Targeting Three Distinct HER2 Domains with a Recombinant Antibody Mixture Overcomes Trastuzumab Resistance

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Disclosure of Potential Conflicts of Interest
All listed authors are current or former employees at Symphogen A/S
No additional conflicts of interest to disclose for any of the authors.

Financial Support
Nothing to disclose

Running Title
Superior HER2 targeting by antibody mixtures

Keywords
HER2, EGFR, antibodies, mixture, trastuzumab, pertuzumab

Notes
Abstract Length: 215 words.
Manuscript Length: 5482 words
No. of Tables: 1
No. of Figures: 5
Abstract

Human epidermal growth factor receptor 2 (HER2) plays an important role in the development and maintenance of the malignant phenotype of several human cancers. As such, it is a frequently pursued therapeutic target and two antibodies targeting HER2 have been clinically approved trastuzumab and, pertuzumab.

It has been suggested that optimal inhibition of HER2 is achieved when utilizing two or more antibodies targeting non-overlapping epitopes. Superior clinical activity of trastuzumab plus pertuzumab combination in metastatic breast cancer supports this hypothesis. Since trastuzumab and pertuzumab were not co-developed, there may be potential for further optimizing HER2 targeting.

The study herein evaluated functional activity of anti-HER2 antibody combinations identifying optimal epitope combinations that provide efficacious HER2 inhibition. High affinity antibodies to all four extracellular domains on HER2 were identified and tested for ability to inhibit growth of different HER2 dependent tumor cell lines.

An antibody mixture targeting three HER2 subdomains proved to be superior to trastuzumab, pertuzumab, or a combination in vitro and to trastuzumab in two in vivo models. Specifically, the tripartite antibody mixture induced efficient HER2 internalization and degradation demonstrating increased sensitivity in cell lines with HER2 amplification and high EGFR levels. When compared with individual and clinically approved mAbs, the synergistic tripartite antibody targeting HER2 subdomains I, II, and IV demonstrates superior anti-cancer activity.
Introduction

The human epidermal growth factor receptor (HER) family comprises EGFR (HER1), HER2, HER3, and HER4. These receptors are able to homo- and hetero-dimerize in response to diverse ligands constituting a complex biological system relaying information from the extracellular milieu across the cell membrane in a cell type- and context-dependent manner often resulting in cellular growth, proliferation and survival (1). Deregulation of the system through mutation, overexpression or gene amplification is frequently associated with development, progression, and acquired resistance in many human malignancies (1,2). For this reason, the four HER receptors have been pursued as targets for anticancer therapy using both small molecule tyrosine kinases inhibitors (TKIs) and monoclonal antibodies (mAbs) (3,4).

Unlike the other HER receptors, no high affinity ligand has been identified for HER2. Structural studies have demonstrated that HER2 adopts a permanently open conformation resembling the conformation of other HER receptors when bound to ligand (5), exposing the dimerization arm making HER2 constitutively available and a preferred dimerization partner for other HER family members (6,7). HER2 is frequently amplified and/or overexpressed in human solid tumors, including breast-, gastric-, endometroid-, lung- and colorectal cancers and is correlated to aggressive disease and poor overall survival (8–15).

Among current approved agents for HER2 overexpressing tumors is trastuzumab (Herceptin™), the first antibody found and approved for treatment of HER2-positive early-stage and metastatic breast- and gastric- cancers (16–19). Mechanistically, trastuzumab’s antitumor activity is unclear, but may involve disruption of HER2 homodimerization and prevention of cleavage of the extracellular domain generating the active truncated receptor p95HER2 (20). In the clinical setting, secondary effector functions may also be involved. Nevertheless, trastuzumab has limited activity against tumors with low HER2 levels driven by ligand-induced heterodimerization with EGFR and HER3.

Pertuzumab (Perjeta™), another antibody, prevents heterodimerization with HER3 and EGFR by binding subdomain II (21). Preclinically, pertuzumab efficiently blocks growth of HER2:HER3 dependent cell lines and xenografts. Clinical activity as a single agent however has been modest, but when combined with trastuzumab antitumor activity in HER2 metastatic breast cancer is superior to that seen when either agent is administered alone (22). The combination also significantly increased pathological CR in neoadjuvant setting (23,24).

Lapatinib (Tykerb™), is a potent inhibitor of HER2 and EGFR (25) and when combined with trastuzumab has been shown to increase PFS and OS of patients who had progressed after previously having received trastuzumab (26).

The above described results suggest that HER2 is not efficiently inhibited by single agents. The combination of trastuzumab and pertuzumab has proven superior to either agent alone in the clinical setting, but
increased activity of the two mAbs has not been observed pre-clinically and hence more efficient mAb combinations may be found. Several preclinical studies have demonstrated superiority of combining two anti-HER2 mAbs over individual antibodies, but a systematic screen combining antibodies against all the different HER2 subdomains in a broader panel of cell lines is yet to be performed (27–32).

In this present study we conducted a systematic screening evaluating the inhibitory activity of antibody mixtures simultaneously targeting at least two HER2 subdomains in various human cancer cell lines and xenografts. Our screen suggested optimal HER2 targeting is accomplished by a tripartite mixture of antibodies targeting domains I, II and IV.
Materials and Methods

Cell Culture and Reagents

All cell lines were obtained from the American Tissue Culture Collection (ATCC, USA) in the period from 2010-2013 except for OE19, obtained from DSMZ (Germany) in 2011, KATOII and OKAJIMA, kindly provided by professor Morag Park in 2013, and GEO, was previously described (33). All cell lines were Mycoplasma free and cultured according to the suppliers’ recommendations. All cell lines were used within six months of resuscitation and regularly tested for authenticity using the cell line authentication service provided by LGC Standards. OE19 and MDA-MB-175-VII clones resistant to trastuzumab and pertuzumab, respectively, were generated by six months of selection in media containing 50 µg/ml of antibody.

Trastuzumab (Herceptin™) was obtained from Roche. A pertuzumab analogue expressing the sequence encoding variable regions of pertuzumab was generated by cloning and validated for functional equivalence to Perjeta® in vitro (Figure S1).

Human HER2 extracellular domain (ECD) (GenBank Accession No.X03363) was synthesized together with a fully murine HER2 ECD (GenBank Accession No.NM_001003817) at Genscript (Piscataway, New Jersey). Chimeric variants were synthesized as well. HER2 domain definitions were previously described (21). Vector constructs were transiently expressed in HEK293 cells and HER2 ECD protein purified by HIS tag affinity chromatography.

Immunizations and generation of antibody repertoires

8-10 weeks old BALB/c or C57BL/6 female mice, were immunized with either soluble HER2-Fc alone or alternating SK-BR-3 cells and soluble HER2-Fc. Spleens were recovered and macerated through a 74 µm cell strainer (Corning, USA) generating single cell suspensions. Antibodies specific for HER2 were cloned using the murine Symplex technology as previously described by Koefoed et al.(34).

Ranking of apparent antibody binding affinity on chimeric human:mouse HER2 ECDs by ELISA.

Ranking of antibody binding to different chimeric human:mouse HER2 ECD constructs was determined by ELISA titrations on HER2 ECD variants or mouse HER2 ECD control antigen coated at 4°C overnight in 96 well plates (Nunc) as per manufacturer’s instructions. Antibodies were serially diluted 5-fold from 50 µg/ml and added in triplicate. Bound antibody was detected with HRP conjugated goat anti-human Kappa Light from AbD Serotec (Star 127P), diluted 1:20.000 in blocking buffer. Apparent affinities expressed as EC50 values were calculated using nonlinear regression (GraphPad Prism 5, USA).

Affinity measurements of anti-HER2 antibodies by Bio-Layer Interferometry
Kinetic binding analysis was performed on an Octet QK384 biosensor (Fortebio, Menlo Park, CA). Briefly, anti-HER2 antibodies were diluted to 1.5 µg/ml and captured on anti-human Fc sensors (Fortebio, Menlo Park, CA), followed by binding to monovalent soluble human HER2 ECD serially diluted 2-fold from 100 nM. The recorded binding responses were fitted to a simple Langmuir 1:1 binding model for calculation of the on-rate ($k_{on}$), off-rate ($k_{off}$) and affinity (KD) constants using double referencing.

**Cell Proliferation and Growth Assays**

A standard 4 day WST-1 viability assay (Roche Diagnostics, Denmark) was used to measure growth and growth inhibition following treatment with mAbs and mAb mixtures and performed as described (30). The number of viable cells was calculated as percentage of untreated control. Antibody mixtures were generated prior to performing experiments and mixed in ratios of 1:1 or 1:1:1 (w/w) and immediately thereafter added to experimental wells.

**Internalization**

Cells were seeded in 384 well plates and allowed to attach overnight. Next day cells were treated with 50 µg/ml of total antibody for 4 hours fixed in 100% methanol, and stained with 1 µM Hoechst (Invitrogen) and 2 µg/ml CellMask Blue (Invitrogen). HER2 was visualized with an anti-HER2 antibody binding at the c-terminal of the receptor (Cell Signaling Technology) and secondary Alexa-488 conjugated goat anti-rabbit F(ab’)_2, (Cell Signaling Technology). Cells were examined using the Opera LX high-content confocal imaging system (Perkin-Elmer) at 400×.

**Live Cell Analysis**

Cells were cultured in 384-well culture plates (Corning Life Science, Tewksbury, MA, USA) according to the manufacturer’s instructions and visualized every four hours using a real-time cell imaging system (IncuCyte™ live-cell ESSEN BioScience Inc., Ann Arbor, Michigan, USA).

**Flow cytometric determination of cell death and cell cycle distribution**

Cells were seeded in normal growth medium, allowed to adhere overnight and treated with 50 µg/ml of the indicated antibodies or mixtures. After 96 hours of treatment, adherent and non-adherent cells were pooled and all cells were counted by trypan-blue exclusion in a Vi-Cell™ XR (Beckman Coulter). Cells were subsequently stained with phycoerythrin-conjugated Annexin V and 7-aminoactinomycin (7AAD) as per manufacturers’ instructions (BD Biosciences). For cell cycle experiments, cells were seeded in normal growth medium and allowed to adhere overnight. Cells were then synchronized in serum-free medium for 24 hours prior to addition of treatment (50 µg/ml of the indicated antibodies or mixtures) in medium
supplemented with 5% FBS. After 24 hours incubation, adherent cells were harvested, permeabilized in 70% ethanol and stained with propidium iodide (Guava cell cycle reagent for flow cytometry, Millipore). 10,000 events were acquired for all flow cytometric studies and analyzed using FlowJo software (Tree Star, Inc.)

**Antibodies and Western blot analyses**

Cells grown under the previously specified conditions were lysed in RIPA buffer. Immunoblotting was conducted using NuPAGE gels (Invitrogen) according to manufacturer’s instructions utilizing anti-HER2, anti-pHER2 (Tyr1248), anti-EGFR, anti-pEGFR (Ser1046), anti-HER3, anti-pHER3 (Tyr1289), anti-ERK1/2, anti-pERK1/2 (Thr202/Tyr204), anti–pAKT (Ser-473), anti–total-AKT and anti–β-actin antibodies (Cell Signaling Technology). Bands were visualized using IRDye® 800CW Secondary Antibodies (LI-COR Biosciences, UK) and band intensities quantified using Image Studio (LI-COR Biosciences, UK). For quantification of total HER2 levels, lysate samples were diluted to a protein concentration of 0.6-1 g/µl in a master mix containing internal fluorescent standards and DTT, and processed under standard conditions in a Sally Simple Western instrument (ProteinSimple). Rabbit monoclonal primary antibody against HER2 (Clone 28D8 Cell Signaling Technology diluted 1:50) was used for detection and all samples were analyzed in duplicate.

**Mouse xenograft in vivo studies**

1x10^6 OE19 or NCI-N87 cells suspended in 0.1 ml PBS were inoculated subcutaneously into the right flank of 6-8 week old BALB/c nu/nu mice. Mice were monitored daily and tumors measured two to three times weekly using calipers. Tumor volume was calculated using the formula: 0.5x(length)x(width)^2. Mice were randomized to treatment when the mean tumor volume reached a pre-determined size. Antibodies were administered at 50 mg/kg total antibody twice weekly by i.p. injection. Data are expressed as mean ±SEM. All in vivo studies were performed in accordance with the Danish law on animal experimentation and approved by the Animal Ethical Committee, Denmark.

**Synergy Evaluation**

For demonstration of antibody synergy we used the concept of non-linear blending as described by Peterson JJ et al. (35)

**Statistical Analysis**

Statistical analysis was performed using the unpaired student t test and p values less than 0.05 were considered statistically significant.
Results

Generation and initial selection of chimeric anti-HER2 mAbs

Mouse-human chimeric IgG1 antibodies were generated from mice immunized with recombinant human HER2 extracellular domain and cancer cells overexpressing HER2 using the murine Symplex™ technology (34). Antibody supernatants were screened for binding to a HER2 overexpressing cell line SK-BR-3 and recombinant soluble HER2. Positive clones were DNA sequenced and VH and VK genes aligned to germline sequences using the ImMunoGeneTics reference sequence directory (http://www.imgt.org/). A total of 156 unique antibodies estimated to originate from 51 distinct V-D-J rearrangements were identified. Forty unique antibodies from distinct V-D-J rearrangements were selected for high-throughput functional evaluation. More than 1300 mixtures were evaluated and ranked according to their ability to inhibit growth of human gastric cancer cell line, NCI-N87, and breast cancer cell lines, BT474, HCC202, and SK-BR-3. Eight mAbs appearing most frequently in the optimal mixtures were selected and further characterized.

Characterization of Individual mAbs

To probe epitope diversity, four chimeric HER2 ECD antigens, where each single domain was sequentially replaced by mouse HER2 were produced and the mAbs were tested for binding to these constructs (Table 1 and Table S1). Analysis revealed that the eight HER2 antibodies could be grouped into four different epitope bins: Antibodies 4380, 4383, 4385, and 4387 were classified as DI. Antibody 4382 and pertuzumab control mAb were directed against DII. Antibody 4384 was directed against DIII and antibodies 4517, 4518 and trastuzumab were directed against DIV. To confirm the antibody subdivision, all antibodies were tested in a pair wise cross-competition analysis using label free BioLayer Interferometry (Table S2). Antibodies directed against specific domains generally blocked the binding of similar antibodies binding the same domain, while not sterically interfering with the binding of other domains. One exception was the DI antibody 4383, which apart from blocking the binding of DI antibody 4380 also blocked the binding of DII antibodies 4382 and pertuzumab. A schematic representation of the binding sites for the ten different anti-HER2 mAbs is presented in Figure 1A.

The binding kinetics of the mAbs were evaluated with label free BioLayer Interferometry analysis by immobilizing IgG on biosensors and measuring the interaction with monovalent HER2 ECD in solution (Table 1, Figure S2). All antibodies had binding affinities in the low nM to high pM range. Due to biphasic dissociation at high antigen concentrations only apparent affinities were estimated for antibodies 4385 and 4387. The KD values for trastuzumab and pertuzumab were estimated to 0.34 nM and 0.3 nM, respectively.
Evaluation of inhibitory activity of antibody mixtures

All possible dual mixtures of the eight anti-HER2 antibodies were tested for the ability to inhibit growth of four cancer cell lines (Figure 1B). Most mAb mixtures containing antibodies with non-overlapping epitopes exhibited superior activity in the gastric cancer cell line NCI-N87, irrespective of the HER2 subdomain they were binding. mAb 4387 was unique in that it showed some agonistic activity in all four cell lines, but worked well in mixtures with DII antibodies. The two breast cancer cell lines, HCC202 and BT474, demonstrated a more heterogeneous response. However, for all subdomain combinations mAb mixtures demonstrated superior activity when compared to individual mAbs. Mixtures targeting either subdomain I+II, I+III or III+IV were optimal. In the SK-BR-3 cell line, dual mAb mixtures were not superior to individual mAbs. Nine dual mAb mixtures, eight three mAb mixtures and two four mAb mixtures were selected and dose-response curves generated in four cell lines (Figure S3). Additional inhibitory activity was obtained by targeting three non-overlapping epitopes compared with two, but adding a fourth antibody did not further improve activity.

mAb mixtures 4384+4517, 4382+4518, 4385+4382+4518 and 4384+4387+4517 were further evaluated and dose-response curves generated for individual mAbs as well as the mixtures in eight cancer cell lines with varying HER2, EGFR and HER3 levels (Figure 1C, Figure S4). Results clearly show the increased activity of the mAb mixtures in the HER2 amplified cell lines, whereas no apparent benefit of mixtures was observed in HER2-HER3 heterodimer driven cell lines MDA-MB-175-VII and MCF7 (Figure 1C). Furthermore, mAb mixtures, specifically the tripartite mixture consisting of the three antibodies 4382+4387+4517, had a broader and, in several cell lines (SK-BR3, OE19, NCI-N87 and HCC202), superior inhibitory profile compared with either trastuzumab, pertuzumab or a mixture of the two (Figure 1C and D). Interestingly, in HCC202, OE19, NCI-N87 and SK-BR3, pertuzumab appeared to antagonize the activity of trastuzumab. The ability of the four mixtures and individual mAbs to induce ADCC and CDC was also investigated. No difference in ADCC induction by individual- versus mAb mixtures was seen but the rate of CDC induction by mAb mixtures was dramatically higher (Figure S5).

In vivo antitumor activity

The most broadly inhibitory two mAb mixture, 4384+4517, and three mAb mixture, 4382+4387+4517, were evaluated for functional activity in the HER2 dependent gastric cancer tumor xenograft models NCI-N87 and OE19. In the OE19 model, both antibody combinations were able to control tumor growth significantly better than trastuzumab (Figure 1E). In the first half of the treatment period, the anti-HER2 combination 4384+4517 induced regression of the OE19 tumors, followed by a slow gain in tumor size. In contrast,
4382+4387+4517 had dramatically enhanced antitumor activity, leading to tumor regression and complete tumor remission in all mice after only four antibody injections. Likewise, a noticeable anti-tumor response induced by the tripartite mixture with tumor regression was observed in the NCI-N87 tumor xenograft model. Treatment with 4384+4517 was able to control NCI-N87 tumor growth during treatment, whereas tumors treated with trastuzumab escaped while on treatment. Following end of treatment, both 4384+4517 and 4382+4387+4517 treated NCI-N87 tumors resumed growth, although the latter with seemingly slower growth kinetics indicating superior antitumor activity of the three mAb mixture.

**Contribution of individual antibodies to activity of the three mAb mixture**

Further evaluation of the efficacy of 4382+4387+4517 was performed in a panel of 67 human cancer cell lines from seven different indications (Figure 2A). Activity of the mixture was seen across all indications, but the most sensitive cell lines had HER2 overexpression. The three mAb mixture exhibited superior activity to both trastuzumab and pertuzumab and the combination in HER2 overexpressing lines. Except for HER2 overexpression, no clear association of response to 4382+4387+4517 with other molecular changes was found.

The contribution of the individual antibodies to the activity of the three mAb mixture was investigated by deconvolution in four selected cell lines (Figure 2B). In all four cell lines, a clear increase in activity was observed when shifting from individual mAbs to three mAbs with the addition of each mAb. The two mAb mixture consisting of 4387 and 4382 binding domain I and II, respectively, was the most potent and efficacious of the two mAb mixtures. Potential synergy of the tripartite mixture was investigated from the dose response curves using the method of non-linear blending in the cell line OE19 and indeed was demonstrated to be highly synergistic (Figure S6). As previously observed (Figure 1C, Figure S6), pertuzumab appears to decrease the activity of trastuzumab in these four cell lines.

Since antibody mixtures are able to cross-link receptors and cause their internalization and degradation (30,33), the level of HER2 internalization and degradation by the different mAbs and mixtures was investigated. Figure 2C shows the level of HER2 internalization in BT474 cells upon antibody treatment. 4382+4517 and 4387+4517, but not 4382+4387, induced HER2 internalization. The three mAb mixture induced the highest level of HER2 internalization, whereas all individual mAbs including trastuzumab and pertuzumab failed to induce HER2 internalization. Similar results were obtained for SK-BR-3 cells. These results correlated well with the level of HER2 degradation induced by the individual anti-HER2 mAbs and mAb mixtures in the two cancer cell lines OE19 and HCC202 (Figure 2D, Figure S7). Individual mAbs failed to induce HER2 degradation, whereas all mixtures except 4382+4387 induced significant HER2 degradation. Again, 4382+4387+4517 induced higher levels of HER2 degradation compared with the two mAb mixtures, including pertuzumab+trastuzumab. The level of HER2 phosphorylation did not correlate with the level of
HER2 degradation for all mixtures. The mAbs binding to domain IV decreased levels of phosphorylated HER2 (pHER2) in the absence of degradation, whereas mAbs binding other domains had less effect on pHER2. 4387+4382 decreased pHER2 without causing HER2 degradation, whereas 4387+4517 induced HER2 degradation but increased pHER2. The other mAb mixtures reduced both HER2 and pHER2 levels with the greatest efficiency achieved by the 3 mAb mixture.

**Cell cycle arrest and cell death**
Viability assays, including levels of apoptosis and cell cycle arrest were performed to determine the cause of the observed cell growth inhibition. As expected, the number of viable cells was lower upon treatment with 4382+4387+4517 when compared with trastuzumab and pertuzumab treatments (Figure 3A). High levels of apoptosis were induced by 4382+4387+4517 in NCI-N87 (Figure 3B and C), whereas more modest levels of cell death were observed in OE19 and BT474 in response to antibody treatment (Figure 3B and C). In contrast, these cell lines appeared to undergo cell cycle arrest in response to anti-HER2 antibody treatment as seen by an increase in the percentage of G0-G1 cells and a decrease of cells in S-phase (Figure 3D).

**Effect of mAb mixture on downstream signaling**
The impact of 4382+4387+4517 and trastuzumab on HER family receptor phosphorylation and downstream signaling was investigated in two gastric and two breast cancer cell lines (Figure 4A) to better define the molecular mechanisms behind the superior activity of the mAb mixtures. Although some heterogeneity was observed across the four cell lines 4382+4387+4517 induced a stronger and more prolonged inhibitory effect on receptors and downstream signaling molecules when compared with trastuzumab. The difference was most evident in the OE19 cell line. Here the trastuzumab effect was fully compensated after 24 hours as seen by restoration of HER2, EGFR, HER3, and AKT phosphorylation to baseline levels, 4382+4387+4517 at the same time point still efficiently inhibited the phosphorylation of these molecules. In the NCI-N87 cell line, trastuzumab had no effect on HER2, pHER2, EGFR and pEGFR levels, but inhibited pHER3, pERK and pAKT, suggesting that in this cell line trastuzumab blocks growth by interfering with the HER2-HER3 interaction. Some compensation was observed at the 24 hour time point. Like in OE19, 4382+4387+4517 reduced the phosphorylation of HER2, EGFR, HER3, ERK and AKT in this cell line. In the two breast cancer cell lines BT474 and HCC202, both trastuzumab and, more potently, 4382+4387+4517 appeared to transiently increase the phosphorylation level of HER2. The transient increase in pHER2 levels did not lead to increased pEGFR, pHER3, pERK or pAKT, which were decreased by antibody treatment. Overall, these results show that 4382+4387+4517 induces slower but more sustained inhibition of HER2, EGFR, HER3 and the downstream signaling effectors ERK and AKT.
To further explore potential differences in the compensatory mechanism induced by treatment with anti-HER2 mAb or mAb mixtures, OE19 or HCC202 cells were treated with antibodies for 48 hours prior to addition of 1 nM EGF or 1 nM HRG for 15 min. Subsequently, levels of HER family receptor phosphorylation and downstream signaling were investigated (Figure 4B). As observed previously, the levels of HER2, pHER2, pEGFR, pHER3, pERK and pAKT were reduced by treatment with 4382+4387+4517 in the absence of ligand stimulation. Trastuzumab had some effect on pHER2, pEGFR, pHER3, pERK and pAKT, whereas pertuzumab had no effect on the phosphorylation levels of these proteins. The combination of trastuzumab and pertuzumab had a profile similar to, yet weaker than 4382+4387+4517. In the HCC202 cell line, pertuzumab appeared to counteract the inhibitory effect of trastuzumab on pEGFR and pERK consistent with the decreased inhibitory activity of the combination of trastuzumab and pertuzumab combination in this cell line (Figure 4B).

As expected, stimulation of cells treated with antibodies for 48 hours with EGF increased pEGFR, pERK and pAKT levels in both cell lines and completely abrogated the effect of trastuzumab on the phosphorylation of these proteins. The 4382+4387+4517 and to some degree the trastuzumab+pertuzumab combination inhibited EGFR phosphorylation, pERK (OE19) and pAKT (HCC202) in the presence of EGF, indicating that EGF mediated EGFR phosphorylation is driven by EGFR-HER2 heterodimerization and not EGFR homodimers in these cell lines. Interestingly, pertuzumab alone could not block this interaction.

Addition of HRG to the anti-HER2 treated cells yielded high levels of HER3 phosphorylation and increased levels of pEGFR, pAKT and pERK levels in both cell lines (Figure 4B). Like EGF, HRG completely abrogated the inhibitory effect of trastuzumab on the phosphorylation of these proteins and also its effect on pHER2. 4382+4387+4517 and the trastuzumab+pertuzumab combination inhibited pHER2, pHER3, pEGFR and pERK (HCC202) but not pAKT in the presence of HRG. Pertuzumab inhibited HRG-induced HER3 phosphorylation, but not EGFR phosphorylation, indicating that HRG mediated HER3 phosphorylation is driven by HER3-HER2 heterodimerization and EGFR phosphorylation by EGFR-HER2 heterodimers that pertuzumab is unable to inhibit.

**Activity of three mAb mixture in cells with acquired resistance to trastuzumab**

The ability of 4382+4387+4517 to overcome acquired resistance to trastuzumab was investigated in two different model systems. In the first model, trastuzumab resistance was induced by adding ligands to cells in the presence of the different mAbs and mixtures. The growth curves (Figure 5A) indicate that in the absence of ligand, trastuzumab inhibited growth of both OE19 and NCI-N87 cells, but the three mAb mixture was clearly superior. Again, pertuzumab appeared to reduce the activity of trastuzumab, as the combination inhibited cell growth less effectively than trastuzumab alone. Addition of EGF had only a
limited stimulatory effect on the growth of these cells, nonetheless diminished trastuzumab activity. The inhibitory activity of 4382+4387+4517 was only marginally affected by EGF in both cell lines. Both cell lines responded strongly to HRG addition and became completely resistant to the inhibitory activity of trastuzumab (Figure 5A), while pertuzumab+trastuzumab and 4382+4387+4517 inhibited cell growth despite HRG addition. 4382+4387+4517 was clearly superior to pertuzumab+trastuzumab at inhibiting growth of HRG stimulated OE19 cells whereas the activity was similar in NCI-N87.

In the second model of trastuzumab resistance, the activity of 4382+4387+4517 was investigated in two trastuzumab resistant clones of OE19 cells generated by continuous passaging of cells in the presence of trastuzumab. As shown, clones OE19TR6 and OE19TR11, resistant to trastuzumab also fail to respond to pertuzumab or trastuzumab+pertuzumab(Figure 5B). 4382+4387+4517 efficiently inhibited resistant clones demonstrating that this mAb mixture has potential to work in human tumors with acquired resistance to trastuzumab.
Discussion

In this study results from a systematic screen identifying the optimal combination of mAbs among more than a thousand potential mAb mixtures simultaneously targeting two, three or four epitopes on HER2 are presented. Other studies have previously investigated activity of mAb mixtures targeting HER2 in vitro and in vivo (27–32). However, the number of mAbs in these studies was limited and information on their binding epitopes unknown or poorly described.

Our results demonstrate that mAb mixtures are clearly superior to individual antibodies when compared across a broad panel of HER2 dependent cancer cell lines. Although mAb mixtures with increased activity could be obtained with mAbs targeting various combinations of HER2 subdomains, increased activity was often limited to one or a few cell lines. Such differences in response most likely reflect differences in the cancer cells dependency on HER2 and crosstalk with other HER family receptors.

Overall, a mixture of three mAbs targeting HER2 extracellular domains I (4387), II (4382) and IV (4517) respectively, was found to have the broadest and most efficient growth inhibitory activity. All three antibodies were necessary for full activity in cell lines such as HCC202, SK-BR-3 and OE19 and synergistic activity was confirmed in the OE19 cell line (Figure S6). In other cell lines such as MDA-MB-175-VII and MCF7 activity of the tripartite mixture was equal to the domain II targeting antibody 4382 when used as a single agent. The latter two cell lines were also effectively inhibited by pertuzumab, suggesting that 4382 has a mechanism of action comparable to that of pertuzumab, preventing HER2 heterodimerization with HER3 (21). We speculated that do to the similar domain specificity of 4382/pertuzumab and 4517/trastuzumab that a mixture of pertuzumab, trastuzumab and the domain I antibody 4387 would be as effective as the tripartite mixture of 4382+4387+4517. Interestingly, this was only the case in one of the tested cell lines OE19 whereas the T+P+4387 was inferior to 4382+4387+4517 in the HCC202 cell line (Figure S8). These results highlights the importance of the fine epitopes of the antibodies and that even small differences in binding can make a critical difference for activity of mAb mixtures.

In two in vivo models of gastric cancer, 4382+4387+4517 was also superior to trastuzumab and the best two mAb mixture, confirming the in vitro findings. The results suggest several possible explanations for the superior activity of the three mAb mixture in vitro and in vivo. First, 4382+4387+4517 is very efficient at inducing HER2 internalization and degradation in all tested cell lines. Second, the domain II binding antibody 4382 is able to prevent HER2-HER3 heterodimerization, which likely is a major mechanism of escape when HER2 levels are decreased. Third, the mAb mixture induces significantly higher levels of complement dependent cytotoxicity compared to individual antibodies and trastuzumab. The role of
complement in killing of xenograft tumors is unclear due to the species difference but we cannot rule out some contribution from CDC to the growth inhibition seen by the tripartite mixture in vivo.

Antibody-mediated target internalization and degradation has previously been demonstrated to correlate with increased growth inhibitory activity in vitro and in vivo (30,33,34). Although, the exact mechanism whereby antibody mixtures induce target internalization and degradation is currently unknown, receptor cross-linking and lattice formation have been suggested, as has inhibition of receptor recycling (29,30,33,36). Irrespective of the molecular mechanisms responsible for HER2 removal from cancer cells, our results demonstrate that HER2 removal prevents rapid feedback compensation and re-phosphorylation of HER2, EGFR and/or HER3 as seen upon treatment with trastuzumab in OE19, NCI-N87, BT474 and HCC202.

It has been suggested that the enhanced activity when combining pertuzumab and trastuzumab is due to the complementary mechanism of actions of the two antibodies (32,37,38). In the current study we see no strong synergy between trastuzumab and pertuzumab in the absence of ligands and in some situations we find that the effect of the combination is inferior to the individual antibodies. However, we do see that trastuzumab+pertuzumab induces HER2 internalization and degradation, although less efficiently than the three mAb mixture. It could be speculated that in some situations the internalization induced by the combination of trastuzumab+pertuzumab interferes with the mechanism of action of trastuzumab. Only in the presence of the HER3 ligand HRG does the combination appear to provide some superior activity over trastuzumab. It is likely that the superior activity of the trastuzumab and pertuzumab combination is most evident in vivo where ligands are present. This is supported by recent data from clinical trials showing that the combination increased the overall survival of patients with HER2-positive metastatic breast cancer when given together with docetaxel compared with trastuzumab and docetaxel alone (39).

The three mAb mixture identified here outperforms or performs as well as trastuzumab, pertuzumab and a combination of the two in the presence and absence of HER family ligands EGF and HRG. In particular, we see superior activity of the three mAb mixture in cell lines with HER2 amplification and high EGFR expression (OE19, NCI-N87, SK-BR-3 and HCC202), as well as in cells with acquired resistance to trastuzumab or pertuzumab. Interestingly, trastuzumab resistant cells are also resistant to the combination of trastuzumab and pertuzumab. In the HER2 amplified and EGFR high cell lines, the superior activity of 4382+4387+4517 correlates with its ability to inhibit HER2 and EGFR activity. Pertuzumab is claimed to be able to block EGFR-HER2 heterodimerization, but no effect of pertuzumab on either cell viability and EGFR or HER2 activity was observed in these cell lines (21). Based on our results and recent literature, it appears that a subset of breast and gastric cancers with HER2 amplification and high EGFR exists, and that these
tumor cells are resistant to the combination of pertuzumab+trastuzumab or either agent alone due to HER2-EGFR crosstalk (40,41). The three mAb mixture, due to its effective induction of HER2 degradation, inhibits crosstalk of HER2 with EGFR and its development should be considered for this group of patients.

Conclusion

Overall, our data show that a mixture of three mAbs against three different extracellular domains on HER2 has superior antitumor activity in vitro and in vivo, most likely accomplished by efficient HER2 internalization and degradation thereby predicting anti-tumor activity in cells driven by HER2 homodimers as well as tumor cells driven by HER2:EGFR and/or HER2:HER3 heterodimers.
References


7. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J. 1997;16:1647–55.


Tables

Table 1: Domain specificity and binding kinetics for selected antibodies binding human HER2.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>HER2 Domain</th>
<th>$k_{on}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
<th>$K_D$ Error (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4385*</td>
<td>1</td>
<td>21.2 x10$^4$</td>
<td></td>
<td>3.0 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>4387*</td>
<td>1</td>
<td>18.3 x10$^4$</td>
<td></td>
<td>3.3 ± 0.07</td>
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<tr>
<td>4380</td>
<td>1</td>
<td>14.3 x10$^4$</td>
<td>1.9 x10$^{-4}$</td>
<td>1.3 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>4383</td>
<td>1</td>
<td>9.2 x10$^4$</td>
<td>1.1 x10$^{-4}$</td>
<td>1.1 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>4382</td>
<td>2</td>
<td>7.2 x10$^4$</td>
<td>1.1 x10$^{-4}$</td>
<td>1.5 ± 0.02</td>
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<tr>
<td>Pertuzumab$^2$</td>
<td>2</td>
<td>23.4 x10$^4$</td>
<td>0.8 x10$^{-4}$</td>
<td>0.3 ± 0.01</td>
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</tr>
<tr>
<td>4384</td>
<td>3</td>
<td>23.7 x10$^4$</td>
<td>1.2 x10$^{-4}$</td>
<td>0.5 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>4518</td>
<td>4</td>
<td>20.7 x10$^4$</td>
<td>9.4 x10$^{-4}$</td>
<td>4.6 ± 0.08</td>
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<tr>
<td>4517</td>
<td>4</td>
<td>16.9 x10$^4$</td>
<td>3.7 x10$^{-4}$</td>
<td>2.2 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Trastuzumab$^3$</td>
<td>4</td>
<td>28.3 x10$^4$</td>
<td>1.2 x10$^{-4}$</td>
<td>0.4 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

* Apparent affinity.


$^3$ 0.5 nM, Bostrom J. et. al. Plos One 2011; 6(4):1-12
Legends to Figures

**Figure 1.** Systematic functional evaluation of anti-HER2 mixtures. A) Schematic overview of HER2 and the approximate binding sites of the selected anti-HER2 antibodies. B) Matrix showing activity of all combinations of the eight antibodies. Numbers shown are average of four replicates. C) Heat map visualization of dose-response curves for selected anti-HER2 antibodies and mixtures in eight cell lines D) The level of growth inhibition at the highest antibody concentration (50 µg/ml). Line and number in boxes indicate median level of inhibition. E) Activity of selected leads and trastuzumab in two gastric cancer models *in vivo.*

**Figure 2.** Functional evaluation and deconvolution of the activity of the best anti-HER2 mAb mixture. A) Heat map showing level of growth inhibition by 10 µg/ml of selected anti-HER2 antibodies and mixtures. B) Dose-response curves from 50 µg/ml for the best anti-HER2 three mAb mixture, the three individual mAbs and the three possible two mAb mixtures. C) Evaluation of HER2 internalization by confocal microscopy after 4 hours of treatment of BT474 cells with 50 µg/ml of the indicated mAbs and mAb mixtures. D) Level of HER2 degradation and HER2 phosphorylation after 48 hours of antibody treatment.

**Figure 3.** Effect of anti-HER2 mAbs and mixtures on apoptosis and cell cycle arrest. A) The number of viable cells was substantially reduced after anti-HER2 treatment. B) Flow cytometric analysis of cell death. C) Number of viable cells was quantified by trypan-blue exclusion and cell death was demonstrated by staining with Annexin-V and 7AAD; average of 3 separate experiments. D) Cell cycle status was investigated by propidium iodide incorporation, and analyzed using FlowJo univariate cell cycle tool. Representative images are shown, presenting the average number of cells in S-phase (n=3). *p<0.05 and **p<0.01.

**Figure 4.** Effect of anti-HER2 mAbs and mixtures on receptor phosphorylation and downstream signaling. A) Time-frame of trastuzumab and 3 mAb mixture-induced inhibition of HER2 signaling. B) Immunoblot analyses of the impact of anti-HER2 mAbs and mixtures on receptor phosphorylation levels and downstream signaling. Cells were treated with 50 µg/ml of the indicated mAbs and mAb mixtures for 48 hours and either left untreated or treated with 1 nM of EGF or 1 nM HRG for 15 minutes.

**Figure 5.** Activity of the 3 mAb mixture in ligand-stimulated cells or cells with acquired resistance to trastuzumab determined by live cell imaging. A) The level of growth inhibition by indicated anti-HER2 mAbs and mAb mixtures in the presence and absence of 1nM EGF or 1nM HRG. B) The level of growth inhibition
in two trastuzumab resistant clones of OE19 (OE19TR6 and OE19TR11) by anti-HER2 mAbs and mAb mixtures.
Molecular Cancer Therapeutics

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Mol Cancer Ther Published OnlineFirst January 22, 2015.

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