Insights into HER2 signaling from step-by-step optimization of anti-HER2 antibodies

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HER2, a ligand-free tyrosine kinase receptor of the HER family, is frequently overexpressed in breast cancer. The anti-HER2 antibody trastuzumab has shown significant clinical benefits in metastatic breast cancer; however, resistance to trastuzumab is common. The development of monoclonal antibodies that have complementary mechanisms of action results in a more comprehensive blockade of ErbB2 signaling, especially HER2/HER3 signaling. Use of such antibodies may have clinical benefits if these antibodies can become widely accepted. Here, we describe a novel anti-HER2 antibody, hHERmAb-F0178C1, which was isolated from a screen of a phage display library. A step-by-step optimization method was employed to maximize the inhibitory effect of this anti-HER2 antibody. Crystallographic analysis was used to determine the three-dimensional structure to 3.5 Å resolution, confirming that the epitope of this antibody is in domain III of HER2. Moreover, this novel anti-HER2 antibody exhibits superior efficacy in blocking HER2/HER3 heterodimerization and signaling, and its use in combination with pertuzumab has a synergistic effect. Characterization of this antibody revealed the important role of a ligand binding site within domain III of HER2. The results of this study clearly indicate the unique potential of hHERmAb-F0178C1, and its complementary inhibition effect on HER2/HER3 signaling warrants its consideration as a promising clinical treatment.

Introduction

Amplification of the human epidermal growth factor receptor 2 (HER2) gene, and therefore the ErbB2 receptor, occurs in ~20–30% of breast tumors and is associated with a more malignant phenotype, resistance to chemotherapy, and poor prognosis.1,4 However, unlike other ErbB receptors, HER2 has no known ligand.3 To date, there are at least 11 different EGF family ligands that bind to HER receptors, including epidermal growth factor (EGF), transforming growth factor α (TGF-α), and neuregulins (NRGs).6,7 The binding of these ligands to their cognate receptors results in the dimerization and activation of the receptor, which transmits downstream growth signals. Although no ligand has been reported that directly binds to HER2, it is considered to be an important coreceptor partner of other HERs. The importance of HER2 has led to the development of targeted therapies that are tumor-selective and effective for extending life expectancy in patients with early and advanced breast cancer, such as trastuzumab (Herceptin®), a humanized monoclonal IgG1 that binds to the ectodomain of HER2, induces clinical responses in HER2-overexpressing breast cancers, and prolongs patient survival when combined with chemotherapy.8 This antibody is now in clinical use for metastatic breast cancer overexpressing ErbB2, but up to 70% of patients are resistant to the treatment.9 The clinical efficacy of trastuzumab appears to be limited to breast cancers that overexpress HER2, as measured by intense membrane staining in the majority of tumor cells with HER2 antibodies (3+ by immunohistochemistry) or excess copies of the HER2 gene determined by fluorescence in situ hybridization. Therefore, HER2 overexpression as detected by immunohistochemistry or fluorescence in situ hybridization is a biomarker that is predictive of a good response to treatment with the antibody. However, many patients with HER2 gene-amplified metastatic breast cancers do not respond to or eventually escape trastuzumab’s effects, suggesting both de novo and acquired mechanisms of therapeutic resistance. Nevertheless, researchers are especially interested in pertuzumab (Perjeta®) for the treatment of HER2-positive breast cancer since its approval by the US Food and Drug Administration in 2012. Pertuzumab is an antibody that binds to the dimerization arm of ErbB2 and...
prevents its function as a coreceptor with the other ErbBs, most notably ErbB3.10,11 The Phase 3 trial that supported the approval of pertuzumab12 was conducted in 808 patients with metastatic breast cancer receiving first-line treatment. The individuals in the treatment group who received the combination of pertuzumab, trastuzumab, and docetaxel showed a significant reduction in the risk of progression or death and an absolute increase in progression-free survival of 6.1 mo. Notably, recent reports suggest that trastuzumab does not block ErbB2 activation, but rather disrupts noncanonical ErbB2–ErbB3 interactions that result in constitutive ErbB3 activation.13 Importantly, the aforementioned clinical results with pertuzumab and trastuzumab validate the laboratory findings. Taken together, these findings suggest that targeting ErbB2 with monoclonal antibodies (mAbs) that have complementary mechanisms of action results in a more comprehensive blockade of ErbB2 signaling. Thus, the development of novel antibodies that may have complementary blocking effects on HER2 is urgently needed.

With the aim of developing a novel therapeutic agent for HER2 signaling inhibition, we developed a novel anti-Herb2 antibody, hHERmAb-F0178, which binds to domain III of HER2 and was enhanced using a step-by-step antibody optimization method. The crystal structure of human HER2 complexed with the final antibody was solved at 3.5 Å resolution to confirm the epitope of this antibody. This novel anti-HER2 antibody exhibits superior efficacy for blocking HER2/HER3 heterodimerization and signaling, and its use in combination with pertuzumab has a synergistic effect. The characterization of this novel antibody reveals the important role of ligand binding domain III of HER2.

Results

ErbB2-specific mAbs cloned from human B cell phage display libraries

The previously described large non-immunized repertoires of human scFv fragments displayed on phage14,15 were used for the selection of single chain antibodies that bound to the human HER2 extracellular domain (ECD) protein. More than 800 distinct HER2-binding scFv fragments were identified and were ranked based on their affinity for binding to recombinant HER2 ECD protein. A panel of 25 candidate scFv fragments that demonstrated the greatest binding affinity and did not compete with trastuzumab and pertuzumab for binding to HER2 were selected for further analysis. These scFv fragments were converted to full IgG molecules (IgG1,κ) and assessed for their ability to inhibit the proliferation of BT474 and MCF-7 cells in an in vitro proliferation assay. Examples of antibodies demonstrating the greatest inhibition activity in this assay as full IgGs are shown in Figure 1. The antibodies demonstrating the best inhibitory profile as full IgG molecules were hHERmAb-F0178 and hHERmAb-F0179 (Fig. 1A); both were demonstrated to specifically recognize HER2 and did not bind to other EGFR family members, including EGFR, HER3, and HER4.

Characterization of anti-ErbB2 antibodies

The molecular weights of the recombinant anti-ErbB2 antibodies hHERmAb-F0178 and hHERmAb-F0179 were determined by SDS-PAGE. Under reducing conditions, each antibody yielded two protein bands with molecular masses of ~55 kDa (heavy chain) and ~25 kDa (light chain). SDS-PAGE analysis under non-reducing conditions showed a single band at ~150 kDa for the F0718 antibody (Fig. 1B). The results suggest that these two antibodies are IgG-like molecules, which are composed of two heavy chains and two light chains held together by disulfide bonds. Competitive binding assays were performed and showed that hHERmAb-F0178 and hHERmAb-F0179 have a high binding affinity for ErbB2, and the epitopes of these two antibodies do not appear to overlap with the epitopes of trastuzumab and pertuzumab on ErbB2 (Fig. 1C). The affinity constant (Kd) of hHERmAb-F0178 and hHERmAb-F0179 for the ECD of ErbB2 (ErbB2-ECD) was determined by an enzyme-linked immunosorbent assay (ELISA). The data shown in Table 1 indicate that these two antibodies have an ErbB2 binding affinity comparable to that of trastuzumab and pertuzumab.

The pharmacokinetics (PK) of trastuzumab, pertuzumab, hHERmAb-F0178 and hHERmAb-F0179 were determined after single-dose intravenous administration to mice. The serum concentrations of these anti-ErbB2 mAbs were measured by ELISA; briefly, we identified the antibodies in mice serum that would bind to ErbB2-ECD immobilized on ELISA plates. As summarized in Table 2, the main PK parameters of hHERmAb-F0178 and hHERmAb-F0179 in mice were very close to those of trastuzumab and pertuzumab. The data presented in Table 2 also showed that hHERmAb-F0178 and hHERmAb-F0179 had PK properties similar to those of a conventional IgG molecule, suggesting that they are highly stable in vivo.

The epitopes of F0178 and F0179 overlap with Fab37

To identify the epitopes recognized by hHERmAb-F0178 and hHERmAb-F0179, phage clones were isolated by panning the Ph.D.-7 phage display peptide library16 with hHERmAb-F0178 and hHERmAb-F0179. Three rounds of selection were performed, and, at each round, the library was pre-cleared with a control mouse IgG2a,κ antibody. After the third round of panning, the binding of the isolated phage clones to hHERmAb-F0178 and hHERmAb-F0179 was determined by ELISA. Sequence analysis of hHERmAb-F0178- and hHERmAb-F0179-positive phage clones identified five and six distinct amino acid sequences, respectively (Fig. 1D). Alignment of these sequences revealed the consensus motifs NRDED and AEVQQN, which could be aligned with the (476) NRPED (480) and (400) ASVFQN (405) sequence located at ECD III of ErbB2. To determine whether these two sequences within ErbB2 are the epitopes recognized by hHERmAb-F0178 and hHERmAb-F0179, alanine substitutions were introduced into ErbB2-ECD at residues N476, R477, E479, D480, V402, Q404, and N405, and the binding of hHERmAb-F0178 and hHERmAb-F0179 to these ErbB2-ECD mutants was measured by ELISA. The results showed that single alanine substitutions at N476, R477, E479, D480, V402, Q404, or N405 significantly reduced hHERmAb-F0178 and hHERmAb-F0179 binding.
affinity for ErbB2 (Fig. 1E). Double alanine substitutions at positions 477 and 479 further reduced the binding affinity of hHERmAb-F0178, and triple alanine substitutions at positions 402, 404, and 405 almost totally abolished hHERmAb-F0179 binding to ErbB2. In contrast, the binding of pertuzumab to these ErbB2-ECD mutants was approximately the same as its binding to wild-type ErbB2-ECD (Fig. 1E). These data demonstrate that the (476) NRPED (480) and (400) ASVFQN (405) sequences within ErbB2 are the hHERmAb-F0178 and hHERmAb-F0179 epitopes.

These epitope mapping and HER2 mutagenesis data revealed that both the hHERmAb-F0178 and hHERmAb-F0179 epitopes were located at ECD III of ErbB2, although they are located a slight distance apart (Fig. 2A). These antibody binding sites appeared to be located opposite to the dimerization interfaces in domains II and IV, implying that these antibodies did not directly disrupt the interaction of HER2 with other HERs according to the current HER2 dimerization model.10 Notably, the epitopes of these two antibodies overlap with an anti-domain III antibody, Fab37, described in a previous study27 (Fig. 2A).
The effect of these antibodies on HER signaling was further examined. Antibodies with neighboring epitopes vary in their ability to block ErbB2 heterodimerization and signaling

We examined the ability of hHERmAb-F0178, hHERmAb-F0179, and Fab37 to disrupt HRG-dependent ErbB2-HER3 heterodimerization in BT-474 and MCF-7 cell lines. Notably, although these antibodies have similar epitopes, in both cell lines, hHERmAb-F0179 was more effective than hHERmAb-F0178 and Fab37 with respect to disrupting HRG-dependent ErbB2/ErbB3 association and subsequent downstream AKT phosphorylation; however, Fab37 had no detectable effect on the HRG-dependent ErbB2/ErbB3 dimerization (Fig. 2B). Although antibody Fc region may contribute this difference, together with the epitope information, a slight epitope shift (Fig. 2A) to domain III appears to elevate the antibodies’ capacity to disrupt HRG-dependent ErbB2/ErbB3 signaling. Optimization of the antibodies using a step-by-step epitope refinement method

Because the epitope shift contributes to the inhibition efficacy of the antibody, we employed a step-by-step antibody optimization method to obtain more effective antibodies. Optimization of the scFv corresponding to the lead candidate hHERmAb-F0178 was performed to identify antibodies with enhanced inhibitory activity. Randomized libraries were generated from the hHERmAb-F0178 sequences in which the terminal 6 residues of the VH complementarity-determining region (CDR)2 or CDR3 or the terminal 6 residues of the VL CDR2 or CDR3 were randomly replaced to increase diversity within the VH and VL domains. Optimized scFvs were again selected for binding to human HER2 ECD. The ability of the selected scFv to inhibit the formation of HER2 heterodimerization on the surface of MCF-7 cells was assessed, and scFvs with improved inhibitory profiles were identified (Fig. 2C). For full-length IgG1 antibodies have better pharmacokinetic profiles and prospect for targeted therapy, the top 8 scFv candidates were converted to full IgG molecules and assayed for inhibition activity in the BT474 and MCF-7 cell proliferation assay. Most full IgG showed increased activity over the scFv form. The most potent antibody in the inhibition assay, hHERmAbF0178-C1, was selected for further characterization (Fig. 2D). The hHERmAbF0178-G3 antibody, which showed the greatest inhibitory activity as a scFv, did not demonstrate the greatest inhibitory activity as a full IgG.

Structure of the ErbB2 extracellular domain in complex with F0178-C1 Fab

Recently, Crystallography study has been employed to investigate precise antibody epitope, to further investigate the antibody function and HER2 inhibition mechanism, the crystal structure of the ErbB2 ECD in complex with hHERmAbF0178-C1 Fab was determined using the molecular replacement (MR) method and refined to 3.5 Å resolution with a final Rwork value of 22.1% (R-free = 25.8%) in the space group P2₁2₁2₁ (Table 3).

One HER-Fab complex molecule exists in the asymmetric unit with a Matthews coefficient of 2.8 Å³/Da, corresponding to 56.5% of the solvent content. Superimposing HER2 in the HER2-Fab complex in its free form yielded a root–mean–square deviation (r.m.s.d.) of 0.3 Å for all the Cα atoms, indicating that no significant overall structural change occurred, except in several key residues at the antibody–antigen interface (Fig. 3A).

The HER2-Fab complex is approximately 135 Å × 100 Å × 90 Å, and 1219 Å² of accessible surface area can be observed at the antibody binding interface. The structure of the ErbB2 molecule in the HER2-Fab complex is very similar to its previously reported structure. The ECD of ErbB2 is composed of four domains, designated I, II, III, and IV (Fig. 3A). Domains I and III are structurally very similar: a repeating sequence of hydrophobic residues, mainly leucines, causes these domains to fold into a “β hairpin” with sides formed by three parallel β sheets. Domains II and IV are also similar in structure, being composed of disulfide-bonded modules (i.e., small structural units held together by one or two disulfide bonds). Domain II contains seven disulfide-bonded modules, with an eighth module that is structurally part of domain I. Unlike domain IV, domain II contains an insertion (resides 247–266) in its central disulfide-bonded module. This insertion forms a β hairpin that protrudes from the rest of the protein.

HERmAbF0178-C1 Fab binds to the pocket formed by domains I and III of HER2, and the functional and structural status of this region has been discussed extensively in previous studies. The antibody Fab presents a canonical β-sandwich immunoglobulin fold, with the heavy chain folding into the VH and CH domains and the light chain folding into the VL and CL domains. The elbow angle, defined as the subtended angle by two pseudo-2-fold axes relating VH to VL and CH to CL of the antibody Fab, was -135°. The CDR comprises loops L1, L2, L3, H1, H2, and H3 of HHERmAbF0178-C1, which belong to Chothia canonical classes12 2, 1, 1, 1, and 3, respectively. All the CDR loops of HHERmAbF0178-C1 form a large, deep pocket to accommodate the epitope (Fig. 3A) and participate in the interaction with HER2.

F0178C1-ErbB2 interactions

The hHERmAbF0178-C1 Fab binds to TNF through a large and highly complementary interface. The epitope of hHERmAbF0178-C1 Fab on HER2 is composed of many discontinuous segments, including residues R12, L13, A15, HER2 E330, R332, L355, HER2 E357, F359, HER2 A364, P369, Q371, A392, D395, S396, and HER2 Q424 (Fig. 3B, C). Both the heavy and light chains of
hHERmAbF0178-C1 participate in the interaction with HER2, with all the contacts coming from CDRs. Among these CDRs, L3 and H3 contribute to a majority of the interactions with the antigen. Additional contributions are made by CDRs L1, L2, H1, and H2, which indicates a strong and stable interaction within this antigen–antibody pair.

The light chain of hHERmAbF0178-C1 interacts with domain III of HER2. Residue mAbD32 of CDR L3 forms one
hydrogen bond with HER2Q371 at domain III of HER2. An extensive network of intermolecular side chain hydrogen bonds between CDR L2 and domain III of HER2 contributes to most of the light chain interactions and positions the side chains of mAbP53, mAbQ54, and mAbP56 of CDR L2 such that they can interact with HER2F359, HER2D395, and HER2S396. CDR L3 additionally contributes to antigen–antibody communication through the hydrogen bond formed between the side chain of mAbW92 and the HER2P363 residue in domain III of HER2 (Fig. 3C).

The interface between the heavy chain of hHERmAbF0178-C1 and HER2 is primarily composed of the residues inside domain I and domain III of HER2. The mAbS31 in CDR H1, mAbY54 in CDR H2, and mAbT74 in CDRH3 form hydrogen bonds with HER2R12 and HER2L13 in domain I. Moreover, the residues mAbN52 and AdaD49 of CDR H2 are in contact with HER2D359, HER2D362, and HER2E357 in domain III, as well as HER2A15 in domain I of HER2 (Fig. 3B).

The combination of F0178-C1 and pertuzumab more effectively blocks ErbB2/ErbB3 heterodimerization and signaling

We evaluated the ability of trastuzumab, pertuzumab, hHERmAbF0178-C1, trastuzumab plus pertuzumab, trastuzumab plus hHERmAbF0178-C1, pertuzumab plus hHERmAbF0178-C1, and trastuzumab plus pertuzumab plus hHERmAbF0178-C1 to inhibit the in vitro proliferation of breast cancer cell lines in the presence of HRG. Our data clearly indicated that the antiproliferative activity of these anti-ErbB2 mAbs was directly related to their ability to block ligand dependent ErbB2 heterodimerization and signaling (Fig. 4A and B), as no downregulation of HER2 can be detected with HER2 antibodies (data not shown). Accordingly, pertuzumab plus hHERmAbF0178-C1 demonstrated far more antiproliferative activity than any of the others (Fig. 4A and B). Next, the therapeutic efficacy of anti-ErbB2 mAbs was examined in nude mice bearing established BT474 or MCF-7 xenograft tumors (Fig. 4C). Pertuzumab and hHERmAbF0178-C1, but not trastuzumab, significantly delayed MCF-7 tumor progression. Furthermore, the antitumor activity of pertuzumab plus hHERmAbF0178-C1 was similar to that of trastuzumab plus pertuzumab in the BT474 xenograft mouse model. Notably, pertuzumab plus hHERmAbF0178-C1 inhibited tumor growth much more effectively than the combination of trastuzumab and pertuzumab in the MCF-7 xenograft mouse model (Fig. 4C).

HER2/HER3 heterodimerization and signaling were disrupted by F0178-C1 through a block of HER-ligand binding

It is surprising that anti-domain III antibodies have the capacity to block HER2 heterodimerization because in the current model, domain III did not participate in the dimerization of HER2. However, a structural comparison
Figure 4. For figure legend, see page 985.
Figure 4 (See previous page). F0178-C1 effectively inhibits HER2/HER3-Dependent Signaling and Tumor Growth in Xenograft Models and Shows Increased Inhibition When Combined with Pertuzumab. (A) BT474 and MCF-7 cells were treated with increasing concentration of trastuzumab (gray line), pertuzumab (wine line), F0178-C1 (blue line), the combination of trastuzumab plus pertuzumab (black line), the combination of trastuzumab plus F0178-C1 (dark yellow line), the combination of pertuzumab plus F0178-C1 (orange line), or the combination of trastuzumab plus pertuzumab plus F0178-C1 (violet line) in the presence of HRG (2 nM). Concentrations shown on the x axis reflect the concentration of the single antibody. Cell proliferation relative to untreated control was measured after 4 d using AlamarBlue staining. (B) MCF-7 cells treated with indicated concentrations of trastuzumab, pertuzumab, F0178-C1 or combination of pertuzumab and F0178-C1 were stimulated with 0.5 nM HRG for 10 min. Co-immunoprecipitation assay with an anti-HER3 antibody and blotted for pHER2, and Cell lysates were and immunoblotted to detect pAKT (Ser473), pERK1/2 (Thr202/Tyr204), and total HER3. (C) Mice with established BT474 and MCF-7 cells xenografts were injected intraperitoneally with single dose of pertuzumab (12.5 mg/kg, wine line), trastuzumab (12.5 mg/kg, violet line), F0178-C1 (12.5 mg/kg, blue line), combination of trastuzumab plus pertuzumab (12.5 mg/kg, light gray line) combination of pertuzumab plus F0178-C1 (12.5 mg/kg, orange line), or vehicle (gray line). Data are presented as mean tumor volume ± SEM.

Table 2. Pharmacokinetic parameters of recombinant anti-ErbB2 antibodies in mice

| Parametera | Trastuzumab-biotin-based competitive ELISA | Pertuzumab-biotin-based competitive ELISA |
|------------|------------------------------------------|------------------------------------------|
| AUC (day μg ml⁻¹) | 419.91 | 399.23 |
| T₁/₂ (day) | 6.12 | 6.14 |
| CL (ml day⁻¹ kg⁻¹) | 11.15 | 10.91 |
| VSS (ml kg⁻¹) | 55.78 | 56.89 |

*Pharmacokinetic parameters were calculated using a noncompartmental analysis. AUC, area under the concentration vs. time curve; T₁/₂, half-life; CL, clearance; VSS, steady-state volume of distribution.

Table 3. Data collection and refinement statistics

| Parameter | ErbB2-F0178C1 complex |
|-----------|------------------------|
| **Data collection statistics** | |
| Cell parameters | σ = 72.6 Å, b = 82.5 Å, c = 110.3 Å, α = 90°, β = 90°, γ = 90° |
| Space group | P 1 2 1 1 |
| Wavelength used (Å) | 1.000 |
| Resolution (Å) | 38.5 (3.66) – 3.5 |
| No. of all reflections | 65,743 |
| No. of unique reflections | 18,262 |
| Completeness (%) | 99.9 (99.9) |
| Average I/σ(I) | 11.8(3.5) |
| Rmerge (%) | 13.6 (47.3) |
| **Refinement statistics** | |
| No. of reflections used (α(F) > 0) | 16,557 |
| Rwork (%) | 22.1 |
| Rfree (%) | 25.8 |
| r.m.s.d. bond distance (Å) | 0.008 |
| r.m.s.d. bond angle (°) | 1.363 |
| Average overall B-value (Å²) | 46.2 |
| Ramachandran plot | |
| Res. in most favored regions | 761 (77.6%) |
| Res. in additionally allowed regions | 136 (13.8%) |
| Res. in outlier region | 83 (8.5%) |

shows that the epitope of hHERmAbF0178-C1 is similar to that of cetuximab, an anti-EGFR antibody that has been used clinically for a decade. Unlike to other HERs, HER2 was reported to lack of a ligand;²⁴ however, recent studies have suggested that HER2 may have activating membrane-associated ligands.²² Because hHERmAbF0178-C1 bound to domain III and its epitope was similar to that of the ligand blocker, cetuximab, we hypothesized that the antibody-HER2 interaction disrupts the binding of HER2 membrane-associated ligands. Thus, we mutagenesis the residues of HER2 at the antibody-HER2 interface. MCF-7 cells transfected with some HER2 variants with mutations lost the capacity to form phosphorylated HER2/HER3 heterodimers (Fig. 5A and B), without any loss of the surface HER2 level (Fig. 5C). These data suggest that HER2 may in fact have a ligand and that hHERmAbF0178-C1 may interrupt this physiological process, which may account for its inhibitory effects.

Discussion

Although anti-ErbB2 antibodies have been widely used in breast cancer and gastric cancer therapy, the mechanism underlying the different clinical benefits caused by different epitopes has not yet been elucidated. The inhibition of HER2 dimerization, which blocks the associated signaling, has been reported previously.²³ For example, trastuzumab binds to domain IV of HER2,¹² and pertuzumab binds to the dimerization arm of HER2.¹⁰ The capacity of these antibodies to block the heterodimerization of HER2 is well-understood, and recent studies have carefully evaluated the relationship between the structural epitope and the inhibitory function of the antibody, as domain II and domain IV have their own roles in HER2 dimerization.²⁶ To date, no other HER2-specific
antibodies have been reported that have dimerization blocking activity; however, a novel anti-ErbB2 antibody, F0178C1, binds to domain III of HER2, which is far from the dimerization interface in domain II and domain IV of HER2. Regardless, F0178C1 shows excellent heterodimerization blocking activity. These data suggest a unique functional role for domain III, which may be employed as a ligand receiving pocket such as EGFR, HER3, and HER4.

Ligand-induced activation of EGFR involves a marked change in the extracellular region from a ‘tethered’ (inactive) to an ‘extended’ (active) configuration, in which an exposed ‘dimerization arm’ in domain II drives the formation of receptor dimers. In tethered EGFR, the dimerization arm is occluded by auto-inhibitory intramolecular interactions between domains II and IV, which are also observed in unliganded ErbB3 and ErbB4 but are absent in ErbB2. ErbB2 is structurally unique, and even without a bound ligand, its extracellular region resembles the extended (EGF-bound) form of EGFR, with the dimerization arm exposed and apparently ‘poised’ to drive receptor–receptor interaction. No known soluble ligand directly regulates ErbB2, and it is the only family member that transforms cells when simply overexpressed (without ligand addition).

Although no exhaustive information on the ligand-independent activation process of HER2 is available, the roles that the regions of HER2 play in this activation process have been reported. For example, recombinant ErbB2 ECD was found to be unable to dimerize in analytical ultracentrifugation studies, interactions within the C-terminal region of ErbB receptors are sufficient for constitutive activation, and amino acid residues that reside in the kinase domain or between the kinase domain and the C-terminus are important for dimerization. Taken together, these observations suggest that segments within the intracellular domain contain sequences necessary for dimerization and the ECD of ErbB2 is more likely to be playing the role of dimerization regulation. Although no genuine soluble ligand for ErbB2 is known, at least one

Figure 5. The F0178C1 epitope may overlap with the HER2-ligand interaction interface. (A) Effects of key residues on the antibody-HER2 interface on HER2 dimerization. (B) Locations of the key residues represent on the right. (C) Fluorescence-activated cell sorting analysis with the 20 ug of FITC labeled antibody pertuzumab. (D) Comparison the relative location of different anti-domain III antibodies. Domains I, II, III, and IV of the ErbB2 ECD protomer are colored as Figure 2. Antibody F0178C1 is colored wheat and Fab37 is colored bluewhite and modeled using PDB ID 3N85, cetuximab is colored yellow and modeled using PDB ID 1YY.
membrane-bound regulator that contains EGF-like domains has been identified. A subunit of Muc4 (ASGP2) was reported to interact with ErbB2 and to promote its tyrosine phosphorylation. An EGF-like domain in the membrane-associated Muc4 might bind between domains I and III of ErbB2 and induce conformational changes that promote the ability of ErbB2 to form homodimers or heterodimers. Similarly, it was shown in Drosophila that Spitz must be palmitoylated (a modification that drives its membrane association) to regulate dEGFR in vivo. Taken together, these data suggest that domain III of HER2 may also play a role as a ligand binding domain. Thus, the inhibition mechanism of anti-domain III antibodies may involve a block of HER2 ligand binding. Confirmation of this mechanism awaits the identification of the ErbB2 ligands. Remarkably, F0178-C1 provides synergistic inhibition of ErbB2 heterodimerization and signaling in combination with pertuzumab, which directly blocks ErbB2 heterodimerization. However, another anti-domain III antibody, Fab 37, did not exhibit the HER2/HER3 dimerization blocking activity, partially because the epitope of Fab 37 is slightly farther from domain II. The epitope of cetuximab, an antibody that binds to domain III of EGF and blocks EGF binding, can illustrate this phenomenon (Fig. 5D). Due to the steric hindrance between domains I and III of HER2, ‘cetuximab-like’ anti-HER2 antibodies cannot bind to the area on HER2 as that of cetuximab on EGF, and F0178-C1 may have attained the best relative binding position. Although Herceptin (trastuzumab) is the backbone of HER2-directed cancer therapy, recently, more and more data point out that trastuzumab does not block ErbB2 activation but rather disrupts noncanonical ErbB2–ErbB3 interactions that result in constitutive ErbB3 activation. In this study, we mainly focused on the HRG-dependent ErbB2 activation, and the impact of F0178-C1 on the whole ErbB2 signaling network is ongoing.

In conclusion, our study defines a new class of ErbB2 targeting antibodies that bind to domain III, the probable ErbB2 ligand binding site. Moreover, we employed a step-by-step method to maximize the inhibition effect of these antibodies, which exhibit synergistic inhibition of ErbB2 in combination with pertuzumab, an anti-ErbB2 antibody that directly disrupts heterodimerization. These results offer interesting mechanistic insights into the structure and function of the HER2 receptor. Importantly, F0178C1 demonstrates a unique ability to function synergistically with pertuzumab, suggesting that it has great potential for clinical use.

Materials and Methods

Cell lines, antibodies, and animals

The human breast cancer cell lines BT474 and MCF-7 were obtained from the American Type Culture Collection (ATCC). The cells were cultured in DMEM medium supplemented with 12% fetal calf serum in 5% CO2 at 37.8 °C in a humidified incubator. Trastuzumab was purchased from Roche Ltd and the pertuzumab antibody was expressed and purified using the method described in previous studies. Six-week-old female BALB/c mice and five-week-old female BALB/c nude mice were obtained from the Beijing Experimental Animal Center of Chinese Academy of Sciences. All animals were treated in accordance with guidelines of the Committee on Animals of the Chinese Academy of Sciences.

Phage display libraries and selections

Nonimmunized human single-chain variable fragment (scFv) phage display libraries were used for lead-scFv isolation. HER2-ECD, at 10 mg/ml in phosphate buffered saline (PBS), was immobilized on immunotubes (Nunc), and HER2-binding phage was isolated by 3 sequential rounds of panning. The optimized variants were isolated by selection from randomized libraries in solution using biotinylated HER2 captured on streptavidin-coated paramagnetic beads (Dynal). The F0178 and F0179 VH CDR3 randomized repertoires were constructed by polymerase chain reaction (PCR) using mutagenic oligonucleotides to replace the last 6 VH CDR3 amino acids with randomized codons. The mutated DNA for both lineages was ligated into the phagemid vector pCANTAB6 and electroporated into Escherichia coli TG1. Libraries of 6 × 108 and 8 × 108 individual clones were generated for F0178 and F0179, respectively. The randomized libraries were subjected to 1 round of panning on 10 mg/ml of immobilized HER2 ECD, followed by 12 rounds of soluble selection using decreasing concentrations of biotinylated HER2 ECD, from 50 nM down to 100 pM.

Construction, expression and purification

Residues 1–624 of the ErbB2-ECD was prepared as described previously except that we used the pcDNA3.1 (+) expressing vector (Invitrogen) and the FreeStyle 293 expression system (Invitrogen). Antibody screening for a human anti-ErbB2 domain III antibody is discussed below. The heavy and light chains of the antibody was cloned to pcDNA3.1 (+) and expression vectors were co-transfected into Chinese hamster ovary K1 cells using a Lipofectamine 2000 reagent (Invitrogen). The stable transfectants were isolated through limiting dilution in the presence of 600 μg/mL G418 and 300 μg/mL zeocin. The culture supernatants from the individual cell clones were analyzed for antibody production through a sandwich ELISA. The assay used goat anti-human IgG Fc (KPL) as the capture antibodies and goat anti-human kappa-horseradish peroxidase (HRP; Southern Biotechnology Associates) as the detecting antibodies. Purified human IgG1/Kappa (Sigma) was used as the standard control. The clones producing the highest amount of recombinant antibodies were selected and grown in a serum-free medium. The recombinant antibodies were purified through protein A affinity chromatography from the serum-free culture supernatant. The antibody concentrations were determined by absorbance at 280 nm, and the purity was confirmed through SDS–PAGE analysis and western blot.

The Fab fragment of the 0718C1 for the crystallographic investigation was obtained through a papain digestion of an antibody. The digested protein sample was loaded onto a Protein A Sepharose 4 FF column (GE Healthcare). The Fab fragment eluted in the flow-through was separated from the Fc fragment and further purified through ion-exchange chromatography using a Q-Sepharose FF column (GE Healthcare). The protein
The diffraction data for the HER2–0178C1 complex was collected at beamline BL5A (Photon Factory) with a resolution of 3.5 Å. The data were processed, integrated, and scaled using the HKL2000 package. The crystals belong to space group P1 with cell parameters \( a = 72.6 \text{ Å}, b = 82.5 \text{ Å}, c = 110.3 \text{ Å}, \alpha = 90^\circ, \beta = 90^\circ, \gamma = 90^\circ \). The statistics of all data collections and structure refinements are summarized in Table 3.

The HER2–0178C1 structure was solved through the molecular replacement method, which employs the crystal structures of HER2 and pertuzumab Fab (PDB code: 1S78) as the initial searching model by using the program Phaser. The clear solutions in both the rotation and translation functions indicated the presence of one complex molecule, including one HER2 and one Fab molecule, in one asymmetric unit. This result is consistent with the Matthews coefficient and solvent content. The inconsistent residues were manually rebuilt in the program Coot under the guidance of the Fo–Fc and 2Fo–Fc electron density maps. The residues were refined in PHENIX, and the respective working R-factor and R-free decreased from 0.41 and 0.48 to 0.22 and 0.26, respectively, for all data from 50.0 Å to 3.5 Å. The refinement was monitored by calculating \( R_{	ext{cc}} \), based on a subset containing 5% of the total reflections. Model geometry was verified using the program PROCHECK. The data collection and refinement statistics are presented in detail in Table 3. All structure figures were prepared using Pymol.
Blots were blocked in 10% BSA/TBST and probed with an anti-Flag antibody (F3165, Sigma) to detect HER2 followed by a peroxidase-conjugated anti-mouse secondary antibody (Amersham) To verify and normalize for expression of transfected constructs between experimental conditions, 50 μg of cell lysate was checked by western blotting with different antibodies. Anti-mouse HRP-conjugated secondary antibody was used for visualization by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

**Phosphorylation assays**

Ligand independent activation of HER2 was detected by a tyrosine phosphorylation assay using an anti-phospho-ErbB2-Tyr1221/1222 antibody (2243; Cell Signaling)

**Cell Proliferation Assay**

Cells were incubated with different concentrations of recombinant anti-ErbB2 mAbs or peptides for 2 h, followed by the addition of HRG. Recombinant human HRG (R&D Systems) were added at a final concentration of 1 nM, respectively. After an additional 4-d incubation, cell proliferation was determined by Cell Titer 96 AQueous One Solution Cell Proliferation Assay (MTS assay) kit (Promega).

**In vivo therapy study**

For BT474 or MCF-7 xenograft studies, 3 × 10⁶ BT474 or MCF-7 cells were inoculated into the mammary fat pad of female BALB/c nude mice. When tumor volumes reached an average of about 100 mm³, the mice were randomly divided into groups of 10 mice each. Mice were injected intraperitoneally with single dose of pertuzumab (12.5 mg/kg, wine line), trastuzumab (12.5 mg/kg, violet line), F0178-C1 (12.5 mg/kg mg/kg, blue line), combination of pertuzumab plus F0178-C1 (12.5 mg/kg, orange line), or vehicle (gray line) Tumors were measured with digital calipers, and tumor volumes were calculated by the formula: volume = length × (width)²/2.

**Phage Display Peptide Library Screening**

The Ph.D.-7 phage display peptide library (PDPL) kit was purchased from New England Biolabs (Beverley, MA). Biopanning of PDPL with mouse anti-ErbB2 mAbs was performed according to the manufacturer’s instructions. Phage clones were isolated by panning the Ph.D.-7 PDPL with F0178 and F0179. After three rounds of panning, reactivity of positive phage clones with antibodies was measured by ELISA. All antibody positive phage clones were subjected to sequence analysis.

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**Phage ELISA**

ELISA screening of phage clones was performed as previously reported.³⁹ Briefly, 100 μl of supernatant containing amplified particles from each phage clone were added to 96-well plates precoated with mouse anti-ErbB2 mAbs. After incubation for 2 h at RT, detection was performed with HRP-conjugated anti-phage M13 mAb (GE Healthcare). Finally, positive phage clones were subjected to DNA sequence analysis.

**Peptide ELISA**

Peptides (Control peptide or peptide 355LPESFDG DPASNTAPLQ PE373) were synthesized and conjugated to KLH by Shanghai Science Peptide Biological Technology. Mass Spectrometry and HPLC were employed to confirm the structure and sequence of the peptide. The reactivity of the antibody with peptides was determined by ELISA assay. Briefly, different concentrations of F0178C1 were added to 96-well plates precoated with KLH-conjugated peptide and incubated for 2 h, followed by the addition of HRP-conjugated goat polyclonal secondary antibody to mouse IgG-H&L (Abcam). After washing, 3, 3', 5, 5'-tetramethylbenzidine (TMB) was added as a substrate and the absorbance was checked by western blotting with different antibodies. For visualization, the Blots were blocked in 10% BSA/TBST and probed with an anti-Flag antibody (F3165, Sigma) to detect HER2 followed by a peroxidase-conjugated anti-mouse secondary antibody (Amersham) To verify and normalize for expression of transfected constructs between experimental conditions, 50 μg of cell lysate was checked by western blotting with different antibodies. Anti-mouse HRP-conjugated secondary antibody was used for visualization by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).
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