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Ellebaek, Sofie; Pedersen, Susanne Brix; Grandal, Michael; Lantto, Johan; Horak, Ivan D.; Kragh, Michael; Tuxen Poulsen, Thomas

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Authors:

Sofie Ellebæk[1], Susanne Brix[2], Michael Grandal[1], Johan Lantto[1], Ivan D. Horak[1], Michael Kragh[1], and Thomas Tuxen Poulsen[1]

Authors' affiliations:

[1]Symphogen A/S, Ballerup, Denmark

[2]Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, Denmark

Corresponding author:

Sofie Ellebæk

Symphogen A/S,

Pederstrupvej 93

DK-2750 Ballerup, Denmark

E-mail: sel@symphogen.com

Tel: +4545265050

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Brief description of the novelty and impact of the paper: A limitation to the success of single HER-targeting mAbs is the development of acquired resistance through mechanisms such as switch in receptor dependencies and altered receptor dimerization patterns. Here we demonstrate that Pan-HER, a novel mAb mixture simultaneously targeting EGFR, HER2, and HER3 lowers basal EGFR homo- and heterodimerization levels and inhibits ligand-induced changes in EGFR dimerization, demonstrating the potential of Pan-HER to circumvent such resistances.

Abstract

The human epidermal growth factor receptor (HER)-family is involved in development of many epithelial cancers. Therefore, HER-family members constitute important targets for anti-cancer therapeutics such as monoclonal antibodies (mAbs). A limitation to the success of single HER-targeting mAbs is development of acquired resistance through mechanisms such as alterated receptor dimerization patterns and dependencies. Pan-HER is a mixture of six mAbs simultaneously targeting epidermal growth factor receptor (EGFR), HER2, and HER3 with two mAbs against each receptor. Pan-HER has previously demonstrated broader efficacy than targeting single or dual receptor combinations also in resistant settings. In light of this broad efficacy, we decided to investigate the effect of Pan-HER compared with single HER-targeting with single and dual mAbs on HER-family cross-talk and dimerization focusing on EGFR. The effect of Pan-HER on cell proliferation and HER-family receptor degradation was superior to treatment with single mAbs targeting either single receptor, and similar to targeting a single receptor with two non-overlapping antibodies. Furthermore, changes in EGFR-dimerization patterns after treatment with Pan-HER were investigated by *in situ* proximity ligation assay and co-immunoprecipitation, demonstrating that Pan-HER and the EGFR-targeting mAb mixture efficiently down-regulate basal EGFR homo- and heterodimerization in two tested cell lines, whereas single mAbs had limited effects. Pan-HER and the EGFR-targeting mAb mixture also blocked EGF-binding and thereby ligand-induced changes in EGFR-dimerization levels. These results suggest that Pan-HER reduces the cellular capability to switch HER-dependency and dimerization pattern in response to treatment and thus hold promise for future clinical development of Pan-HER in resistant settings.

Introduction

The human epidermal growth factor receptor (HER)-family is one of 20 subfamilies of human receptor tyrosine kinases (RTKs) comprising epidermal growth factor receptor (EGFR) (ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Many cancers are driven by HER-family dependencies where signal transduction pathways of the HER-family receptors are deregulated leading to increased proliferation and escape from apoptosis [1–3].

EGFR is one of the most studied HER-family receptors and a recognized key oncogenic driver in many epithelial cancers. Upon activation by ligands such as epidermal growth factor (EGF) two monomeric EGFRs dimerize to form an actively signaling complex. EGFR also associates in heterodimers with other RTKs, such as HER2 and HER3 [2], [4]. It is well known that heterodimerization of and cross-talk between HER-family receptors play an important role in primary and acquired resistance to targeted treatments [5–7]. Furthermore, accumulating evidence suggests that dimerization is not always ligand-dependent and that when EGFR is overexpressed, a significant fraction of receptors exists in preformed homo- and heterodimers [8–10]. Also, there is a correlation between overexpression of EGFR and activation of downstream signaling molecules such as AKT and ERK1/2 [11], suggesting that the preformed dimers are actively signaling. This phenomenon is also known for other RTKs such as HER2 and MET [12], [13].

As mentioned above, EGFR forms heterodimers with both HER2 and HER3, with HER2 being the preferred heterodimerization partner for the other HER-family members [2], [14], [15]. Furthermore, no ligand for HER2 has been identified and HER2 activation therefore requires heterodimerization with other receptors to become ligand-activated. HER3 is activated upon binding of heregulin (neuregulin), but since HER3 contains an impaired tyrosine kinase, the receptor can most potently signal in heterodimers [16], [17]. Upon EGF-induced activation of EGFR homodimers, the receptors undergo fast internalization and degradation compared to ligand-induced internalization of other HER-family members [18–20]. In this regard, it has been shown that EGFR in heterodimers with HER2 or HER3, and overexpression of for instance HER2 results in prolonged and enhanced signaling, through increased recycling and internalization compared to EGFR in homodimers [21–23].

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Several agents targeting HER-family receptors including tyrosine kinase inhibitors and monoclonal antibodies (mAbs) are clinically approved for treatment of various cancers. Five therapeutic mAbs targeting HER-family receptors are currently on the market; cetuximab (Erbitux®), panitumumab (Vectibix®), and nimotuzumab (various brandnames) targeting EGFR and trastuzumab (Herceptin®) and pertuzumab (Perjeta®) targeting HER2 [24]. While no HER3 targeting antibodies have yet reached clinical approval, the fully human mAb MM-121 (Seribantumab) is currently being evaluated in late clinical trials [25], [26].

However, acquired resistance towards single receptor targeting treatments greatly limits the therapeutic potential of single HER-targeting therapies. Mechanisms of resistance toward single HER-family targeting mAbs include receptor mutations, increased ligand production, compensatory receptor up-regulation, switch in receptor dependency, and increased receptor cross-interactions by heterodimerization [6]. Specifically with regards to cetuximab resistance, mechanisms such as increased EGFR expression and co-activation of other RTKs through heterodimerization with EGFR has been observed [27]. Resistance mechanisms to trastuzumab treatment also include increased EGFR expression, activation of EGFR-HER2 heterodimers, and increased levels of EGFR and HER3 ligands [28], [29].

It has been shown that mixtures of mAbs targeting a single HER-family member have superior inhibitory efficacy when it comes to cell growth, down-regulation of single receptors and heterodimers, and decreased downstream signaling compared to a single mAb targeting the same receptor [30–34]. Furthermore, it has been shown that resistance toward single targeting mAbs as a result of switch in receptor dependency and receptor heterodimerization can be overcome by targeting the activated receptor [27]. For example cetuximab resistances, due to ligand-induced activation of HER3 and prosurvival pathways, can be abrogated by treatment with MM-121 or dual EGFR-HER2 targeting [26], [35], [36]. Also, increased expression of HER3 due to pertuzumab treatment can be abrogated by targeting HER3 [37]. Together, this indicates a strong rationale for combinatorial targeting of HER-family receptors with mAb mixtures to circumvent resistance, via heterodimerization and compensatory receptor up-regulation, toward single HER-family targeting mAbs.

Pan-HER is a mixture of six mAbs comprising a pair of mAbs targeting non-overlapping epitopes of each of EGFR, HER2, and HER3 in a balanced ratio. Pan-HER was recently reported to demonstrate superiority to single mAbs targeting HER-family receptors delaying both primary and acquired resistance due to tumor heterogeneity and plasticity [38].

Here we investigated HER-family dimerization patterns upon treatment with single HER-targeting mAbs, mixtures of two mAbs targeting a single receptor, and Pan-HER in HER-dependent cell lines. The results show that Pan-HER and the single targeting mAb mixtures decrease proliferation, downstream signaling, and total receptor levels of EGFR, HER2, and HER3 more effectively than single mAbs, but also that multitargeting of all three receptors with Pan-HER is essential to have the broadest efficacy. Furthermore, Pan-HER and the single targeting mAb mixtures lower basal EGFR homo- and heterodimerization levels in absence of ligand proportionally to decreased receptor levels and inhibits EGF-induced changes in EGFR dimerization by blocking EGF binding, whereas the single mAbs have almost no effect. However, simultanous targeting of all three HER-family receptors by treatment with Pan-HER, may provide a more efficacious therapeutic strategy by means of multiple receptor targeting in HER dependent tumors.

Materials and Methods

Cell lines

PK-1 and OVCAR-8 cell lines were obtained from RIKEN Cell Bank and National Cancer Institute,
respectively and grown for limited numbers of passages in RPMI1640+10% Fetal Bovine Serum (FBS)+1%
Penicillin/Streptomycin (Life Technologies) at 37°C, 5% CO_2, and serum starved 24-48 hours prior to
experiments. All cell lines were routinely tested for mycoplasma infection and cell line authenticity was
documented by Human Cell Line Authentication (LGC Standards).

Therapeutic antibodies

The six antibodies used for the 2-mAb mixtures and Pan-HER were generated as described elsewhere [38].
Cetuximab (Erbitux®) and trastuzumab (Herceptin®) were obtained from Merck KGaA and Roche,
respectively. Analogues of pertuzumab and MM-121 were generated in house and tested, along with a mAb
binding non-human IgG used as negative control, as previously specified [34], [38].

Detection of cell surface expressed receptors

Flow cytometry was used to quantify surface expressions of EGFR, HER2, and HER3 in cancer cell lines
using Quantum Simply Cellular beads (Bangs Laboratories) conjugated with capture antibodies (as detailed
below). The output Antibody Binding Capacity (ABC=$ABC_{detection\ antibody}$-$ABC_{isotype\ control}$) of each cell line
correlates with the number of receptors on the cell surface. Cells were dissociated using Cell Dissociation
Reagent (Life Technologies) and filtrated in 70μm Cell Strainers (BD Biosciences). $1.0x10^5$ cells per well
were washed and stained using anti-EGFR, anti-HER2, and anti-HER3 detection and isotype control
antibodies (Abcam, clone #: ICR10 and RTK2758 and R&D Systems, clone #: 191924, 133303, 66223, and
11711), prior to analysis on FACSVerse Flow cytometer (BD Biosciences) with Kaluza flow analysis
software (Beckman Coulter).

Proliferation assay by live cell imaging

PK-1 and OVCAR-8 cells were plated in medium containing 0.5% FBS and 50 μg/mL antibody and proliferation was followed for 160 hours using IncuCyte ZOOM (Essen Bioscience) to obtain pictures every fourth hour. IncuCyte ZOOM 2015A software (Essen Bioscience) was used for data analysis.

Cell treatment and harvest of lysates

Cells were grown to approximately 80% confluency prior to antibody treatment for 24 hours. 15 minutes prior to harvest, cells were stimulated with 1 nM EGF (R&D Systems) or left unstimulated. Ice cold 1x Lysis Buffer (Cell Signaling Technology) containing Protease Inhibitor Cocktail kit (Thermo Scientific) and Phosphatase Inhibitor Cocktail set II (Calbiochem) was added, cells harvested with a cell scraper, and cleared by centrifugation at 4°C. Protein concentration measurements were carried out using Pierce BCA Protein Assay kit (Thermo Scientific) prior to co-immunoprecipitation (Co-IP) and/or Western blot analysis.

Western blot analysis

Effects of antibody treatments on total and phospho-protein levels were investigated by Western blot analysis. Primary antibodies: Rabbbit anti-EGFR, rabbit anti-HER2, rabbit anti-HER3 (Cell Signaling Technology, clone #: D38B1, 29D8, and D22C5), mouse anti-HER3 (Millipore, clone #: 2F12), rabbit anti-AKT, rabbit anti-pAKT (Ser473), rabbit anti-ERK1/2, rabbit anti-pERK1/2 (Thr202/204), and rabbit anti-β-actin (Cell Signaling Technology, clone #: D9E, D13.14.4E, and 13E5) diluted in Odyssey Blocking Buffer (LI-COR Biosciences) were used for detection, and membranes were scanned on Odyssey Scanner (LI-COR Biosciences) using Odyssey IR Imaging System (LI-COR Biosciences).

Duolink in situ proximity ligation assay

Duolink proximity ligation assay (PLA) was used for detection, quantification, and visualization of EGFR homo- and heterodimers, using EGFR, HER2, and HER3 detection antibodies generated as described elsewhere [38]. PLUS and MINUS PLA probes were made from these antibodies according to the manufacturer's instructions (Olink Bioscience). 5,000 cells/well were plated in 2% FBS 48-72 hours prior to measurements. 24 hours prior to measurements, cells were treated with 40 μg/mL Pan-HER, cetuximab, trastuzumab, pertuzumab, MM-121 or negative control mAb. For ligand stimulation, cells were incubated

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with 1 nM EGF for 15 minutes at 37°C, prior to fixation with 3.7 % formaldehyde for 10 minutes and permeabilization with 0.1 % Triton X-100 for 5 minutes. Cells were blocked and probed with anti-EGFR-, anti-HER2-, and anti-HER3-PLA probes, added in PLUS-MINUS pairs, followed by ligation, rolling circle amplification, and washing. Cytosols and nuclei were stained with Cell Mask Blue and Hoechst (Life Technologies) before confocal microscopy analysis using Opera high content screening system (Perkin Elmer, ver. 2.0.1) for visualizing PLA signals at 40X magnification and Acapella high content imaging and analysis software (Perkin Elmer, ver. 2.6) for quantifying PLA signals and cells. The number of positive spots/cell was calculated based on analysis of 15 pictures in each of three replicate wells. Statistical significance was calculated using Student's t-test. In the experiment with EGF stimulation, the number of PLA signals/cell was normalized to the total level of EGFR determined by Western blot analysis in order to exclude the effect of degradation due to the antibody treatments.

Detection of labelled EGF to PK-1 cells

5,000 PK-1 cells/well were plated in 2% FBS 48h prior to 30minutes of Pan-HER treatment and stimulation with 2ug/mL Alexa-647 labelled EGF (Thermo Fisher Scientifc) for 3min at 4°C to limit ligand-induced internalization. Cells were fixed, permeabilized, and blocked as described above prior to staining with FITC-labelled anti-EGFR antibody (Abcam, Clone ICR10) for 1h. Cytosols and nuclei were stained with Cell Mask Blue and Hoechst (Life Technologies) followed by confocal microscopy analysis.

Co-Immunoprecipitation

Indirect Co-IP with streptavidin-conjugated beads was performed according to manufacturers instructions (Thermo Scientific), using 2 mg total protein from each lysate sample and biotinylated capture or isotype control antibody (R&D Systems and Cell Signaling Technology, clone MOPC-21), and incubated for two hours with tilting at room temperature. 0.5 mg beads per sample were prewashed prior to incubation with Ab-antigen samples for one hour with tilting at room temperature. Finally, samples were washed, incubated in low pH Elution Buffer for 5 minutes at room temperature, and subjected to Western blot analysis.

Results

Selection and characterization of HER-dependent cancer cell lines for studying EGFR dimerization

Seeking to evaluate the effect of Pan-HER on EGFR homo- and heterodimerization, we aimed to identify EGFR high expressing cancer cell lines with sensitivity to more than one HER-targeted therapeutic mAb, because such models could be expected to be driven by HER-family heterodimerization.

15 cancer cell lines sensitive to Pan-HER treatment [38] (and data not shown) were screened for total and surface expression levels of EGFR, HER2, and HER3 by Western blot analysis and flow cytometry, respectively. The 15 cell lines displayed highly varying receptor levels, even though the majority expressed rather high levels of EGFR (Fig. 1A). Based on the protein levels in Fig. 1A and a previous screen of HER-dependence [38], two cell lines (PK-1 and OVCAR-8) were selected for studying sensitivity to single HER-targeting and Pan-HER. Both PK-1 and OVCAR-8 expressed very high cell surface levels of EGFR and both cell lines expressed low but detectable levels of HER2 (Fig. 1B). Furthermore, PK-1 expressed a very low level of HER3, while OVCAR-8 was chosen because it expressed the highest level of HER3 of all the analyzed cell lines.

Next, the cell line sensitivity to single HER-targeting mAbs and Pan-HER was investigated by live cell imaging. As shown in Fig. 2A, both PK-1 and OVCAR-8 demonstrated varying sensitivity to single HER-targeting mAbs, mAb mixtures, and Pan-HER. Pan-HER most effectively inhibited the proliferation of PK-1 closely followed by both the HER3-targeting mAb mixture and MM-121. Whereas in OVCAR-8, Pan-HER, MM-121, and the EGFR- and HER3-targeting mAb mixtures inhibited the proliferation to the same extent. This effect of Pan-HER (Suppl. Fig. 1) and the other HER-targeting mAbs demonstrates that targeting of the receptors with more than one antibody effectively decreases proliferation, and that more than one HER-family member contributes to the growth of both cell lines, which were therefore considered suitable *in vitro* models for studying alterations in HER-dimerization patterns upon Pan-HER treatment.

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Treatment with Pan-HER has previously been reported to cause simultaneous down-regulation of EGFR, HER2, and HER3 [38], so in order to evaluate effect on downstream signaling, the effect of Pan-HER and single targeting mAbs on total HER-family receptor levels and phosphorylation levels of the major downstream signaling molecules AKT and ERK1/2 was investigated in both cell lines. The mixtures targeting single receptors caused down-modulation of their respective target, while Pan-HER simultaneously reduced the protein levels of all three receptors (Fig. 2B). Cetuximab and pertuzumab treatment did not alter the receptor levels, however, trastuzumab and MM-121 were able to decrease the HER2 and HER3 levels, respectively in both cell lines but not to the same extent as Pan-HER and the HER2- and HER3-targeting mixtures. Furthermore, signs of compensatory up-regulation of EGFR, HER2 or HER3 were observed upon single targeting treatments. For example the HER2- and HER3-targeting mAb mixtures slightly increased EGFR levels in PK-1, MM-121 increased HER2 levels in both cell lines, and pertuzumab treatment increased HER3 levels in OVCAR-8. In contrast, Pan-HER effectively decreased the expression of all three receptors, thus preventing compensatory up-regulation. Furthermore, the level of pAKT in both cell lines was decreased upon HER3-targeting (MM-121, HER3 and Pan-HER). In PK-1 pERK1/2 was slightly decreased by cetuximab and more abundantly by the EGFR-targeting mAb mixture and Pan-HER. In OVCAR-8 MM-121, the HER3-targeting mAb mixture, and Pan-HER all decreased the pERK1/2 level, whereas, both the EGFR- and HER2–targeting mAb mixture slightly increased pERK1/2 levels. Neither total AKT or ERK1/2 levels were altered upon any of the treatments.

In summary, both PK-1 and OVCAR-8 exhibited sensitivity to targeting of both single and multiple HER-family receptors, suggesting that HER-family heterodimerization might play a growth promoting role in both cell lines. The two cells lines were used to study changes in EGFR dimerization in response to treatment with single HER-targeting mAbs, mixtures, and Pan-HER.

Pan-HER down-regulates basal EGFR homodimer levels in PK-1 and OVCAR-8 cells

PLA was applied to investigate EGFR dimerization in cells treated with single HER-targeting mAbs, mixtures or Pan-HER. Since PLA is an antibody based detection method, it is essential that the detection

antibodies and the therapeutic antibodies bind non-overlapping epitopes. Previous epitope binning and binding competition studies have confirmed that the epitopes of the detection antibodies against EGFR, HER2, and HER3 did not overlap with the therapeutic mAbs (cetuximab, trastuzumab, and pertuzumab) and the mAbs comprising Pan-HER, and that the detection antibodies had single target specificity with regard to binding and cross-reactivity to EGFR, HER2, and HER3 [34], [39], [40] (Suppl. Fig. 2).

EGFR is known to associate in preformed homodimers in absence of ligand [8–10] and since PK-1 and OVCAR-8 both express high levels of EGFR, the level of EGFR homodimers in these cell lines was investigated using PLA. In contrast to cetuximab, which did not alter the EGFR homodimer level, both the EGFR-targeting mAb mixture and Pan-HER significantly decreased the basal EGFR homodimer level in both PK-1 and OVCAR-8 upon 24 hours of treatment ($p< 0.001$, $p< 0.01$, and $p< 0.05$) (Fig. 3). These results correlate with the decrease in total EGFR levels detected by Western blot analysis upon treatment with the EGFR-targeting mAb mixture and Pan-HER but not upon cetuximab treatment (Fig. 2B). The decrease in homodimers is therefore likely due to receptor degradation. This is further supported by the fact that no significant receptor degradation and no change in basal EGFR homodimer and EGFR-HER2 heterodimer levels were observed upon one hour treatments (data not shown). Furthermore, it could be speculated that treatment with single mAbs targeting other HER-family members could push the dimerization balance towards an increase in EGFR homodimers. However, treatment with trastuzumab, pertuzumab, and MM-121 did not affect EGFR homodimerization levels (data not shown).

Pan-HER down-regulates basal EGFR heterodimer levels in PK-1 and OVCAR-8 cells

EGFR has been shown to associate in preformed heterodimers with HER2 or HER3 [8], [9], and therefore the basal levels of HER-family heterodimers in PK-1 and OVCAR-8 was also investigated after treatment (Fig. 4). Collectively, the results in Fig. 4 demonstrate that the single mAbs had very little effect and did not alter the EGFR heterodimer levels in PK-1 and OVCAR-8 cells. On the other hand, Pan-HER and some of the single HER-targeting mAb mixtures effectively decreased both basal EGFR-HER2 and EGFR-HER3 heterodimer levels in both PK-1 and OVCAR-8, most likely through receptor degradation. However, while

the EGFR-targeting mAb mixture reduced both EGFR-HER2 and EGFR-HER3 levels in both cell lines the HER2- and HER3-targeting mAb mixtures did not significantly alter the EGFR-HER2 and EGFR-HER3 dimer levels in OVCAR-8, respectively, despite the fact that the receptors are being degraded (Fig. 2B). Furthermore, trastuzumab and MM-121 treatments resulted in degradation of HER2 in PK-1 and OVCAR-8 cells and HER3 in OVCAR-8 cells, respectively (Fig. 2B), but neither treatment altered the EGFR-HER2 and EGFR-HER3 heterodimer levels (Fig. 4). This indicates that targeting of more than one receptor in the dimer is more likely to succesfully down-regulate preformed HER-family dimer levels, and that Pan-HER has the potential to prevent cancer cells from evading treatment by switching HER-family dependency.

Pan-HER prevents EGF-induced changes in EGFR dimerization in PK-1 cells

Increased EGFR expression, increased EGFR-HER2 heterodimerization, and increased levels of EGF all play a role in acquired resistance toward cetuximab and trastuzumab [27], [28]. Therefore, it was of interest to investigate the effect of treatment on ligand stimulation and ligand-induced HER-family dimerizations. This was done by investigating the ability of Pan-HER to interfere with EGF binding to EGFR and by studying the EGFR homo- and EGFR-HER2 heterodimerization levels upon treatment with single targeting mAbs, mixtures, and Pan-HER for 24 hours followed by 15 minutes of EGF stimulation in PK-1 cells. The PLA signals were normalized to total EGFR protein levels as determined by western blot analyies after 24 hours of treatment, to exclude the effect of protein degradation on the PLA dimerization signal.

In Figure 5A, the immunofluorescence stainings showed that Pan-HER effectively blocks EGF binding to EGFR, thereby possibly preventing EGF from inducing changes in EGFR dimerization levels (Fig. 5A). Furthermore, using PLA it was shown that 15 minutes of EGF stimulation lowered the basal EGFR dimer levels in PK-1, likely due to ligand-induced receptor activation and internalization resulting in decreased detection by PLA. However, cetuximab, the EGFR-targeting mAb mixture, and Pan-HER maintained EGFR dimer levels at baseline levels regardless of EGF stimulation (Fig. 5B) supporting the result in Figure 5A, that the EGFR-targeting mAbs of Pan-HER interfer with EGF-induced EGFR dimer dynamics. On the other

hand, none of the other single mAbs inhibited the EGF-induced decrease in the dimer levels, and neither did the HER2-targeting mAb mixture shown by an even larger decrease in the detected dimer level (Fig. 5B).

To confirm and support these results, Co-IP for detection of EGFR homo- and EGFR-HER2 heterodimers was performed on whole cell PK-1 lysates. As shown in Fig. 5C, untreated cells stimulated for 15 minutes with EGF resulted in an increase in the EGFR dimer level in PK-1. However, Pan-HER treatment resulted in both a decrease in basal EGFR homodimer level and also triggered reduction of homo- and heterodimer levels upon EGF stimulation. Finally, as shown in Fig. 5D, Pan-HER was also able to decrease pEGFR levels and phosphorylation of the downstream signaling molecules AKT and ERK1/2 when cells were stimulated for 15 minutes with EGF, indicating that Pan-HER inhibit EGF-induced activation of EGFR dimers. Together, these results demonstrate that Pan-HER effectively reduces the level of both preformed EGFR dimers and ligand-induced dimerization and activation of EGFR.

Discussion

Previous studies have shown that increased EGFR expression and activation of HER2 and HER3 through heterodimerization with EGFR are involved in acquired resistance towards cetuximab and trastuzumab [27], [28]. Here we demonstrate the potential of the antibody mixture Pan-HER to overcome such resistance, since Pan-HER was found to down-regulate both total HER-family receptor levels, basal EGFR homo- and heterodimer levels, and prevent EGF-induced changes in EGFR dimerization in two EGFR overexpressing cell lines.

Pan-HER and the single HER-targeting mAb mixtures were shown to decrease the total receptor levels more effectively than the single mAbs in both PK-1 and OVCAR-8 cells (Fig. 2B). It has previously been shown that two antibodies targeting a single HER-family receptor more potently down-regulate the receptors than a single mAb targeting the same receptor [30–34], [38]. In the current studies all treatments were performed at equal total antibody concentrations, meaning that since Pan-HER contains two antibodies targeting each of EGFR, HER2, and HER3, the concentration of antibodies targeting each receptor is 1/3 of the concentration of the single mAbs and the single HER-targeting mAb mixtures. Thus, the superior receptor down-regulating effect of Pan-HER compared to the single targeting mAbs and mixtures underlines the added benefit of combining mAbs against multiple receptors. A possible explanation for this synergy is that targeting with multiple mAbs results in crosslinking of receptors inducing efficient receptor down-regulation even when associated in preformed dimers, and that the effect of crosslinking is greater when multiple receptors are targeted [31]. Furthermore, compensatory receptor up-regulation was observed upon treatment with some of the single receptor targeting mAbs and mixtures (Fig. 2B), while the ability of Pan-HER to simultaneously decrease the level of EGFR, HER2, and HER3 prevents compensatory HER-family receptor up-regulation as a resistance mechanism.

The effect of mAb mixtures targeting more than one HER-family receptor on heterodimerization has only received limited focus so far [32], [41], [42]. Nevertheless, the effect of Pan-HER on HER-family dimerization shown here is consistent with previously reported effects of the combined targeting of two receptors. For example the effect of trastuzumab in combination with pertuzumab on down-regulating

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EGFR-HER2 heterodimerization in the presence of EGF [32] is consistent with the effect of Pan-HER shown in Fig. 5, and the ability of cetuximab plus trastuzumab to down-regulate basal EGFR-HER2 heterodimers is consistent with the effect of Pan-HER shown in Fig. 4 [41], [42]. Common for these results is also that the single mAbs have almost no effect by themselves compared to the multitargeting when it comes to altering the HER-family dimerization levels.

The preformed EGFR homo- and heterodimers may drive resistance development in two ways. First, the preformed dimers may be able to potently signal in a ligand-independent manner when overexpressed, as is the case with HER2 and MET overexpression [12], [13], or secondly they might be positioned in dormant receptor complexes which will be activated upon increased ligand production, which is also a documented resistance mechanism [26], [28], [43]. In both cases, the ability of Pan-HER (but not the single mAbs) to remove preformed EGFR homo- and heterodimers, might reduce the risk of development of such resistances.

Phosphorylation of both AKT and ERK1/2 was down-regulated upon treatment with Pan-HER (Fig. 2B), indicating inhibition of both signaling pathways. In contrast, the effect of the single mAbs on downstream signaling varied in the two cell lines. The pAKT level was decreased upon treatment with HER3-targeting in both cell lines, correlating with the fact that HER3 activates AKT. Furthermore, the pERK1/2 level was decreased by treatment with EGFR-targeting in PK-1 and HER3-targeting in OVCAR-8, the latter indicating that signaling through EGFR-HER3 heterodimers is inhibited, decreasing the activation of ERK1/2 [2]. Even though trastuzumab treatment resulted in down-regulation of total HER2 levels in both cell lines, neither pAKT nor pERK1/2 levels were altered (Fig. 2).

Pertuzumab has previously been shown to block HRG-induced dimerization of HER2 and HER3 and also down-regulate basal HER2-HER3 dimer levels in HER2-high expressing cancer cells [44], [45]. Furthermore, pertuzumab has been shown to disrupt EGFR-HER2 heterodimers and push the dimer distribution toward EGFR homodimerization when stimulated with EGF [46], [47]. Surprisingly, we found that pertuzumab was neither able to down-regulate basal EGFR-HER2 dimer levels, nor prevent EGF-induced down-regulation of EGFR-HER2 heterodimers using PLA. However, this might be explained by the

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relatively low HER2 expression in these two cell lines, as pertuzumab might have a different effect on dimer distribution in HER2 overexpressing and/or amplified cell lines. Furthermore, cetuximab has been shown by others to slightly reduce both basal and EGF-induced EGFR-HER2 dimerization [42]. However, only the ability of cetuximab to block EGF-induced down-regulation of EGFR dimers was observed in this study. This is in line with the finding that cetuximab partially blocks the EGF binding site and locks EGFR in its closed conformation [48].

The effect of EGF on EGFR dimerization was investigated using two different assays: PLA and Co-IP. Using PLA, 15 minutes of EGF stimulation resulted in a decrease in EGFR dimer levels after normalization to total EGFR levels, indicating rapid internalization of EGFR dimers in line with previous reports by others [18–20]. Co-IP-based analysis of the effect of EGF on whole cell EGFR-dimer levels demonstrated that 15 minutes of EGF stimulation resulted in an increase in EGFR homo and heterodimer levels, correlating with previous use of Co-IP to detect EGFR-HER2 dimers upon EGF stimulation [47]. This difference in the effect of EGF might be explained by differences in the two assays. For example, if PLA primarily detects dimers at the cell surface level, the loss of EGFR dimer signals would be explained by internalization but not degradation of the dimers, which is expected to take more than 15 minutes. This explains why an increase in EGFR dimer levels was observed using Co-IP, which detects both intra- and extracellular dimer complexes. Therefore, we decided to normalize the EGFR dimer levels with and without EGF stimulation to the total EGFR levels, to study the effect of antibody treatments on EGFR dimerization dynamics while excluding the effect of receptor degradation. This showed that, Pan-HER, the EGFR targeting mAb mixture, and cetuximab were all able to prevent the EGF-induced dimer formation. This was further supported by the fact that Pan-HER was found to completely block EGF binding to EGFR (Fig. 5A). However, it is important to notice that even though all three treatments effectively blocked EGF, only the EGFR targeting antibodies of Pan-HER resulted in single receptor and dimer degradation.

In summary, the data presented here demonstrate superiority of the novel antibody mixture Pan-HER compared to single reference mAbs with regards to inhibition of proliferation, enhanced receptor degradation, and down-regulation of EGFR homo- and heterodimers with and without ligand stimulation.

The results imply that Pan-HER by these means will be able to overcome acquired resistance that cannot be sufficiently prevented by single targeting mAbs, whether the resistance mechanism is based on increased expression of HER-family receptor, switches in HER-family dependency and dimerization patterns, or increased ligand production. Our data support clinical evaluation of Pan-HER in patients with HER-family dependent epithelial tumors.

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Figure legends

Figure 1. Detection of EGFR, HER2, and HER3 in 15 cancer cell lines by western blot analysis and flow cytometry using Quantum Simply Cellular beads. **A.** Total and surface expressed levels of EGFR, HER2, and HER3. β-actin was used as loading control for western blot analyses. **B.** Flow histograms and mean fluorescence intensity of EGFR, HER2, and HER3 surface expression in PK-1 and OVCAR-8 cells. Grey peaks are detections with isotype control antibodies.

Figure 2. Pan-HER antibodies inhibit proliferation, down-regulate total receptor levels, and inhibit down-stream signaling in PK-1 and OVCAR-8 cells. **A.** Cellular proliferation based on live cell imaging of PK-1 and OVCAR-8 upon treatment with 50 μg/mL cetuximab, trastuzumab, pertuzumab, MM-121, HER-targeting mAb mixture (EGFR, HER2, and HER3), Pan-HER or negative control antibody for 160 hours. Data represent means ± SEM (n = 6). Representative pictures showing percent confluency at t=160 hours of negative control mAb and Pan-HER treated cells. **B.** Protein levels of EGFR, HER2, HER3, AKT, pAKT (Ser473), ERK1/2, and pERK1/2 (Thr202/204) upon 24 hours of treatment with 50 μg/mL cetuximab, trastuzumab, pertuzumab, MM-121, mAb mixtures targeting EGFR, HER2 or HER3, and Pan-HER or negative control antibody as determined by western blot analyses. β-actin was used as loading control.

Figure 3. Pan-HER down-regulates basal EGFR homodimer levels in PK-1 and OVCAR-8 cells. **A.** EGFR homodimers in PK-1 and representative pictures of negative control antibody, cetuximab, EGFR-targeting mAb mixture (EGFR) or Pan-HER treated cells. **B.** EGFR homodimers in OVCAR-8 and representative pictures of negative control antibody, cetuximab, EGFR-targeting mAb mixture (EGFR) or Pan-HER treated cells. EGFR homodimers were quantified upon 24 hours of antibody treatment at 40 μg/mL, measured as mean number of spots/cell in 15 pictures. Data represent means ± SD (n = 3). ***P < 0.001, **P < 0.01, *P < 0.05. Top pictures: PLA signals shown in red and nuclei and cytosol in blue. The scaling is fixed to negative control pictures. Bottom pictures: Positive PLA signals included in quantification shown in colors and excluded PLA signals in white.

Figure 4. Pan-HER down-regulates basal EGFR heterodimer levels in PK-1 and OVCAR-8 cells. **A.** EGFR-HER2 heterodimers in PK-1. **B.** EGFR-HER3 heterodimers in PK-1. **C.** EGFR-HER2 heterodimers in OVCAR-8. **D.** EGFR-HER3 heterodimers in OVCAR-8. EGFR-HER2 and EGFR-HER3 heterodimers were quantified upon 24 hours of treatment with 40 μg/mL cetuximab, trastuzumab, pertuzumab, MM-121, HER-targeting mAb mixtures (EGFR, HER2, and HER3), Pan-HER or negative control antibody, measured as mean number of spots/cell in 15 pictures. Data represent means ± SD (n = 3). **P < 0.01, *P < 0.05.

Figure 5. Pan-HER prevents EGF-induced changes in and activation of EGFR dimerization by blocking EGF in PK-1. **A.** Detection of EGF(red) and EGFR(green) +/- 30 minutes of treatment with 50ug/mL Pan-HER followed by 3 minutes of stimulation with 2ug/mL Alexa 647-labelled EGF at 4°C using Opera high content screening system. Nuclei and cytosol are shown in blue and the color scaling is fixed to the untreated picture stimulated with EGF for 3 minutes. **B.** Basal and EGF-induced changes in EGFR homo- and EGFR-HER2 heterodimer levels in PK-1 normalized to total EGFR levels. EGFR homo- and EGFR-HER2 heterodimers were quantified upon 24 hours of treatment with 40 μg/mL cetuximab, trastuzumab, pertuzumab, MM-121, EGFR-targeting mAb mixture (EGFR), HER2-targeting mAb mixture (HER2), Pan-HER or negative control antibody +/- 15 minutes of stimulation with 1 nM EGF, measured as mean number of spots/cell in 15 pictures using PLA. Data represent means ± SD (n = 3). ***P < 0.001, **P < 0.01. **C.** EGFR homo- and EGFR-HER2 heterodimer detection by Co-IP in PK-1 lysates upon 24 hour Pan-HER treatment +/- 15 minutes of stimulation with 1 nM EGF along with total receptor levels of EGFR and HER2 as controls of equal amounts of EGFR and HER2 in IP samples and IP isotype control samples. **D.** Protein levels of EGFR, pEGFR (Tyr1068), AKT, pAKT (Ser473), ERK1/2, and pERK1/2 (Thr202/204) upon 24 hours of treatment with 50 μg/mL Pan-HER or negative control antibody as determined by western blot analyses. β-actin was used as loading control.

Supplementary Figure 1. Cell proliferation video of PK-1 and OVCAR-8 cells treated for 160 hours with 50 μg/mL negative control mAb or Pan-HER using live imaging. The video is generated from 41 pictures taken at four hours intervals.

Supplementary Figure 2. Binding and cross-reactivity of the three detection antibodies (1260, 4385 and 5259) used in PLA, the six therapeutic Pan-HER antibodies (1277, 1565, 4384, 4517, 5038, and 5082), and the negative control antibody to recombinant EGFR (**A**), HER2 (**B**), and HER3 (**C**) measured as optical density (OD) by ELISA.
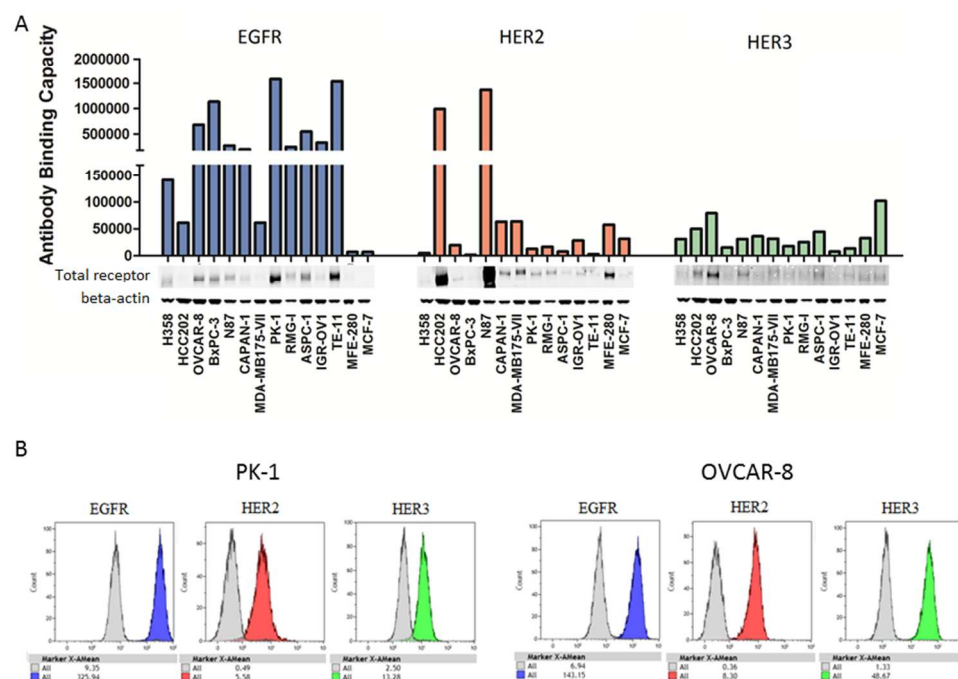
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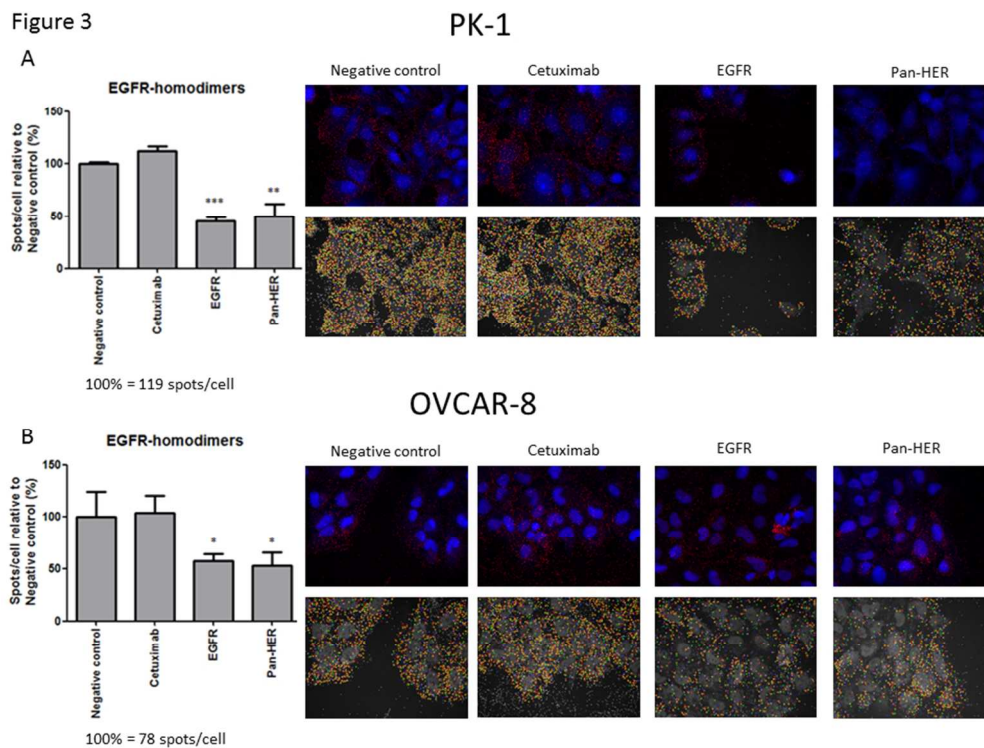254x190mm (96 x 96 DPI)
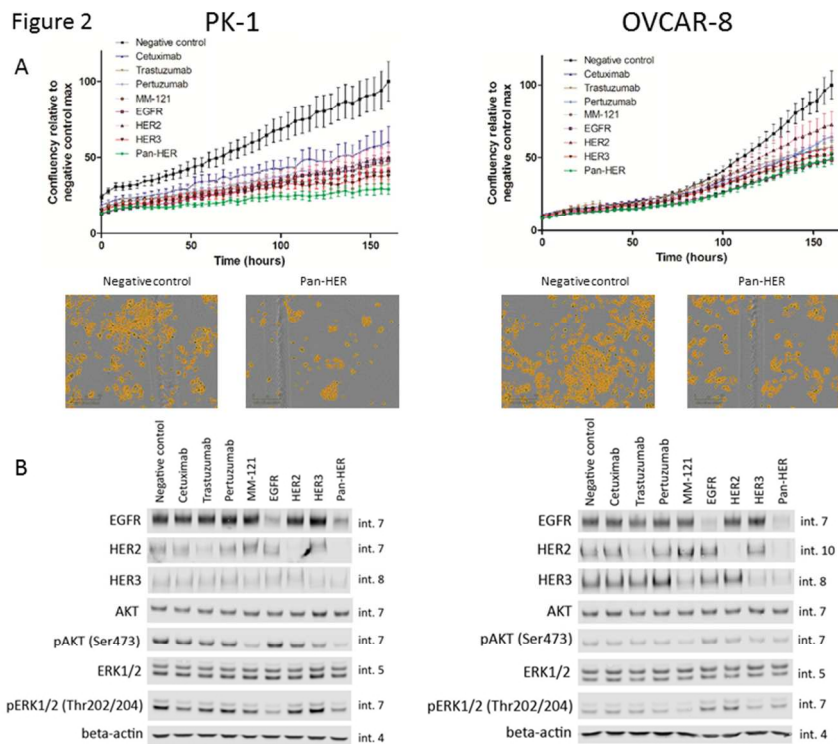
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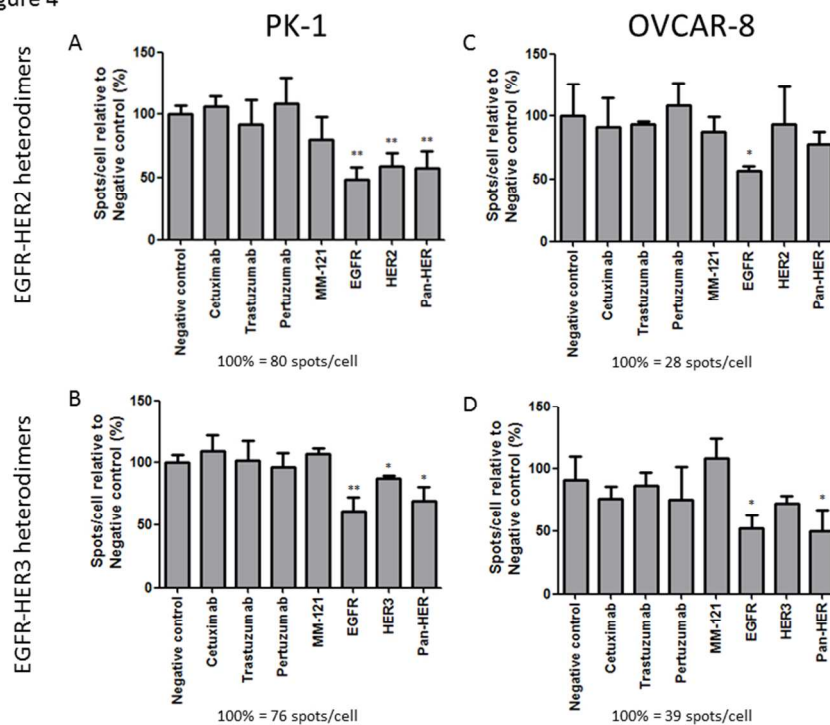254x190mm (96 x 96 DPI)

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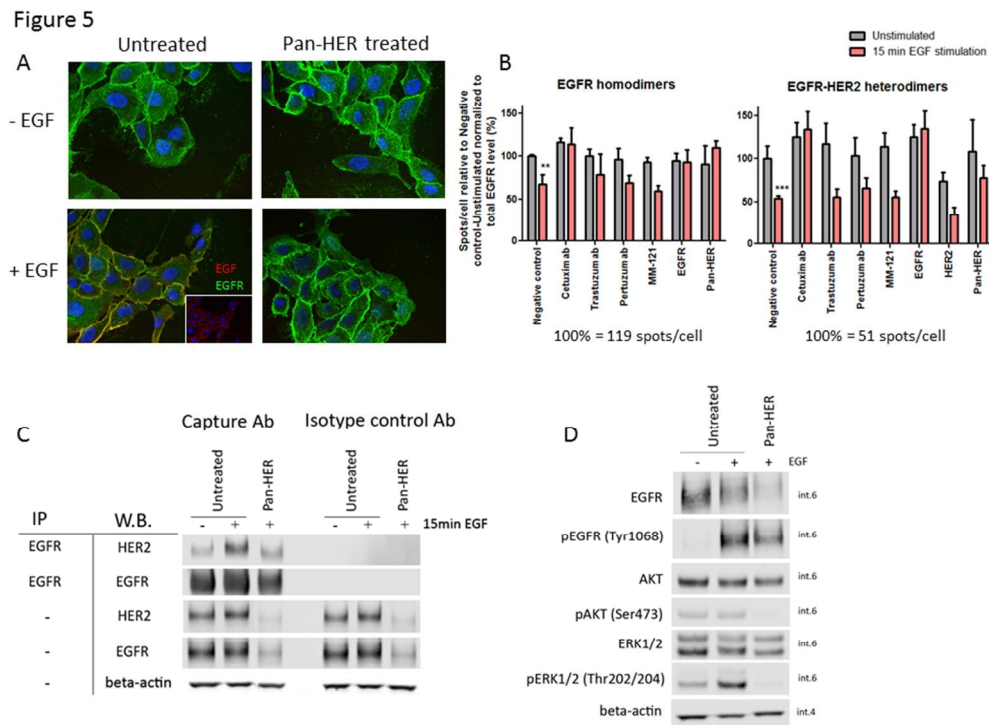254x190mm (96 x 96 DPI)

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