Bispecific Antibody to ErbB2 Overcomes Trastuzumab Resistance through Comprehensive Blockade of ErbB2 Heterodimerization

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Abstract

The anti-ErbB2 antibody trastuzumab has shown significant clinical benefits in metastatic breast cancer. However, resistance to trastuzumab is common. Heterodimerization between ErbB2 and other ErbBs may redundantly trigger cell proliferation signals and confer trastuzumab resistance. Here, we developed a bispecific anti-ErbB2 antibody using trastuzumab and pertuzumab, another ErbB2-specific humanized antibody that binds to a distinct epitope from trastuzumab. This bispecific antibody, denoted as TPL, retained the full binding activities of both parental antibodies and exhibited pharmacokinetic properties similar to those of a conventional immunoglobulin G molecule. Unexpectedly, TPL showed superior ErbB2 heterodimerization-blocking activity over the combination of both parental monomeric antibodies, possibly through steric hindrance and/or inducing ErbB2 conformational change. Further data indicated that TPL potently abrogated ErbB2 signaling in trastuzumab-resistant breast cancer cell lines. In addition, we showed that TPL was far more effective than trastuzumab plus pertuzumab in inhibiting the growth of trastuzumab-resistant breast cancer cell lines, both in vitro and in vivo. Importantly, TPL treatment eradicated established trastuzumab-resistant tumors in tumor-bearing nude mice. Our results suggest that trastuzumab-resistant breast tumors remain dependent on ErbB2 signaling and that comprehensive blockade of ErbB2 heterodimerization may be an effective therapeutic avenue. The unique potential of TPL to overcome trastuzumab resistance warrants its consideration as a promising treatment in the clinic. Cancer Res; 73(21); 6471–83. ©2013 AACR.

Introduction

Overexpression of HER2 (or ErbB2), a member of the ErbB family of receptor tyrosine kinases, is found in 25% to 30% of human breast cancers (1, 2). Trastuzumab, a humanized monoclonal antibody (mAb) directed against ErbB2, is the first anti-ErbB2 treatment approved for clinical use for patients with ErbB2-overexpressing metastatic breast cancer (3). The use of trastuzumab has led to significant improvements in survival in ErbB2-positive breast cancer (4–8). However, many patients still do not respond to trastuzumab treatment (de novo resistance; ref. 8), and the majority of trastuzumab-responsive patients develop resistance within 1 year of treatment initiation (acquired resistance; refs. 9, 10). Novel therapeutic approaches are, therefore, needed to overcome de novo and acquired resistance to trastuzumab therapy.

The mechanism of trastuzumab resistance is not well understood at the moment, but cross-talk between different ErbB family receptors is believed to be associated with resistance to trastuzumab therapy (10–12). Although no specific ligand for ErbB2 has been identified, it is the preferred heterodimerization partner of the ErbB family (13). ErbB2 forms heterodimers with both ligand-free and ligand-bound forms of the other three ErbB family members (EGFR, ErbB3, and ErbB4), which activates ErbB receptors and downstream MAPK and AKT signaling pathways, thereby promoting cell proliferation and survival (3, 14–19). Previous studies have shown that trastuzumab only partially inhibits ErbB2-containing heterodimer formation (16, 19, 20). Thus, ErbB2 heterodimerization may still initiate signaling events that confer resistance when ErbB2 is inhibited by trastuzumab (11, 12).

Pertuzumab is another ErbB2-specific humanized antibody that binds to a distinct epitope from trastuzumab (21–25). Pertuzumab efficiently inhibited ErbB2–ErbB3 complex

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formation when cells were stimulated with ErbB3 ligand (20). In contrast, trastuzumab had little effect on ligand-mediated ErbB2/ErbB3 association (20). Interestingly, in the absence of ErbB3 ligand, the abilities of these two antibodies to inhibit ErbB2/ErbB3 heterodimer formation were reversed: the amount of ErbB3 associating with ErbB2 was clearly reduced when cells were treated with trastuzumab, whereas pertuzumab only caused a minor decrease in ErbB3 in complex with ErbB2 (16). The combination of these two anti-ErbB2 antibodies that have complementary mechanisms of action synergistically inhibits the in vitro and in vivo growth of ErbB2-overexpressing breast cancer cell lines (24, 25). In a phase II study, the activity of combination treatment with pertuzumab and trastuzumab was investigated in patients with ErbB2-positive breast cancer with documented progression on trastuzumab as prior therapy (26). The clinical data showed that combination therapy was well tolerated and the clinical benefit rate (CBR) was 50% (26). Another clinical study was designed to further evaluate whether the encouraging activity observed with the combination of pertuzumab and trastuzumab actually required the presence of trastuzumab (27). The results indicated that pertuzumab monotherapy showed modest efficacy in patients with HER2-positive metastatic breast cancer who had experienced progression during earlier trastuzumab-based therapy, with a CBR of 10.3% (27). In comparison, after reintroduction of trastuzumab, the combination of pertuzumab and trastuzumab showed much greater activity than pertuzumab alone, with a CBR of 41.2% (27). These studies suggest that ErbB2 remains a valid therapeutic target even after cancers have progressed on multiple ErbB2-directed therapies and that comprehensive blockade of ErbB2 heterodimerization may overcome trastuzumab resistance.

Despite the effectiveness of trastuzumab and pertuzumab combination therapy in patients with ErbB2-positive breast cancer whose disease progressed after prior trastuzumab-based therapy, the objective response rate is only 24.2%, and less than 8% of patients experience a complete response (26). Thus, there is still an urgent need to improve ErbB2-directed therapy. Here, we report a bispecific anti-ErbB2 antibody that exhibits superior efficacy over the combination of trastuzumab and pertuzumab.

Materials and Methods

Cell lines and animals

The human breast cancer cell lines BT-474, SK-BR-3, HCC1954, MDA-MB-231, MDA-MB-468, and MCF-7 and the Chinese hamster ovary cell line CHO-K1 were obtained from the American Type Culture Collection. All the cell lines were authenticated twice by morphologic and isoenzyme analyses during the study period. Cell lines were routinely checked for contamination by Mycoplasma using Hoechst staining and consistently found to be negative. Six-week-old female BALB/c mice and 5-week-old female BALB/c nude mice were obtained from the Shanghai Experimental Animal Center of Chinese Academy of Sciences. All animals were treated in accordance with guidelines of the Committee on Animals of the Second Military Medical University.

Construction, expression, and purification

The extracellular domain of ErbB2 (ErbB2-ECD) was prepared as described previously (23), except that we used the pcDNA3.1(+) expressing vector (Invitrogen) and the FreeStyle 293 expression system (Invitrogen). Trastuzumab was purchased from Roche Ltd. The pertuzumab antibody (21) was expressed and purified using the method described in our previous studies (28, 29). Recently, Wu and colleagues described a new approach for producing bispecific tetravalent antibody that exhibits excellent, immunoglobulin G (IgG)-like physicochemical and pharmacokinetic properties (30). The bispecific tetravalent antibody can be efficiently produced by conventional mammalian expression systems as a single species for easy manufacturing and purification (30). Here, we used this approach to construct monospecific or bispecific tetravalent antibodies. Briefly, the VH and VL of the antibody X were respectively fused in frame to the S’ terminus of the heavy chain and light chain of the antibody Y via short (or long) linkers, which were respectively selected from the N termini of human CH1 and Ck, where X and Y represent either trastuzumab (31) or pertuzumab (21). The sequences of the short linkers (SL) are AST (between the two VH) and TVA (between the two VL). The long linker (LL) sequences are ASTKGPVSF (between the two VL) and TVAAPSVFI (between the two VH). We generated six tetravalent antibodies: TP1 (Vtrastuzumab-SL-Vpertuzumab-constant), TP2 (Vpertuzumab LL-Vpertuzumab-constant), PT3 (Vpertuzumab-SL-Vtrastuzumab-constant), PT4 (Vpertuzumab-LL-Vpertuzumab-constant), TP5 (Vtrastuzumab-LL-Vtrastuzumab-constant), and PP1 (Vpertuzumab-LL-Vpertuzumab-constant). The resultant heavy-chain and light-chain genes for the tetravalent antibody were respectively cloned into the pCDNA3.1(+) vector (Invitrogen), yielding the heavy-chain and light-chain expression vectors. The tetravalent antibody was expressed and purified using a similar method as described in our previous report (28). Briefly, the heavy-chain and light-chain expression vectors were cotransfected into CHO-K1 cells. After transfection, the stable transfectants were isolated by limiting dilution in the presence of G418 (500 μg/mL). The cell clone producing the highest amount of antibodies was grown in serum-free medium. Finally, the recombinant antibody was purified by affinity chromatography on Protein A-Sepharose (GE Healthcare). The purified antibodies were analyzed on 8% SDS-PAGE under nonreducing conditions and on 12% SDS-PAGE under reducing conditions, followed by Coomassie Brilliant Blue staining.

Competitive binding assay

Cells at 1 × 10⁶ cells/mL were incubated with a subsaturating concentration of the indicated Alexa Fluor 488-conjugated anti-ErbB2 mAbs and increasing concentrations of purified competing antibodies for 1 hour at 4°C. Then, the cells were washed and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson). The IC₅₀ values of competitors were calculated using a four-variable algorithm.

Affinity measurement

The affinities of anti-ErbB2 antibodies for ErbB2-ECD were determined as described previously (31, 32). Briefly, each mAb
was incubated with increasing concentrations of ErbB2-ECD for an hour. The concentration of free antibody was then measured by ELISA using immobilized ErbB2-ECD and was used to calculate affinity ($K_d$).

**Pharmacokinetics**

Groups of 6-week-old female BALB/c mice were injected with 5 mg/kg body weight of anti-ErbB2 mAb via the tail vein. Blood samples were taken every day by retro-orbital bleeding and collected in tubes coated with heparin to prevent clotting. Four mice were used for every time point, and each mouse was bled only once. After centrifugation to remove the cells, the plasma samples were stored at −80°C until analysis. Serum concentrations of anti-ErbB2 mAbs were measured by competitive ELISAs. Briefly, serial dilutions of serum samples were incubated with a subsaturating concentration of trastuzumab–biotin or pertuzumab–biotin on ErbB2–ECD-coated ELISA plates at 37°C for 1 hour. Detection was carried out with alkaline phosphatase-conjugated avidin. Pharmacokinetic parameters were calculated using a noncompartmental analysis.

**Immunoprecipitation**

The association of ErbB2 with ErbB3 cannot be detected using standard immunoprecipitation methods in the absence of ligand stimulation. To detect ligand-independent ErbB3/ErbB2 heterodimer, Junttila and colleagues (16) used a reversible chemical cross-linking procedure with 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP). This reagent cross-links extracellular proteins through amino groups. Even weakly interacting protein complexes can then be coimmunoprecipitated by using this reagent (16). In this study, we detected the ligand-independent ErbB2-containing heterodimers using the reversible chemical cross-linking procedure described by Junttila and colleagues (16), with minor modifications. Briefly, cells were incubated with the indicated antibodies for 1 hour at 37°C. After washing twice with ice-cold HEPES/NaCl buffer (50 mmol/L HEPES pH 7.2, 150 mmol/L NaCl), the cells were incubated with 2 mmol/L DTSSP (Thermo Scientific) dissolved in HEPES/NaCl buffer for 1 hour at 4°C. The cells were then washed three times with ice-cold 25 mmol/L Tris (pH 7.1), 150 mmol/L NaCl and lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors. For the determination of the expression levels of EGF receptor (EGFR) and ErbB3 in cell lysates, total cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies against EGFR (sc-03; Santa Cruz Biotechnology), ErbB2, and ErbB3.

**siRNA transfection**

Cells were transfected with 100 pmol siRNA using DharmaFECT 4 transfection reagent (Dharmacon). Sequences for interfering RNAs were: 5′-CTA GGA GGA CCG TCG C-3′ (EGFR); 5′-GAT CTT TGG GAG CCT GGC A-3′ (ErbB2); 5′-ACC ACG GTA TCT GGT CAT AAA-3′ (ErbB3).

**Cell proliferation assay**

Cells were incubated with different concentrations of recombinant anti-ErbB2 mAbs for 2 hours, followed by the addition of ErbB ligands or not. Recombinant human EGF and HRG were added at a final concentration of 5 and 1 nmol/L, respectively. After an additional 4-day incubation, cell proliferation was determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay) Kit (Promega).

**In vivo therapy study**

For HCC-1954, MDA-MB-231, or MCF-7 xenograft studies, 3 × 10^6 HCC-1954, MDA-MB-231, or MCF-7 cells were inoculated into the mammary fat pad of female BALB/c nude mice. For BT-474 or BT-474-TraR xenograft studies, female BALB/c nude mice were implanted with 0.72 mg 60-day release 17β-estradiol pellets (Innovative Research of America). After 6 days, 1 × 10^7 BT-474 or BT-474-TraR cells were injected into the mammary fat pad in a 1:1 PBS:Matrigel suspension (BD Matrigel; BD Biosciences). When tumor volumes reached an average of approximately 100 mm^3, the mice were randomly divided into groups of 10 mice each. Treatments consisted of twice weekly intravenous injection of different anti-ErbB2 mAbs for 4
consecutive weeks. Control mice were given vehicle (IgG) alone. Tumors were measured with digital calipers and tumor volumes were calculated by the formula: volume = length \times (width)^2/2.

**Real-time quantitative PCR**

Total RNA was isolated using the TRIZol reagent (Invitrogen) and reverse transcribed with the PrimeScript RT Reagent Kit (Takara). The real-time quantitative PCR was carried out on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the SYBR Premix Ex Taq kit (Takara). β-Actin was used as an endogenous control to normalize expression levels. The primers used were as follows. EGF: (F) 5′-TGG ATG TGC TTG ATA AGC GG-3′, (R) 5′-ACC ATG TTT CCA GTG TGT-3′; TGFα: (F) 5′-AGG TCC GAA AAC ACT GTG AGT-3′, (R) 5′-AGC AAG CGG TTC TTC CCT TC-3′; HB-EGF: (F) 5′-ATC GTG GGG CTT CTC ATG TTT-3′, (R) 5′-TTA GTC ATG CCC AAC TTC ACT TT-3′; AR (amphiregulin): (F) 5′-TAT GCT GCT GGA TTG GAC CTC-3′, (R) 5′-GTT CAC ACC AGG GCG TTA TG-3′; BTC (β-cellulin): (F) 5′-CCT GGG TCT AGT GAT CCT TCA-3′, (R) 5′-GAG GCC ATT AGT TTC AGG ACT TC-3′; EPRG (epiregulin): (F) 5′-GTTG CCA TCA TGA TCG AGG TGG GGA-3′, (R) 5′-TCT CGT CCA AGG CAT CCA GGA-3′; HRG: (F) 5′-TGG CTG ACA GCA GCA GGA-3′, (R) 5′-CTG GCC TGG ATT TTC AGG TGG GGA-3′; b-actin: (F) 5′-CTC CAC ACC AGG GCG TTA TG-3′, (R) 5′-TCT CCC AGC TAG CAG TCC TTC TT-3′.

**Statistical analysis**

Statistical analysis was conducted by Student unpaired t test to identify significant differences unless otherwise indicated. Differences were considered significant at \( P < 0.05 \).

**Results**

**Characterization of anti-ErbB2 bispecific antibodies**

We engineered two monospecific tetravalent antibodies (TP\(_L\) and PP\(_L\)) and four bispecific tetravalent antibodies (TP\(_L\), TP\(_S\), PT\(_S\), and PT\(_L\)) using trastuzumab and pertuzumab (Fig. 1A). The molecular weights of these recombinant anti-ErbB2 mAbs were determined by SDS-PAGE. Under reducing conditions, each of the six tetravalent antibodies (TP\(_L\), TP\(_S\), PT\(_S\), PT\(_L\), TT\(_L\), and PP\(_L\)) yielded two protein bands with a molecular mass of approximately 65 kDa (heavy chain) and approximately 35 kDa (light chain), respectively (Fig. 1B). The SDS-PAGE analysis under nonreducing conditions showed a single band at approximately 200 kDa for each of the six tetravalent antibodies (Fig. 1B). These results suggest that these tetravalent 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 conducted to examine the relative binding affinity of TP\(_L\), TP\(_S\), PT\(_S\), or PT\(_L\) for the trastuzumab epitope on ErbB2 and the pertuzumab epitope on ErbB2. The results showed that out of the four bispecific antibodies, only TP\(_L\) retained the full binding activities of both parental antibodies (Fig. 1C and Supplementary Fig. S1). The relative binding affinity (mean IC\(_{50} \pm \) SD) of TP\(_L\) for the trastuzumab epitope was similar to that of trastuzumab (Supplementary Table S1) and the relative binding affinity of TP\(_L\) for the pertuzumab epitope was similar to that of pertuzumab (Supplementary Table S2). Therefore, we selected TP\(_L\) for further investigation. The affinity constant (\( K_d \)) of TP\(_L\) for the extracellular domain of ErbB2 (ErbB2-ECD) was determined by an ELISA. The data shown in Supplementary Table S3 indicated that TP\(_L\) had an ErbB2-binding affinity comparable with that of trastuzumab and much higher than that of...
pertuzumab. Moreover, the affinity constant ($K_d$) of TTL or PPL for ErbB2-ECD was measured and the results showed that these two monospecific tetravalent antibodies had binding affinities similar to those of their respective parental antibodies (Supplementary Table S3).

The pharmacokinetics of trastuzumab, pertuzumab, TTL, PPL, and TPL were determined after single-dose intravenous administration to mice. The serum concentrations of these anti-ErbB2 mAbs were measured by competitive ELISA in which they competed with biotin-conjugated trastuzumab (trastuzumab–biotin) and/or biotin-conjugated pertuzumab (pertuzumab–biotin) for binding to ErbB2-ECD immobilized on ELISA plates. As summarized in Supplementary Table S4, the main pharmacokinetic parameters of TTL and PPL in mice were very close to those of their respective parental mAbs, trastuzumab and pertuzumab. The serum concentrations of TPL were determined by both trastuzumab–biotin-based and pertuzumab–biotin-based competitive ELISAs. The pharmacokinetic parameters calculated based on the two different competitive ELISA methods were very similar (Supplementary Table S4), indicating that the TPL molecule was intact and capable of binding both the trastuzumab epitope and the pertuzumab epitope in the presence of serum. The data presented in Supplementary Table S4 also showed that TPL had pharmacokinetic properties similar to those of a conventional IgG molecule, suggesting that it is highly stable in vivo.

TPL blocks ErbB2 heterodimerization more effectively than the combination of trastuzumab and pertuzumab

The expression of ErbB receptors in human breast cancer cell lines used in this study was determined and shown in Supplementary Table S5. BT-474 and SK-BR-3 cell lines were trastuzumab-sensitive, and HCC-1954, MCF-7, MDA-MB-231, and MDA-MB-468 cell lines were de novo resistant to trastuzumab (Fig. 2A). Next, we examined the capability of trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TTL, PPL, TTL plus PPL, or TPL to disrupt ErbB2 heterodimerization in BT-474 and MCF-7 cell lines. In both cell lines, trastuzumab

![Figure 2](https://example.com/f2.png)

**Figure 2.** TPL blocks ErbB2 heterodimerization in the absence and presence of ErbB ligand. A, trastuzumab response in breast cancer cell lines. Cells were incubated with 100 nmol/L of trastuzumab for 4 days. Cell proliferation was determined by MTS assay. Sensitivity was defined as a growth inhibition rate of more than 20%. B, coimmunoprecipitation assay was conducted to evaluate the ability of 100 nmol/L of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TTL, PPL, TTL plus PPL, or TPL to disrupt the ligand-independent association of ErbB2 with EGFR or ErbB3 in BT-474 and MCF-7 cell lines. C, coimmunoprecipitation assay assessing the effects of 100 nmol/L of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TTL, PPL, TTL plus PPL, or TPL pretreatment on EGF-induced ErbB2/EGFR and HRG-induced ErbB2/ErbB3 heterodimerization in BT-474 and MCF-7 cell lines.
was more effective than pertuzumab in disrupting ligand-independent ErbB2/ErbB3 association (Fig. 2B). The combination of trastuzumab and pertuzumab caused a marked decrease in the amount of ligand-independent ErbB2/ErbB3 complex compared with either mAb alone (Fig. 2B). Both trastuzumab and pertuzumab were effective in inhibiting EGF-stimulated ErbB2/EGFR dimerization, although pertuzumab exhibited a stronger effect (Fig. 2C). However, only pertuzumab, but not trastuzumab, was able to disrupt HRG-mediated ErbB2/ErbB3 complex formation (Fig. 2C). The addition of trastuzumab to pertuzumab led to a decrease in EGF-induced ErbB2/EGFR heterodimer but not in HRG-induced ErbB2/ErbB3 heterodimer compared with pertuzumab alone (Fig. 2C). TP$_1$ and PP$_1$, either alone or in combination, was as effective as the parental mAbs, trastuzumab and pertuzumab (Fig. 2B and C). Remarkably, TP$_1$ was far more potent in blocking both ligand-independent and ligand-induced ErbB2 heterodimerization than all other anti-ErbB2 mAbs and mAb combinations tested (Fig. 2B and C). More strikingly, the amount of ligand-independent ErbB2/EGFR heterodimer was markedly reduced only when cells were treated with TP$_1$ (Fig. 2B).

**TP$_1$ potently inhibits ErbB2 signaling in both trastuzumab-sensitive and -resistant breast cancer cell lines**

We examined the inhibitory effects of anti-ErbB2 mAb treatment on the activation of ErbB2 and downstream MAPK and AKT signaling pathways. Our results indicated that the ability of anti-ErbB2 mAbs to inhibit ErbB2 signaling corresponded with their capacity to block ErbB2 heterodimerization (Figs. 2 and 3). In the absence of ligand stimulation, treatment of trastuzumab-sensitive BT-474 cells with either trastuzumab or pertuzumab caused a decrease in ErbB3, MAPK and AKT phosphorylation, but trastuzumab showed a much stronger effect (Fig. 3A). The addition of pertuzumab to trastuzumab further reduced the phosphorylation of ErbB3, MAPK, and AKT (Fig. 3A). Notably, TP$_1$ inhibited the phosphorylation of ErbB3, MAPK, and AKT far more effectively than did all other anti-ErbB2 mAbs and mAb combinations tested (Fig. 3A). It is particularly noteworthy that significant dephosphorylation of EGFR was observed only when cells were treated with TP$_1$ (Fig. 3A). Moreover, in trastuzumab-resistant MDA-MB-231 and HCC-1954 cell lines, only TP$_1$ treatment effectively inhibited ligand-independent ErbB2 signaling, whereas all other treatments had only a very minor effect (Fig. 3B and Supplementary Fig. S2A).

We next sought to determine the ability of TP$_1$ to block ligand-induced ErbB2 signaling. The results showed that the phosphorylation of EGFR, ErbB2, and MAPK was dramatically increased in BT-474 cells after EGF stimulation and this activation could be significantly inhibited by pretreatment with both trastuzumab and pertuzumab, although pertuzumab appeared to be a more potent inhibitor (Fig. 3C). We also showed that the phosphorylation of ErbB2, ErbB3, MAPK, and AKT was substantially enhanced after HRG treatment in BT-474 cells (Fig. 3C). Pertuzumab effectively inhibited the HRG-induced enhancement of ErbB2, ErbB3, MAPK, and AKT phosphorylation, whereas trastuzumab had no effect (Fig. 3C). The combination of trastuzumab with pertuzumab resulted in a marked reduction in EGF-induced, but not in HRG-induced, ErbB activation when compared with pertuzumab alone (Fig. 3C). Of note, TP$_1$ showed the most potent inhibitory effect, almost completely abrogating ligand-stimulated phosphorylation of EGFR, ErbB2, ErbB3, MAPK, and AKT in BT-474 cells (Fig. 3C). Similar results were obtained with trastuzumab-resistant HCC-1954, MDA-MB-468, MDA-MB-231, and MCF-7 cell lines (Fig. 3D and Supplementary Fig. S2B).

**TP$_1$-Fab is as effective as TP$_1$ in inhibiting ErbB2 heterodimerization and signaling in breast cancer cell lines**

To determine whether TP$_1$ must be tetravalent to prevent ErbB2 heterodimerization and block ErbB2 activation, we assessed the properties of a Fab version of TP$_1$–TP$_1$-Fab (Fig. 4A). TP$_1$-Fab, which is bivalent and bispecific, was obtained by papain digestion of TP$_1$ and purified as described previously (33). In the absence of ErbB ligand stimulation, TP$_1$-Fab was as effective as TP$_1$ in disrupting the association of ErbB2 with either EGFR or ErbB3 (Fig. 4B). Both TP$_1$ and its Fab version almost completely blocked the formation of EGF-mediated ErbB2/EGFR heterodimer and HRG-induced ErbB2/ErbB3 heterodimer (Fig. 4C). Likewise, TP$_1$-Fab showed a similar ability as TP$_1$ to inhibit both ligand-independent and ligand-induced phosphorylation of MAPK and AKT (Fig. 4D and E). We next compared the effects of TP$_1$ and its Fab version on breast cancer cell proliferation. The results indicated that in the absence or present of ErbB ligand, TP$_1$ and TP$_1$-Fab were equally potent in suppressing the proliferation of both BT-474 and MCF-7 cell lines (Fig. 4F). Together, these data suggest that the ability of TP$_1$ to block ErbB2 heterodimerization and signaling is independent of tetravalency.

**TP$_1$ has superior in vitro antitumor activity compared with the combination of trastuzumab and pertuzumab**

We evaluated the ability of trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TP$_1$, PP$_1$, TT$_1$ plus PP$_1$, and TP$_1$ to inhibit the in vitro proliferation of trastuzumab-sensitive (BT-474 and SK-BR-3) and trastuzumab-resistant (HCC-1954, MCF-7, MDA-MB-231, and MDA-MB-468) breast cancer cell lines. Our data clearly indicated that the antiproliferative activity of these anti-ErbB2 mAbs was directly related to their ability to block ErbB2 heterodimerization and signaling (Fig. 5A). Notably, trastuzumab was much more effective than pertuzumab in suppressing breast cancer cell proliferation in the absence of ErbB ligand (Fig. 5A). In contrast, pertuzumab exhibited a greater antiproliferative activity than trastuzumab against HRG- and EGF-stimulated cell lines (Fig. 5A). The inhibitory effects of TT$_1$ and PP$_1$, either alone or in combination, were similar to those of trastuzumab and pertuzumab (Fig. 5A). Remarkably, TP$_1$ showed far greater antiproliferative activity than that of any of the others both in the absence and presence of ErbB ligand (Fig. 5A). To rule out potential off-target activity of TP$_1$, we examined the effects of ErbB2 RNAi knockdown on MAPK/AKT pathways and growth in the low-ErbB2–expressing cell line MDA-MB-231. Our data showed that knockdown...
of ErbB2 effectively inhibited the phosphorylation of MAPK and AKT (Supplementary Fig. S3A) and resulted in growth inhibition (Supplementary Fig. S3B). Therefore, it can be concluded that the antitumor activity of TPL is associated with an ErbB2-dependent mechanism.

**TPL suppresses the in vivo growth of both trastuzumab-sensitive and -resistant breast tumor xenografts**

The therapeutic efficacy of trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TTL, PPL, TPL pretreatment on EGF- or HRG-induced EGFR, ErbB2, ErbB3, AKT, and MAPK in MDA-MB-231 cells in the absence of ErbB ligand. B, immunoblots examining the ability of 100 nmol/L of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TTL, PPL, TPL to inhibit the phosphorylation of EGFR, ErbB2, ErbB3, AKT, and MAPK in MDA-MB-231 cells in the absence of ErbB ligand. C, immunoblots assessing the effects of 100 nmol/L of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TTL, PPL, TPL pretreatment on EGF- or HRG-induced EGFR, ErbB2, ErbB3, AKT, and MAPK phosphorylation in BT-474 cells. D, immunoblots evaluating the EGF- or HRG-stimulated activation of MAPK and AKT in MDA-MB-231 and HCC-1954 cell lines pretreated with 100 nmol/L of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TTL, PPL, TPL.
MCF-7 xenograft mouse models (Fig. 5B). The antitumor activity of TTL plus PPL was similar to that of trastuzumab plus pertuzumab (Fig. 5B). Notably, TP L inhibited tumor growth much more effectively than the combination of trastuzumab and pertuzumab in all four tumor models (Fig. 5B). Importantly, TPL treatment resulted in complete regression of all BT-474, MDA-MB-231, and MCF-7 tumors and 40% of HCC-1954 tumors, whereas no tumor eradication was observed in tumor-bearing mice treated with all other anti-ErbB2 antibodies. Moreover, our data also indicated that TPL inhibited breast cancer cell growth in a dose-dependent manner both in vitro and in vivo (Supplementary Fig. S4).

TPL overcomes acquired resistance to trastuzumab

We modeled the development of acquired resistance in patients by treating BT474 and SK-BR-3 cell lines with 10 

µg/mL of trastuzumab for 9 months to obtain the trastuzumab-resistant (TraR) sublines, BT-474TraR and SK-BR-3TraR. The TraR sublines were significantly more resistant to trastuzumab treatment than the parental cell lines both in vitro and in vivo (Fig. 6A and B). In addition, we found that the amount of EGFR and EGFR/ErbB2 heterodimers was dramatically enhanced in the TraR cells compared with the parental cells (Fig. 6C and D). Consistent with this, the TraR cells showed a marked increase in EGFR and MAPK phosphorylation (Fig. 6C). In addition, the TraR cells expressed higher levels of ErbB ligands (EGF, HRG, and BTC) than did the parental cells (Fig. 6E). These data indicate that overexpression of EGFR and ligands for EGFR and ErbB3 may be associated with trastuzumab-resistant phenotypes. Next, we examined the effect of siRNA knockdown of EGFR or ErbB3 on the BT-474TraR cell line. Transfection of EGFR siRNA and ErbB3 siRNA dramatically downregulated the amount of EGFR and ErbB3 in BT-474TraR cells, respectively (Supplementary Fig. S5A and S5B). Treatment with EGFR siRNA inhibited ligand-independent EGFR signaling (Supplementary Fig. S5C, left) and considerably resensitized BT-474TraR cells to trastuzumab treatment (Supplementary Fig. S5D, left). Further data indicated that EGFR
siRNA effectively inhibited EGF-induced EGFR signaling (Supplementary Fig. S5C, middle) and cell growth (Supplementary Fig. S5D, middle). Our data also showed that treatment with ErbB3 siRNA resulted in effective inhibition of HRG-mediated ErbB3 signaling (Supplementary Fig. S5C, right) and cell growth (Supplementary Fig. S5D, right) in the BT-474TraR cell line. Together, these data further suggest that enhanced ligand-independent EGFR signaling and ligand-activated ErbB signaling may be acquired trastuzumab resistance mechanisms.

TPL has been shown to be able to block both ligand-independent and ligand-induced ErbB2 receptor heterodimerization and signaling. Next, we asked whether it could overcome acquired resistance to trastuzumab. As observed in the parental cells, TPL potently inhibited both ligand-independent and ligand-induced ErbB2 receptor heterodimerization and signaling in the TraR cells (Fig. 6F–I). Accordingly, the extent of in vitro growth inhibition by TPL was similar for the parental and TraR cells (Fig. 6A). TPL treatment eradicated established TraR tumors in all tumor-bearing mice (Fig. 6B), whereas trastuzumab treatment failed to lead to significant tumor shrinkage. We also showed that trastuzumab plus pertuzumab was significantly less effective than TPL in inhibiting the in vitro proliferation of BT-474TraR cells (Fig. 6J). Consistent with this, the in vivo antitumor activity of the two mAbs in combination was much lower than that of TPL in the BT-474TraR xenograft mouse model (Fig. 6K).

Figure 5. TPL inhibits the in vitro and in vivo growth of both trastuzumab-sensitive and -resistant breast cancer cell lines. A, MTS assay examining the effects of 100 nmol/L of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TTL, PP, TTL plus PP, or TPL on breast cancer cell proliferation in the absence or presence of ErbB ligand (EGF or HRG). Results are shown as percentage of control cell proliferation. Error bars, SD. *, P < 0.05; **, P < 0.001; ***, P < 0.0001. B, tumor volume of BT-474, HCC-1954, MDA-MB-231, and MCF-7 breast tumor xenografts after treatment with control IgG (10 mg/kg), trastuzumab (10 mg/kg), pertuzumab (10 mg/kg), trastuzumab plus pertuzumab (5 mg/kg each), TTL, plus PP (5 mg/kg each), or TPL (10 mg/kg). Data are shown as means ± SEM. *, P < 0.05; **, P < 0.001; ***, P < 0.0001, Mann–Whitney test.
Discussion

Here, we constructed the bispecific antibody TP4 from pertuzumab and trastuzumab. To our knowledge, this is the first study to report a bispecific antibody targeting distinct ErbB2 epitopes and to show that this bispecific antibody has exceptionally potent antitumor activity. The superior heterodimerization-blocking activity of TP4 over trastuzumab plus pertuzumab suggested that, in addition to the regions recognized by trastuzumab and pertuzumab, other regions in ErbB2 might be involved in ErbB2 heterodimerization, and that TP4 might block the interaction of these regions with other ErbBs through steric hindrance and/or inducing ErbB2 conformational change. The strategy for the generation of a bispecific antibody against different epitopes on the same antigen has been reported by Lu and colleagues, who developed an anti-VEGFR-2 bispecific antibody (34). The extracellular domain of VEGFR-2 consists of seven Ig-like domains, and VEGF binds to the second and third Ig-like domains (35). Lu and colleagues engineered a bispecific diabody using two anti-VEGFR2 single-chain antibodies (scFvs; ref. 34). One scFv binds to an epitope that is located on VEGFR-2 domain 1, and the other scFv binds to an epitope on domains 6 and 7. Neither of the two scFvs blocks VEGFR-2 from binding to VEGF, or has any effect on VEGF-induced receptor activation (34). It is rather unexpected that the bispecific diabody effectively blocks VEGFR-2–VEGF interactions, and inhibits both VEGF-induced activation of the receptor and mitogenesis of human endothelial cells (34). Their results suggest that this diabody is most likely to exert its VEGFR-2–VEGF blocking activity by steric hindrance and/or by causing major conformational changes of VEGFR-2 (34). Taken together, our results and those of Lu and colleagues suggest that a bispecific antibody directed against two different epitopes with the same antigen may exert unique effects through steric hindrance and/or causing major conformational changes of the antigen.

The mechanism of trastuzumab resistance is not yet fully elucidated, but compensatory signaling downstream of ErbB2-containing heterodimers is believed to play a critical role in driving trastuzumab resistance (10–12). A deeper understanding of signaling pathways that may still be active in the presence of trastuzumab and further understanding of ErbB2 heterodimerization will offer unique opportunities to enhance the sensitivity of antibody-based therapy. Here, we show that trastuzumab is ineffective at blocking ligand-independent EGFR–ErbB2 complex formation and ligand-induced ErbB2 heterodimerization. In addition, our results indicate that signaling from ligand-independent ErbB2/EGFR heterodimer and ligand-mediated ErbB2-containing heterodimers may contribute to resistance to trastuzumab. Thus, for some, most, or all ErbB2-positive cancers, ErbB2 itself continues to represent a major vulnerability. The challenge is determining the optimal method to capitalize on this vulnerability (38). One strategy to overcome trastuzumab resistance is to continue trastuzumab therapy but while combining it with an alternative chemotherapy regimen. Trastuzumab emtansine (T-DM1) is an antibody–drug conjugate comprising trastuzumab and DM1, a microtubule polymerization inhibitor (39, 40). In a phase 2 study, T-DM1 monotherapy led to an objective response rate of 34.5% in patients with ErbB2-overexpressing metastatic breast cancer who had prior treatment with trastuzumab, lapatinib, an anthracycline, a taxane, and capecitabine (41). Another strategy to overcome trastuzumab resistance is to combine trastuzumab with another anti-ErbB2 antibody that has different mechanisms of action from trastuzumab. Trastuzumab and pertuzumab mainly interfere with ligand-independent and ligand-induced ErbB2 heterodimerization, respectively (16, 20). The combination of these two antibodies that have complementary mechanisms of action is active in patients with ErbB2-positive breast cancer who had experienced progression during prior trastuzumab or pertuzumab monotherapy (26, 27). In this study, TP4 has been shown to provide a more comprehensive blockade of ErbB2 heterodimerization and signaling and result in greater antitumor activity than trastuzumab plus pertuzumab in trastuzumab-resistant breast tumor models. Thus, it can be concluded that compared with combinatorial therapy with trastuzumab and pertuzumab, TP4 treatment may lead to a better therapeutic outcome for patients with ErbB2-positive breast cancer who do not respond to trastuzumab treatment. In conclusion, the data shown here suggest that trastuzumab-resistant breast tumors remain dependent on signaling downstream of ErbB2-containing heterodimers and that comprehensive blockade of ErbB2 heterodimerization may...
circumvent resistance to trastuzumab. The potent ErbB2 heterodimerization blocker, TPL, shows a unique ability to overcome trastuzumab resistance, suggesting that it has the great potential to be translated to the clinic.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: B. Li, Y. Guo
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Bispecific Antibody to ErbB2 Overcomes Trastuzumab Resistance through Comprehensive Blockade of ErbB2 Heterodimerization

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