Targeting the HER Family with Pan-HER Effectively Overcomes Resistance to Cetuximab

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Abstract

Cetuximab, an antibody against the EGFR, has shown efficacy in treating head and neck squamous cell carcinoma (HNSCC), metastatic colorectal cancer, and non–small cell lung cancer (NSCLC). Despite the clinical success of cetuximab, many patients do not respond to cetuximab. Furthermore, virtually all patients who do initially respond become refractory, highlighting both intrinsic and acquired resistance to cetuximab as significant clinical problems. To understand mechanistically how cancerous cells acquire resistance, we previously developed models of acquired resistance using the H226 NSCLC and UM-SCC1 HNSCC cell lines. Cetuximab-resistant clones showed a robust upregulation and dependency on the HER family receptors EGFR, HER2, and HER3. Here, we examined pan-HER, a mixture of six antibodies targeting these receptors on cetuximab-resistant clones. In cells exhibiting acquired or intrinsic resistance to cetuximab, pan-HER treatment decreased all three receptors’ protein levels and downstream activation of AKT and MAPK. This correlated with decreased cell proliferation in cetuximab-resistant clones. To determine whether pan-HER had a therapeutic benefit in vivo, we established de novo cetuximab-resistant mouse xenografts and treated resistant tumors with pan-HER. This regimen resulted in a superior growth delay of cetuximab-resistant xenografts compared with mice continued on cetuximab. Furthermore, intrinsically cetuximab-resistant HNSCC patient-derived xenograft tumors treated with pan-HER exhibited significant growth delay compared with vehicle/cetuximab controls. These results suggest that targeting multiple HER family receptors simultaneously with pan-HER is a promising treatment strategy for tumors displaying intrinsic or acquired resistance to cetuximab.

Introduction

The HER family of receptor tyrosine kinases (RTK) play a prominent role in cell physiology, development, and cancer pathophysiology. This family consists of four members: EGFR, HER2, HER3, and HER4. These receptors are activated on the cell membrane by the binding of specific ligands, which leads to receptor homo- and hetero-dimerization with other HER family members. Dimerization of HER family receptors results in the activation of each receptor’s tyrosine kinases, and subsequently the activation of multiple downstream effector molecules (1, 2). Specifically, EGFR regulates the MAPK and PI3K/AKT signaling pathways, both of which have been associated with increased cellular proliferation, survival, angiogenesis, and invasion (3).

Overexpression or activation of the EGFR has been linked to poor prognosis in several cancers including metastatic colorectal cancer (mCRC), head and neck squamous cell carcinoma (HNSCC), non–small cell lung cancer (NSCLC), and brain cancer (4–6). Therefore, EGFR has been pursued as a molecular target over the last three decades in many cancer treatment strategies. One approach uses mAbs to target the extracellular domain of the EGFR to block natural ligand binding. Cetuximab (ICM-225, Erbitux) is a human:mouse chimeric monoclonal antibody (mAb) that binds to the EGFR. Cetuximab is approved for the treatment of patients with mCRC and HNSCC (7, 8), and more recent reports have indicated that it may, additionally, have clinical applications in the treatment of NSCLC (9, 10). Unfortunately, clinical data suggest that the majority of patients who initially respond to cetuximab eventually acquire resistance (11, 12).

To understand the mechanisms of acquired resistance to cetuximab, we previously developed models of cetuximab resistance by treating the cetuximab-sensitive NSCLC line H226 and HNSCC line UM-SCC1 with increasing concentrations of cetuximab until single-cell–resistant clones emerged (13). Detailed exploration of these model systems revealed that cetuximab-resistant clones have increased steady-state expression and activation of HER family receptors (EGFR, HER2, and HER3) due to impaired internalization and degradation (13). It was also demonstrated that the activation of HER3 is dependent upon the activation of EGFR and HER2, and this promotes survival in cetuximab-resistant clones (13).

In accordance with known HER family interdependency, it has also been demonstrated that targeting both the EGFR (with cetuximab) and the HER3 (with U3-1287) signaling axes is

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necessary to achieve a significant antitumor response in tumors refractory to cetuximab therapy (14). This indicates the importance of inhibiting more than one HER family receptor to maximally block the signaling network emanating from the HER family (14). Similar patterns of HER family interdependency have been demonstrated in studies of combination antibody therapies for other cancers and found to elicit antitumor response. Acquired resistance to trastuzumab in breast cancer cell lines was correlated with increased expression levels of EGFR and HER3. These cells could be sensitized with combined treatment of gefitinib and cetuximab or U3-1287 (15). Yonesaka and colleagues demonstrated that subsequent depletion of HER2 in resistant A431 cells restored sensitivity to cetuximab (16). Extensive preclinical studies and initial clinical data also demonstrated that combination inhibitory EGFR and HER2 mAbs had additive or even synergistic antitumor activity (review; see refs. 17, 18). Collectively, these findings suggest that molecular therapeutics only targeting one receptor may not have suitable antitumor effect, rather, a combination of antibodies against multiple receptors is necessary.

These findings have led to the development of therapeutics that target multiple members of the HER family. Pan-HER is a mixture of six mAbs targeting EGFR, HER2, and HER3 (19). In this study, we hypothesized that NSCLC and HNSCC cell-based models and tumors with intrinsic or acquired cetuximab resistance may have an extensive response to pan-HER. We found that proliferation in both intrinsic and acquired cetuximab-resistant NSCLC and HNSCC cells was effectively inhibited by pan-HER treatment. In addition, consistent attenuation of downstream signaling of both cellular proliferation and survival pathways was observed. Analysis of pan-HER–treated acquired cetuximab-resistant NSCLC xenograft tumors as well as intrinsically cetuximab-resistant HNSCC PDX tumors showed reduction of EGFR, HER2, and HER3 expression with tumor growth delay. These observations suggest that using pan-HER may be an effective approach for overcoming both intrinsic and acquired resistance to cetuximab.

**Materials and Methods**

**Cell lines**

The human NSCLC cell line H226 was provided by Drs. J. Minna and A. Gazdar (University of Texas Southwestern Medical School, Dallas, TX). The human NSCLC cell line H358 was purchased from ATCC. The H226 and H358 cells were maintained in 10% FBS in RPMI1640 (Mediatech, Inc) with 1% penicillin and streptomycin. The development of cetuximab-resistant NSCLC and HNSCC cells was effectively inhibited by pan-HER treatment. In addition, consistent attenuation of downstream signaling of both cellular proliferation and survival pathways was observed. Analysis of pan-HER–treated acquired cetuximab-resistant NSCLC xenograft tumors as well as intrinsically cetuximab-resistant HNSCC PDX tumors showed reduction of EGFR, HER2, and HER3 expression with tumor growth delay. These observations suggest that using pan-HER may be an effective approach for overcoming both intrinsic and acquired resistance to cetuximab.

**Antibodies**

All antibodies were purchased from commercial sources as indicated below: EGFR, HER2, HER3, and HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Santa Cruz Biotechnology, Inc. pMAPK (T202/Y204), pAKT (S473), AKT, p-S6rp (S235/236), S6rp, and Ki67 were obtained from Cell Signaling Technology. α-Tubulin was purchased from Calbiochem.

**Cell proliferation (CCK8) assay**

This assay was performed as described previously (22). All treatments were performed in quadruplicate.

**Immunoblot analysis**

Whole-cell protein lysate was obtained using Tween-20 lysis buffer [50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L β-glycerothosphate (BGP), and 10 μg/mL leupeptin and aprotinin]. Immunoblot analysis was conducted as described previously (14).

**Bromodeoxyuridine cell-cycle distribution analysis**

Cells were plated at a density of 8 × 103 cells per 100-mm2 plate and allowed to adhere overnight. The cells were treated with vehicle, 20 μg/mL cetuximab, or 20 μg/mL pan-HER for 24 hours. Bromodeoxyuridine (BrdUrd) cell distribution analysis was performed as described previously (20).

**Mouse xenograft model and patient-derived xenografts**

Athymic nude mice (4- to 6-week-old males or females) were obtained from Harlan Laboratories. All animal procedures and maintenance were conducted in accordance with the institutional guidelines of the University of Wisconsin. H226 or UWSCC1 cells were injected in the dorsal flank of the mouse at respective day 0 (2 × 106 cells). Once tumors reached 200 mm3, mice were randomized into treatment or control groups and started on their respective treatments (IgG, cetuximab, pan-HER). The dose of cetuximab and pan-HER for the experiment was 50 mg/kg and twice a week by intraperitoneal injection. Tumor volume measurements were evaluated by digital calipers and calculated by the formula (p)/6 × (large diameter) × (small diameter)2. The patient-derived xenograft (PDX) model was established as described previously (23).

**Mouse cetuximab-resistant human tumor xenografts**

Mice were injected with H226 (2 × 106 cells), and tumors were allowed to grow to 100 mm3. All mice were randomized to treatment or control groups and treated with 50 mg/kg of either cetuximab or IgG i.p. twice weekly. Tumors were monitored for cetuximab resistance that was defined as marked tumor growth in the presence of continued cetuximab therapy. Once cetuximab-resistant tumors reached a volume of approximately 800 mm3, mice were grouped according to similar time points of resistance. At this point, each mouse was treated with either cetuximab or pan-HER (50 mg/kg) i.p. twice weekly. Tumor volume measurements were evaluated by digital calipers and calculated by the formula (p)/6 × (large diameter) × (small diameter)2.
Xenograft tumor collection and protein isolation

Tumors were collected 24 hours after the last cetuximab or pan-HER treatment. Details of tumor collection and protein isolation were described previously (20). Protein quantitation and immunoblot analysis were performed as stated above.

IHC

Tumor tissue samples were collected from xenograft tumors, fixed in 10% neutral-buffered formalin, paraffin-embedded, and cut into sections. The sections were heated in 10 mmol/L citrate buffer (pH 6.0) for EGFR, HER2, and Ki67 or in EDTA buffer (pH 8.0) for HER3 with a decloaking chamber. Samples were incubated with rabbit anti-EGFR (Abcam; ab52894, 1:200), anti-HER2 (Cell Signaling Technology; cs4290, 1:200), anti-HER3 (Cell Signaling Technology; cs12708, 1:50), or anti-Ki67 (Cell Signaling Technology; cs9027, 1:800). Sections were stained by VECTASTAIN Universal Kit/HRP (Vector Laboratories). Antibody binding was revealed by addition of 3,3′-diaminobenzidine substrate. Tissues were counterstained with Mayer hematoxylin (Thermo Fisher Scientific) and were examined using an Olympus BX51 microscope.

Statistical analysis

Student t test was used to calculate the statistical significance of values provided by the different assay as described above. P ≤ 0.05 was considered as statistically significant. Paired t test analysis was performed for in vivo study and P ≤ 0.01 was considered significant at the 99% confidence level as shown by the asterisks (**).

Results

Pan-HER inhibits proliferation of cetuximab-resistant NSCLC clones

We previously reported a model of acquired cetuximab resistance using the cell line H226. Analysis of individual clones with acquired resistance to cetuximab (HC1, HC4, and HC8) indicated increased expression, activity, and dependency of the HER family relative to the cetuximab-sensitive parental control (HP; refs. 13, 14). We have also demonstrated that EGFR increased association with HER2 and HER3. Simultaneous inhibition of EGFR and HER2 decreased HER3 activation as well as inhibited cell proliferation (13). Furthermore, dual knockdown of EGFR and HER3 by siRNA led to potent antiproliferative effects in cetuximab-resistant clones (14). From these results, we hypothesized that pan-HER, a mixture of six antibodies targeting each of EGFR, HER2, and HER3, would provide an antiproliferative response in cetuximab-resistant clones. Cell proliferation analysis was performed following treatment with either cetuximab or with increasing concentrations of pan-HER for 72 hours (Fig. 1A). All cetuximab-resistant clones demonstrated statistically significant and dose-dependent growth inhibition upon treatment with pan-HER. Treatment with 20 µg/ml pan-HER reduced proliferation rate by approximately 40% compared with the vehicle control. To determine the efficacy of pan-HER on targeting the HER family of receptors, cetuximab-resistant clones were treated with pan-HER (0.1, 1, 20, 100 µg/ml) for 24 hours and the expression levels of HER family receptors were examined. Total protein levels of EGFR, HER2, and HER3 were effectively decreased by pan-HER treatment at concentrations from 1 µg/ml in all cetuximab-resistant clones tested (Fig. 1B). In addition, phosphorylation levels of AKT, S6rp, and MAPK were robustly decreased with increasing doses of pan-HER. In a time-course experiment, cetuximab-resistant clones were treated with pan-HER (20 µg/ml) for 24, 48, or 72 hours (Fig. 1C). The expression levels of HER family receptors were downmodulated by pan-HER as early as 24 hours after treatment onset, with maintained downregulation observed out at the 72-hour time point. Overall, these findings indicated that pan-HER can (i) effectively target HER family receptors in cetuximab-resistant clones for prolonged periods of time and (ii) significantly impact downstream signaling, which may explain the observed decrease in proliferation in cetuximab-resistant clones.

Pan-HER effectively downmodulates HER family receptors and inhibits the proliferation of cetuximab-resistant clones as compared with cetuximab

To investigate the impacts of concurrent targeting of HER family receptors in cetuximab-resistant NSCLC clones by cetuximab compared with pan-HER, we compared the expression levels of EGFR, HER2, and HER3 as well as the phosphorylation levels of downstream signaling molecules in cells treated with 20 µg/ml cetuximab or pan-HER for 24 hours (Fig. 2A). Pan-HER treatment resulted in the downmodulation of EGFR, HER2, and HER3, whereas cetuximab treatment had no effect on HER family levels in cetuximab-resistant clones. Furthermore, the phosphorylation level of AKT, S6rp, and MAPK was significantly diminished after pan-HER treatment in all cetuximab-resistant NSCLC clones, whereas cetuximab treatment had little inhibitory effect on these signaling pathways.

Pan-HER–treated cells also showed potent cell-cycle inhibition in cetuximab-resistant clones after pan-HER treatment when compared with those treated with cetuximab (Fig. 2B). In addition, pan-HER–treated cells had a greater inhibition of S-phase than cetuximab-treated cells in acquired cetuximab-resistant NSCLC cells. Interestingly, compared with cetuximab, pan-HER induced more G1 arrest in HP cetuximab-sensitive cells. We utilized a second model of acquired resistance in HNSCC to further investigate whether pan-HER would be effective in other models of acquired cetuximab resistance (13). The proliferation of the parental UMSCC1 cell line (UMSCC1-P) was inhibited by treatment with 20 µg/ml cetuximab, while the three HNSCC cetuximab-resistant clones (C2, C5, and C11) were completely refractory to cetuximab (Fig. 3A). Evaluation of cellular proliferation 72 hours posttreatment indicated statistically significant inhibition in HNSCC cetuximab-resistant clones by 20 µg/ml pan-HER (Fig. 3A). Immunoblot analysis mirrored the findings in the NSCLC-acquired resistant model (Figs. 1 and 2), showing that the expression of HER family members and activation of MAPK, AKT, and S6rp signaling pathways were inhibited by pan-HER treatment (Fig. 3B). The fact that MAPK activity in the cetuximab-resistant HNSCC cells was blocked by cetuximab treatment, suggests that MAPK plays a minor role in resistance. Collectively, these results suggest that pan-HER can effectively overcome cetuximab resistance in two independent models of acquired resistance and impact this resistance by attenuating signaling cascades involving MAPK, AKT, and S6rp.

Pan-HER delays the growth of tumors with acquired cetuximab resistance

To expand our in vitro findings to the in vivo setting, pan-HER was tested in a cell line–derived xenograft model with confirmed HER family dependence. Thirty mice were injected with the NSCLC H226 cells in the dorsal flank on day 0 (2 × 106 cells),
and once tumors reached an average volume of 200 mm³ (∼18 days), mice were randomized and treatment initiated. Cetuximab or pan-HER was administered through an intraperitoneal injection at a dose of 50 mg/kg twice weekly for ten consecutive weeks. Mouse weight was measured weekly, and no discernible toxicity was observed in either the cetuximab or pan-HER treatment.

Figure 1.
Pan-HER inhibits the proliferation of cetuximab-resistant clones. A, pan-HER inhibits the proliferation of cetuximab-resistant clones. Cetuximab-sensitive cells (HP) and cetuximab-resistant clones (HC1, HC4, and HC8) were plated and treated with vehicle, cetuximab (20 μg/mL), or pan-HER (0.1, 1, 20, or 100 μg/mL) for 72 hours prior to measuring the proliferation by CCK8 assay. Data points are represented as mean ± SEM (n = 4); **, P < 0.001. B, pan-HER downregulates HER family receptors and decreases downstream AKT and MAPK signaling. Cells were treated with vehicle or pan-HER (0.1, 1, 20, or 100 μg/mL) for 24 hours. C, HER family receptors remain downregulated in cetuximab-resistant clones at 72 hours after pan-HER treatment. Cells were treated with vehicle or pan-HER (20 μg/mL) for 24, 48, or 72 hours. Cells were lysed and fractionated on SDS-PAGE, followed by immunoblotting for the indicated proteins. α-Tubulin was used as a loading control.
group. Treatment with pan-HER showed statistically significant tumor growth delay and exhibited superior antitumor activity compared with cetuximab treatment over 60 days (Fig. 4A). Analysis of tumor lysates harvested from each treatment group indicated that expression levels of EGFR, HER2, and HER3 were strongly and uniformly reduced in all tumors from pan-HER–treated mice, whereas IgG- or cetuximab-treated tumors retained significant expression levels of HER family receptors (Fig. 4B).

Next, a series of mouse xenograft studies of de novo acquired resistance to cetuximab were established to evaluate the efficacy of pan-HER in overcoming acquired resistance to cetuximab in vivo. Fifty mice were inoculated with the NSCLC H226 cell line individually with $2 \times 10^6$ tumor cells in the dorsal flank. Tumors were allowed to grow to 100 mm$^3$, at which time 40 mice were treated with cetuximab (50 mg/kg) and 10 mice were treated with IgG control (50 mg/kg) twice weekly by intraperitoneal injection. IgG–treated tumors grew rapidly, whereas cetuximab–treated tumors showed delayed growth. Tumors were monitored for the development of cetuximab resistance, defined as marked tumor growth in the presence of continued cetuximab therapy. Once cetuximab–resistant tumors reached a volume of approximately 800 mm$^3$, mice were grouped according to tumor size at the time of resistance. Cetuximab–resistant tumors were observed in 28 of 40 mice (70%), which is in line with previous studies (14, 20, 21). Mice were then randomly subdivided into 13 cetuximab–resistant xenograft groups (28 mice in total), 9 of which are shown in Fig. 5A. One mouse per group was maintained on cetuximab therapy, while the other mouse (or mice) was removed from cetuximab treatment and started on 50 mg/kg pan-HER (i.p. twice weekly). Fifteen mice were treated with pan-HER and 13 mice were maintained on cetuximab therapy. The average tumor volume of mice treated with IgG alone is included in all groups for comparison purposes. Eleven of 15 (73%) cetuximab–resistant tumors treated with pan-HER demonstrated an antitumor response compared with the cetuximab–treated group, whereas 4 of 15 (27%) tumors showed limited response to pan-HER treatment. In Fig. 5A, mice treated with pan-HER in groups 1,
2, 5, and 6 demonstrated more pronounced antiproliferative responses than those maintained on cetuximab. In addition, this antitumor response was maintained for more than 30 days in the pan-HER–treated mice. Mice treated with pan-HER in groups 3, 4, and 7 showed antiproliferative response after 15–20 days of pan-HER treatment. In contrast, tumors treated with Pan-HER in groups 8 and 9 did not exhibit delayed tumor growth compared with the tumors treated with cetuximab.

To determine whether pan-HER effectively downregulated the HER family of receptors in vivo, receptor levels in harvested tumors were investigated by immunoblot analysis (Fig. 5B). Analysis of tumor lysates harvested from each treatment group indicated that pan-HER–treated cetuximab-resistant tumors had low or undetectable levels of EGFR, HER2, or HER3. Cetuximab-treated cetuximab-resistant tumors, however, retained significant expression levels of the HER family receptors. These findings were similar to the results presented in Fig. 2A. Interestingly, examination of pan-HER–treated tumors in groups 8 and 9 indicated that HER family receptors were effectively downmodulated upon treatment despite a minimal decrease in tumor growth. In a complementary approach, we verified these findings in tissue sections of the tumors by immunohistochemical analysis of EGFR, HER2, and HER3 as well as through measurement of markers for cell proliferation (Ki67; Fig. 5C). Strong membrane and intracellular HER family member staining was observed in cetuximab-treated cetuximab-resistant tumors (6c from group 3). In contrast, pan-HER–treated cetuximab-resistant tumors showed moderate intracellular HER family member staining, and limited to no membrane staining in the majority of tumors (7p from group 3). Furthermore, pan-HER–treated cetuximab-resistant tumors had lower expression of Ki67 than cetuximab treatment groups (Fig. 5C). Collectively, these findings show that pan-HER can effectively and consistently target the HER family of receptors in tumors that acquire resistance in vitro or in vivo and significantly impact tumor growth.

Pan-HER effectively decreased HER family receptor expressions in intrinsically cetuximab-resistant NSCLC and HNSCC cell lines

The data presented strongly suggest that pan-HER can effectively overcome cetuximab resistance in several models of acquired resistance to cetuximab. Intrinsic resistance to
cetuximab, however, in NSCLC and HNSCC remains a major clinical hurdle. To determine whether pan-HER can overcome intrinsic resistance to cetuximab, intrinsically resistant NSCLC (H358) and HNSCC (UMSCC-6, UMSCC-4 and UMSCC-11A) cell lines were treated with 20 μg/mL of cetuximab or pan-HER for 72 hours (Fig. 6A). Pan-HER treatment resulted in statistically significant cell growth inhibition while cetuximab treatment had little effect. In addition, while cetuximab treatment exhibited mild inhibition of proliferation in H358 and UMSCC11A, pan-HER treatment showed comparatively greater inhibition. After the establishment of these data, we investigated which pathways were inhibited by pan-HER in those cell lines. Four intrinsically cetuximab-resistant NSCLC and HNSCC cell lines were treated with vehicle, cetuximab, or pan-HER for 24 hours. Expression levels of HER family receptors as well as AKT, MAPK, and S6rp phosphorylation levels were decreased by pan-HER treatment compared with vehicle or cetuximab treatment (Fig. 6B). Total HER3 levels in three UMSGCC cell lines did not show superior downmodulation after pan-HER treatment due to low total HER3 expression. Overall, these data suggest that pan-HER treatment impacts proliferation in intrinsically cetuximab-resistant NSCLC and HNSCC cell lines by reducing HER family receptor expression. In addition, these data show that pan-HER could significantly decrease cell growth inhibition while cetuximab treatment represents a major clinical challenge.

To elucidate potential mechanisms of resistance to cetuximab in NSCLC and HNSCC, we developed models of cetuximab resistance using the cetuximab-sensitive NSCLC line H226 and HNSCC line UMSCC1 (13). In these models, we found that cetuximab-resistant clones had increased expression and activity of the HER family, including EGFR, HER2, and HER3, compared with cetuximab-sensitive HP cell line. These findings suggested that cetuximab resistance could manifest by constitutive overexpression and activity of the HER family (13). We previously found that cetuximab-resistant clones had dependency on EGFR for enhanced proliferative potential when EGFR was targeted genetically (21, 33). To investigate whether cetuximab-resistant tumors would be sensitive to different EGFR antibody, Sym004, a

Pan-HER treatment leads to decreased tumor growth in an intrinsically resistant PDX model

To expand these findings to a clinically more relevant model, the effects of pan-HER treatment were assessed in a PDX model established directly from a HNSCC patient (UW-SCC1). Previous analysis indicated that UW-SCC1 was resistant to cetuximab therapy (24, 25). Twelve mice were then injected with UWSSCC-1 PDX in the flank and tumors were allowed to grow to 200 mm³. All mice were randomized to treatment or control groups and treated with either vehicle, cetuximab, or pan-HER i.p. twice weekly. Mouse weight was measured weekly, and no discernible toxicity was observed in either the cetuximab or pan-HER treatment group. Treatment with pan-HER significantly delayed tumor growth of UWSSCC-1 PDX compared with either vehicle or cetuximab treatment (Fig. 7A). We examined expression levels of HER family members in individual tumors by immunoblot analysis to further investigate the potential of pan-HER to effectively target HER family receptors in tumors in vivo (Fig. 7B). Pan-HER–treated UWSSCC-1 PDX tumors had essentially undetectable levels of EGFR, HER2, or HER3, whereas vehicle or cetuximab-treated UWSSCC-1 PDX tumors retained significant levels of these receptors. These findings were consistent with the results presented in Fig. 5B. Next, we verified these findings in tissue sections of the same tumors by immunohistochemical (IHC) analysis of EGFR, HER2, and HER3. IHC analysis confirmed the immunoblot results by demonstrating downmodulation of membrane and intracellular HER family receptor levels in pan-HER–treated tumors (Fig. 7C). Together, these data demonstrate that pan-HER treatment resulted in an impactful and sustained antitumor effect in an intrinsically cetuximab-resistant HNSCC PDX model through the targeting of multiple HER family members.

**Discussion**

EGFR is one of the most highly targeted receptors in oncology due to its frequent overexpression and aberrant activation in numerous cancers (26). Cetuximab is a chimeric human-murine monoclonal IgG1 antibody that binds to the extracellular domain of EGFR and inhibits ligand binding, dimerization, and ultimately its activation. Cetuximab has shown efficacy in treating numerous cancers (26). EGFR is one of the most highly targeted receptors in oncology due to its frequent overexpression and aberrant activation in numerous cancers (26). Cetuximab is a chimeric human-murine monoclonal IgG1 antibody that binds to the extracellular domain of EGFR and inhibits ligand binding, dimerization, and ultimately its activation. Cetuximab has shown efficacy in treating numerous cancers (26).
mixture of two anti-EGFR mAbs that leads to EGFR degradation, was tested. Sym004 led to rapid internalization and degradation of EGFR in cetuximab-resistant H226 clones with subsequent loss of proliferative effects (20). In line with these data, Pedersen and colleagues reported that Sym004 synergistically inhibited skin, lung, and HNSCC cell growth through efficient target internalization and degradation (34). Huang and colleagues also utilized lung and HNSCC tumors and demonstrated that Sym004 inhibited DNA damage repair as well as augmented radiation response in these cancers (35). Furthermore, Ferraro and colleagues showed that the combination of noncompetitive anti-EGFR antibodies (panitumumab + mAb111) accelerated receptor endocytosis, enhanced receptor internalization, and impaired recycling in triple-negative breast cancer (TNBC) cells. This mixture of mAbs showed strong G1 arrest in TNBC cells and inhibited these tumors’ growth, better than either antibody alone (36). Perera and colleagues demonstrated that two EGFR-targeted antibodies (mAbs 806 and 528) showed synergistic antitumor activity in glioma and squamous cell cancer xenograft models (37).

Given the robust expression of EGFR, HER2, and HER3 in cetuximab-resistant clones, we targeted EGFR and HER3 genetically or with the combination of cetuximab and U3-1287, a mAb directed against HER3 in cetuximab-resistant clones (14). Abrogation of HER3 activity, either by genetic approaches or U3-1287, led to diminished cell proliferation accompanied by impaired MAPK and AKT signaling (14). Interestingly, HER2 was also strongly inactivated by dual mAb therapy, suggesting that this treatment regimen could block signaling from all three HER family receptors. These studies demonstrate that targeting HER family receptors with antibody-based combinatorial therapies can overcome acquired resistance to cetuximab. Similar findings have been made by other groups in the field (18, 38, 39). Thus, targeting multiple HER family receptors is necessary for complete inhibition of the HER family signaling network. In this study, we took a systematic approach, using an innovative antibody combination called pan-HER. Pan-HER is a mixture of six mAbs targeting EGFR, HER2, and HER3, and proliferation (Ki67) IHC.
We found that pan-HER inhibited proliferation in NSCLC and HNSCC cell lines with both intrinsic and acquired cetuximab resistance (Figs. 1A, 3A, and 6A). Similar findings were reported by Jacobsen and colleagues, where pan-HER was tested on various cancer cell lines such as lung, breast, or pancreatic with a known dependency on EGFR, HER2, or HER3. Challenging these cell lines with pan-HER broadly inhibited signaling from the HER family, resulting in abrogated cell proliferation (19). Furthermore, Mancini and colleagues reported that simultaneous targeting of EGFR (mAb565 or cetuximab), HER2 (mAb12 or trastuzumab), and HER3 (mAb33) was very effective for inhibition of T790M+ erlotinib-resistant NSCLC cells (40). We also found that pan-HER antibody mixture resulted in a decrease of all three receptors as well as reduced cell proliferation pathway activation compared with cetuximab alone (Figs. 2A, 3B, and 6B). Similarly, Mancini and colleagues showed that triple combination of mAbs targeting EGFR, HER2 and HER3 reduced the detection of all three on the surface of NSCLC cells and prevented increased MAPK activation (40). Francis and colleagues also demonstrated that pan-HER might be more efficacious in cell lines that overexpress EGFR, HER2, and/or HER3 in NSCLC, HNSCC, or colorectal cancer cell lines (41).

The inhibition of proliferation by pan-HER was demonstrated using several mouse models. Pan-HER exhibited superior tumor growth delay in H226 lung xenograft models with downmodulation of three HER family receptors as compared with cetuximab (Fig. 4A and B). Our data are consistent with other investigators indicating that pan-HER is an effective treatment for pancreatic, squamous, and gastric tumors (19, 42). Next, we utilized two different model systems; de novo acquired cetuximab-resistant and intrinsically cetuximab-resistant PDX xenograft models (Figs. 5A and 7A). Both de novo and PDX models are clinically relevant systems and offer a platform for the investigation of novel therapeutics. In the de novo resistant model, acquired cetuximab-resistant xenograft tumors showed significant growth delay (73%) after pan-HER treatment compared with cetuximab-resistant tumors that were continued on cetuximab treatment (Fig. 5A). In an intrinsically cetuximab-resistant HNSCC PDX model, pan-HER strongly suppressed tumor growth (Fig. 7A). Jacobsen and colleagues also showed a strong effect of pan-HER treatment

Figure 6.
Pan-HER effectively decreases HER family receptors in intrinsic cetuximab-resistant lung and HNSCC cell lines. A, cells were plated and allowed to adhere for 24 hours prior to vehicle, cetuximab (20 μg/mL), or pan-HER (20 μg/mL) treatment. Cell proliferation assays are described in Materials and Methods. Data points are represented as mean ± SEM (n = 6); **, P < 0.001. B, pan-HER degrades HER family receptors and inhibits downstream AKT and MAPK signaling. Cells were treated with vehicle, cetuximab (20 μg/mL), or pan-HER (20 μg/mL) for 24 hours. The cells were then lysed and fractionated on SDS-PAGE for the indicated proteins. α-Tubulin was used as a loading control.

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in a panel of PDX models including ovarian, colorectal, lung, and pancreatic (19). Furthermore, pan-HER could augment radiation response in HNSCC PDX tumors (41). Recently, there are a few reports, in which targeting the HER family receptors, rather than any one receptor, in various cancers corroborated with our data. The four-in-one antibody FL518, which is a potent inhibitor of EGFR, HER2, HER3, and VEGF, inhibited their receptor phosphorylation and had superior antitumor activity in colorectal, breast, or gastric cancer (43). Preclinical data suggested that inhibition of EGFR, HER2, and HER3 by combined treatment with lapatinib, trastuzumab, and U3-1287 showed maximum effect in trastuzumab-resistant breast cancer (44). Mancini and colleagues showed strong inhibitory effect on tumor growth in erlotinib-resistant NSCLC cells (H1975) by triple combinatorial treatment with anti-EGFR, anti-HER2, and anti-HER3 antibodies (40). Notably, treatment with pan-HER clearly decreased the expression of all three receptors in both de novo and PDX cetuximab-resistant NSCLC and HNSCC tumors by immunoblot and immunohistochemical analyses (Figs. 5B and C and 7B and C). Jacobsen and colleagues confirmed that pan-HER treatment induced simultaneous downmodulation of all three receptors in BxPC3 tumors by Western blot analysis and immunohistochemical analysis (19). Nielsen and colleagues demonstrated that HER family receptors were downregulated in BxPC3 xenograft model by only a single dose of pan-HER treatment using immunohistochemical analysis (42). Interestingly, pan-HER–treated cetuximab-resistant tumors in group 8 and 9 showed superior HER family downmodulation; however, pan-HER treatment did not show any growth delay in these tumors (Fig. 5A). It has been exhibited that cetuximab-resistant clones have increased not only EGFR, HER2, HER3, and HER4, but also other receptor tyrosine kinases (RTK) such as cMET, AXL, and IGF1R (13, 45, 46). Recently, mutations in the genes encoding other RTKs, such as FGFR1 or PDGFRA, were found in CRC PDX models with intrinsic resistance to cetuximab (47). Nakamura and colleagues also found HER4 mutation in cetuximab-resistant esophageal squamous cell carcinoma (ESCC) or HNSCC cells (48). These RTK crosstalk mechanisms associated with resistance have also been reported in other tumors such as NSCLC, breast cancer, or melanoma (49). These data may explain why some of the cetuximab-resistant tumors did not respond to pan-HER treatment. It is also notable that many other RTK crosstalks may be involved in the mechanisms of drug resistance and a detailed investigation is necessary. Accordingly, combination of other RTK antibodies/
inhibitors with pan-HER could be a powerful combination treatment in an antitumor effect for tumors with acquired resistance to cetuximab.

Our current study clearly demonstrated that pan-HER effectively targets the HER family receptors in models of intrinsic or acquired resistance to cetuximab and can overcome cetuximab resistance. Our previous reports and numerous examples from other investigators have demonstrated that treatment of tumors with single agents such as anti-RTK antibodies or TKIs frequently leads to rapid resistance [see review (50)]. Several mechanisms of resistance are associated with overexpression of HER family receptors. Recently, Mancini and colleagues demonstrated that the triple combination of mAbs could be a feasible pharmacologic option for treating lung cancer patients who inevitably develop resistance to EGFR TKIs (40). Nakade and colleagues also reported that triple inhibition of EGFR, Met, and angiogenesis by erlotinib plus TAS-115 efficiently controlled growth of HGF-triggered, EGFR-TKI–resistant tumors harboring EGFR mutations (51). Collectively, these studies suggest that completely blocking the signaling of HER family receptors and RTK crosstalk is a focus of future antibody development strategies. Synergistic antibody mixtures targeting the HER family and beyond are likely to show great promise for the future of RIK targeting in cancer.

Disclosure of Potential Conflicts of Interest

I.D. Horak has ownership interest (including patents) in Symphogen. D.L. Wheeler reports receiving a commercial research grant from Symphogen. No potential conflicts of interest were disclosed by other authors.

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