would modulate target receptor levels, three selected cell lines of various tissue origins were treated with Pan-HER, the single-receptor targeting antibody pairs, cetuximab, trastuzumab, or MM-121, and receptor levels were quantified by Simple Western analysis. Cetuximab treatment decreased EGFR levels in MDA-MB-175-VII cells, whereas the total EGFR level was not
significantly decreased in OVCAR-8 and BxPC3 cells (Fig. 4A). Trastuzumab and MM-121 treatment resulted in HER2 and HER3 downmodulation, respectively, in all three cell lines. Treatment with the antibody pairs against each receptor consistently resulted in potent degradation of the targeted receptor, which was in many cases significantly superior to that of single antibodies (Fig. 4A). Notably, Pan-HER treatment caused effective, simultaneous downmodulation of all three receptors in all cell lines tested (Fig. 4A).

Interestingly, targeting a single HER family member was often found to increase levels of one or both of the other receptors. For instance, treatment with trastuzumab or the HER2 targeting antibody pair resulted in upregulation of EGFR in BxPC3 cells and a similar compensatory upregulation of HER2 was observed upon HER3 targeting in all three cell lines. Trastuzumab treatment also appeared to induce elevated HER3 levels in all cell lines (Fig. 4A). Compensatory receptor upregulation in response to single receptor targeting was also observed for EGFR upon HER3 targeting by MM-121 in A431NS cells and for HER2 upon EGFR targeting in H292 cells (Fig. 4B, left; and data not shown). In contrast, Pan-HER uniformly and significantly decreased the expression of all three receptors in all tested cell lines, thereby effectively preventing compensatory receptor upregulation (Fig. 4A and B).

The phosphorylation status of EGFR, HER2, and HER3 upon antibody treatment was assessed in three cell lines with overexpression and constitutive baseline phosphorylation of EGFR, HER2, and HER3. The antibody pairs and, to a lesser extent, the reference antibodies were found to inhibit phosphorylation of the target receptor, whereas Pan-HER decreased phosphorylation of all three targets (Fig. 4B). In general, the phosphorylation level upon treatment correlated well with total receptor levels, suggesting that the decrease in receptor phosphorylation by Pan-HER is due to receptor elimination.

To investigate whether Pan-HER was able to prevent ligand-mediated receptor activation and downstream signaling, BxPC3 and MDA-MB-175-VII cells were treated with Pan-HER followed by exposure to EGFR- and HER3-activating ligands EGF and neuregulin. Total protein and phosphorylation levels of EGFR or HER3 and downstream signaling mediators ERK1/2 and AKT were analyzed (Fig. 4C). Whereas individual targeting of EGFR or HER3 to some extent inhibited phosphorylation of the targeted receptor...
Figure 3.
Pan-HER retains efficacy upon ligand stimulation and in cancer cells with acquired resistance to HER-targeting antibodies. A, heatmaps showing maximum inhibition of treated cells as percentage metabolic activity of untreated cells in the absence of ligand in 44 cancer cell lines either stimulated with EGF (+EGF) or neuregulin (+NRG; for full figure annotation see Fig. 2A). B, scatter plot showing maximum inhibition of metabolic activity in 75 cell lines after treatment in the presence of EGF or neuregulin (NRG). Red bars, mean metabolic activity of all included cell lines. C, dose–response curves after treatment with Pan-HER or single antibodies in two cell lines (CAPAN-1 and OE19) in the absence and presence of either EGF or neuregulin (NRG). For all curves, results are normalized to metabolic activity in the absence of ligand (set to 100%). EGF and NRG (on y-axis) denote cell metabolic activity upon exposure to EGF or neuregulin, respectively, in the absence of therapeutic antibodies. D, dose–response curves after treatment with Pan-HER and representative monoclonal antibodies in a cetuximab-resistant HN5 cell clone, a trastuzumab-resistant OE19 cell clone, and a pertuzumab-resistant clone of MDA-MB-175-VII. Error bars, SEM. Data are representative of two independent experiments.
and downstream signaling proteins, only Pan-HER treatment resulted in simultaneous inhibition of receptor phosphorylation and consistently attenuated downstream signaling in the presence of ligand. Pan-HER likely inhibits receptor phosphorylation not only by preventing ligand binding, but also by inducing receptor internalization and degradation.

Pan-HER effectively inhibits tumor growth in a panel of xenograft models

To expand our in vitro findings to the in vivo setting, the Pan-HER antibody mixture was initially tested in cell line–derived xenograft models with confirmed HER family dependence. The results showed that Pan-HER was superior to single-receptor targeting by cetuximab and trastuzumab in both A431NS, known to be EGFR dependent (27), and N87, known to be HER2 dependent (ref. 34; Fig. 5A). Next, the effect of Pan-HER treatment was assessed in PDX models originating from four different cancer indications: ovary, colorectal, lung, and pancreas (Fig. 5B). Among the tested models were hard-to-treat tumors with known resistance to targeted therapies and models harboring mutations in known oncogenes, including KRAS (Supplementary Table S2). Pan-HER treatment resulted in a striking and sustained antitumor effect in all four indications, with tumor regression observed in three of the models (Fig. 5B).
Pan-HER displays superior in vivo activity due to synergistic effects of targeting EGFR, HER2, and HER3.

To further address the specific contribution of the individual target specificities in the Pan-HER antibody mixture and to assess potential synergies, single-, dual-, and triple-receptor targeting was tested in two xenograft models, BxPC3 and Calu-3, previously used to demonstrate synergistic effects of combined HER family receptor targeting (35–38).

In the BxPC3 pancreatic cancer model, antibody pairs targeting EGFR or HER2 were unable to control tumor growth, but induced

Figure 5.
Pan-HER in vivo efficacy. A, in vivo efficacy of Pan-HER in the A431NS and N87 tumor xenograft models. Cetuximab and trastuzumab were included as comparators. B, effect of Pan-HER treatment in established PDX models originating from ovarian, colorectal, lung, and pancreatic cancer. Gray areas, treatment period. Data, means ± SEM.

Pan-HER Overcomes Tumor Heterogeneity and Plasticity
a marginally slower growth rate when compared with the control group (Fig. 6A). The HER3-targeting antibody pair inhibited tumor growth more effectively than antibody pairs targeting either EGFR or HER2. Simultaneous targeting of any two HER family receptors controlled tumor growth better than targeting of any individual receptor, and the effect was synergistic when EGFR+HER2 or EGFR+HER3 were targeted simultaneously (Supplementary Fig. S3A). The groups treated with antibodies against EGFR+HER3 or Pan-HER both showed pronounced tumor growth inhibition and tumor regression. In a separate experiment, it was demonstrated that the same level of tumor growth inhibition is also achieved with a 10-fold lower dose of Pan-HER (Supplementary Fig. S4).

Figure 6.
Pan-HER in vivo efficacy and effect on receptor modulation in the BxPC3 and Calu-3 tumor xenograft models. A, in vivo efficacy of Pan-HER and its constituent antibody pairs in the BxPC3 and Calu-3 tumor xenograft models. Gray areas, treatment period. Data, means ± SEM. B, Simple Western analysis of EGFR, HER2, and HER3 protein levels in BxPC3 tumor lysates (N = 3/group). Data, means ± SEM. C, IHC images showing examples of EGFR, HER2, and HER3 staining in vehicle and Pan-HER-treated BxPC3 tumors harvested 3 days after final treatment. Bar graphs, means of the IHC scoring of EGFR, HER2, and HER3 in all treatment groups (N = 3/group). Error bars, SEM. The antibody pairs against each receptor are denoted as EGFR, HER2, and HER3.

In the HER2-amplified, yet trastuzumab-resistant Calu-3 lung cancer model, single-receptor targeting, either by reference antibodies or antibody pairs, demonstrated limited effect on tumor growth, whereas each dual-receptor targeting antibody mixture induced synergistic tumor growth inhibition (Fig. 6A...
BxPC3 tumors were investigated by Simple Western analysis. In accordance with the simultaneously. Although small-molecule TKIs are often capable currently in development, fail to capture the full complexity of the these approaches, including bi- or dual-specific antibody formats and/or survival pathways is also well established and causing acquired resistance to targeted therapies, are well known to provide compensatory signaling in human cancers, thereby translating to the in vivo setting. Receptor levels in excised BxPC3 tumors were investigated by Simple Western analysis. EGFR, HER2, and HER3 were all downmodulated in vitro, either when targeted individually or in combination with another receptor (Fig. 6B), but Pan-HER was the only treatment that induced simultaneous downmodulation of all three receptors. In accordance with the in vitro findings (Fig. 4A), HER2 protein levels were upregulated in tumors treated with antibodies targeting HER3, suggesting compensatory HER2 upregulation as an escape mechanism in the BxPC3 model.

IHC analysis of the same BxPC3 tumors confirmed these results by demonstrating simultaneous downmodulation of all three targeted receptors in Pan-HER–treated tumors (Fig. 6C). Although the IHC results did not show the same pronounced differences in EGFR levels between the different treatment groups as the Simple Western, the trend was similar with decreased EGFR levels in the groups treated with antibodies targeting EGFR (Fig. 6B and C). Decreased HER2 levels were observed in all treatment groups compared with the vehicle control group, but most prominently in the groups treated with a HER2-targeting component (Fig. 6C). HER3 levels were also decreased in tumors treated with HER3-targeting antibodies alone or in a mixture (Fig. 6C). Total HER3 IHC scores and levels of HER3 determined by Simple Western were comparable.

Effective downmodulation of all three receptors was also observed in Pan-HER–treated Calu-3 tumors (data not shown). IHC analysis showed that in the tumors most efficiently inhibited by antibody treatment, up to 60% of the tumor volume was comprised of stromal host cells (Supplementary Fig. S5A), suggesting that the remaining tumors in the Pan-HER group (Fig. 6A) primarily consist of fibrous tissue derived from necrosis and resulting scarring. Furthermore, Pan-HER–treated Calu-3 tumors had lower expression of the proliferation marker Ki67 versus all other treatment groups (Supplementary Fig. S3B). This result supports the in vitro findings of enhanced cell cycle arrest in Calu-3 cells treated with Pan-HER versus single-receptor targeting (Supplementary Fig. S6D).

Discussion

The plasticity of the HER family members and their ability to provide compensatory signaling in human cancers, thereby causing acquired resistance to targeted therapies, is well known (12–17). The notion of combining treatments that target different receptors and/or survival pathways is also well established and combined targeting of two HER family members, such as EGFR and HER2 (35, 36, 38), EGFR and HER3 (37), and HER2 and HER3 (39), has been described previously. However, most of these approaches, including bi- or dual-specific antibody formats currently in development, fail to capture the full complexity of the HER family plasticity as only two of the receptors are targeted simultaneously. Although small-molecule TKIs are often capable of inhibiting multiple kinases simultaneously, they are unable to directly address HER3 as very limited kinase activity is associated with this member of the HER family.

To investigate and address these challenges and limitations, we have combined three synergistic pairs of antibodies against EGFR, HER2, and HER3 into a potent HER family–targeting antibody mixture, Pan-HER. As described previously (26–28) and herein, combinations of two antibodies binding to non-overlapping epitopes on their target effectively induce receptor internalization and degradation and in most cases are superior to individual antibodies in terms of cancer cell growth inhibition and prevention of ligand binding (27). Targeting each receptor with a pair of antibodies may also prevent the emergence, or diminish the effect, of mutations in the extracellular domain of the targets, which may confer resistance to monoclonal antibodies (40–42).

The in vitro data presented here demonstrate that Pan-HER potently inhibits a broad range of cancer cell lines of diverse tissue origin and genetic background, including cell lines with acquired resistance to cetuximab, trastuzumab, or pertuzumab. In the presence of HER family ligands (EGF and neuregulin), the effect of single receptor–targeting agents is diminished, whereas Pan-HER remains highly efficacious. Whereas combined EGFR and HER2 targeting in the presence of EGF is comparable with targeting all three receptors by Pan-HER, none of the dual-receptor-targeting mixtures are as efficacious as Pan-HER in the presence of neuregulin. The sustained antiproliferative effect of Pan-HER is likely due to the effective, simultaneous downmodulation of all three receptors, rendering them unavailable for ligand binding and incapable of forming signaling competent heterodimers. Although HER2 does not appear to have a high-affinity ligand, it is constitutively available for dimerization due to its permanently open conformation (43, 44). HER2 has also been shown to be the preferred heterodimerization partner of the other HER family members (45), with the HER2–HER3 dimer being reported as the most potent signaling module (46, 47). Nevertheless, an enhanced effect of targeting HER2 and HER3 simultaneously in the absence of ligand is not evident from the experiments herein, possibly due to the limited effect of HER3 targeting in vitro (Fig. 2). However, in the presence of the HER3 ligand neuregulin, removal of HER2 strongly contributes to the overall efficacy of the Pan-HER mixture in vitro (Fig. 3). Thus, Pan-HER is capable of effectively blocking ligand-induced cell proliferation in cancer cell lines exposed to both EGF and neuregulin, suggesting that it will also be capable of overcoming acquired resistance due to increased ligand production in the clinical setting.

Concurrent targeting of EGFR, HER2, and HER3 with Pan-HER not only results in receptor downmodulation, but also prevents compensatory receptor upregulation, another known mechanism of resistance to single-receptor–targeting therapies observed both in preclinical models and in the clinic (12–17). In the study presented here, compensatory receptor upregulation was observed in multiple cell lines in vitro and the involvement of alternative receptors, including HER3, has also been demonstrated in the cetuximab resistant cell line utilized here (Kønr and colleagues, manuscript in preparation). In addition, studies by others have shown that resistance to trastuzumab may be due to upregulation of EGFR and/or HER3 (14), a compensatory response that was also observed upon pertuzumab treatment in the current study (Fig. 4A). The unpredictable nature of the compensatory response contributes to the difficulty in identifying the most efficacious target combination. However, Pan-HER
effectively prevents the compensatory response in all cell lines examined and appears to be able to control HER family plasticity. No correlation between RAS status and activity of Pan-HER is evident as Pan-HER was active against both cell lines and xenografts with RAS mutations (Fig. 2A and Supplementary Table S2). This finding is exciting, but not surprising, as RAS status is not a strong predictor of resistance to anti-EGFR, anti-HER2, and anti-HER3 antibodies in preclinical studies. Whether or not Pan-HER will provide a benefit in patient tumors with mutations in RAS genes remains to be demonstrated in clinical trials.

Cancer cell inhibition by Pan-HER is mediated by both apoptosis and cell-cycle arrest (Supplementary Fig. S6). In Calu-3 cells, a substantial degree of cell-cycle arrest was observed, correlating well with the significantly decreased Ki67 expression in Pan-HER–treated Calu-3 tumor xenografts (Supplementary Fig. S5B). It appears that the HER family receptor dependency in each cell line determines the treatment outcome, with some cell lines being more prone to cell death and others to cell-cycle arrest. Clearly, in the Calu-3 model system, Pan-HER eradicated tumor cells as well as inhibited proliferation of the remaining cells both in vitro and in vivo. These results are consistent with previous observations indicating that combined targeting of two HER family receptors enhances cell death and cell-cycle arrest (39, 48). Although IHC analysis of the apoptosis marker cleaved caspase-3 did not show enhanced apoptosis in Pan-HER–treated BxPC3 or Calu-3 tumors (data not shown), the possibility that apoptotic cell death may have contributed to the effect earlier during the treatment period cannot be excluded.

The superior ability of Pan-HER to downregulate EGFR, HER2, and HER3 simultaneously and inhibit cancer cell growth in vitro also translates into efficacious tumor growth suppression in vivo. Pan-HER strongly suppresses tumor growth in a range of tumor xenograft models with varying dependency on EGFR, HER2, and HER3, including PDX models of ovarian, colorectal, lung, and pancreatic cancer, thus further confirming its broad in vivo anti-cancer activity. In accordance with previous observations (35–38), the BxPC3 and Calu-3 xenograft studies showed that there is a clear benefit of combining HER family target specificities as compared with single-receptor targeting. Importantly, all three target specificities were shown to contribute to the efficacy of Pan-HER in vivo. In the BxPC3 model, all three dual-receptor–targeting antibody mixtures showed an increased efficacy as compared with single-receptor targeting, with the EGFR+HER3–targeting mixture and Pan-HER being equally efficacious. In the Calu-3 model, synergistic effects were observed for all dual-receptor–targeting mixtures, but the combined targeting of all three receptors with Pan-HER was superior to all other treatments in this model. The BxPC3 study also provided an example of compensatory upregulation in vivo as HER2 levels increased substantially in response to HER3 targeting. Pan-HER effectively prevented this upregulation by downmodulating all three targets simultaneously. The in vivo results demonstrate that Pan-HER is capable of effectively dealing with the extensive plasticity in terms of HER family dependency displayed by these xenograft models of human cancer.

Preclinical and clinical evidence suggests that effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) may contribute to the effect of therapeutic antibodies, including rituximab, trastuzumab, and cetuximab, and efforts have been made to further increase effector functions by Fc engineering (49). Multiple antibodies binding simultaneously to cellular targets can also result in enhanced effector functions, presumably by increasing the antibody density on the cell surface. Pan-HER induces a level of ADCC in vitro similar to that of cetuximab or trastuzumab, whereas the level of CDC is clearly enhanced (Supplementary Figs. S7 and S8). As reported previously (50), nonengineered anti-HER3 antibodies of the IgG1 subtype induce very limited ADCC (Supplementary Fig. S7), most likely due to low HER3 expression in most cell lines.

The data presented herein demonstrates that targeting EGFR, HER2, and HER3 with a mixture of antibodies induces simultaneous downmodulation of the three receptors and effectively prevents compensatory receptor upregulation, a well-known mechanism of resistance to HER family–targeted therapies. This mechanism of action translates into broad and potent activity across a range of cancer models with varying expression and dependency on these receptors both in vitro and in vivo. The broad inhibitory activity is even more evident in the presence of HER family ligands, which may contribute to ligand dependent resistance known to emerge in some patients upon treatment with HER-targeted therapies. Our data suggest that concurrent inhibition of EGFR, HER2, and HER3 with Pan-HER will be superior to existing targeted therapies in dealing with both primary and acquired resistance due to HER family expression heterogeneity and plasticity. Pan-HER may thus provide an attractive strategy to treat various HER family-dependent epithelial tumors. Pan-HER is being evaluated in cynomolgus monkeys to assess potential adverse effects of combined HER targeting.

Disclosure of Potential Conflicts of Interest

H.J. Jacobsen, I. Kjær, and J. Lantto are co-inventors on a patent owned by Symphogen A/S that describes the Pan-HER composition. No potential conflicts of interest were disclosed by the other authors.

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