Bispecific antibody to ErbB2 overcomes trastuzumab resistance through comprehensive blockade of ErbB2 heterodimerization

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Running title: An anti-ErbB2 BsAb Circumvents Trastuzumab Resistance

Keywords: ErbB2; heterodimerization; trastuzumab resistance; bispecific antibody; breast cancer

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.
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 TP_L, retained the full binding activities of both parental antibodies and exhibited pharmacokinetic properties similar to those of a conventional IgG molecule. Unexpectedly, TP_L showed superior ErbB2 heterodimerization-blocking activity over the combination of both parental mAbs, possibly through steric hindrance and/or inducing ErbB2 conformational change. Further data indicated that TP_L potently abrogated ErbB2 signaling in trastuzumab-resistant breast cancer cell lines. We also demonstrated that TP_L 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, TP_L 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 TP_L to overcome trastuzumab resistance warrants its consideration as a promising treatment in the clinic.
Introduction

Overexpression of human epidermal growth factor receptor-2 (HER2 or ErbB2), a member of the ErbB family of receptor tyrosine kinases, is found in 25-30% 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) (8), and the majority of trastuzumab-responsive patients develop resistance within one year of treatment initiation (acquired resistance) (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 demonstrated 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 (21-23). Pertuzumab efficiently inhibited ErbB2/ErbB3 complex 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 2 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 prior 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 ErbB2-positive breast cancer patients 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, HCC-1954, 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 (ATCC). 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 five-week-old female BALB/c nude mice were obtained from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). 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, 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 employed this approach to construct monospecific or bispecific tetravalent antibodies. Briefly, the \( V_H \) and \( V_L \) of the antibody X were respectively fused in frame to the 5’ 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 C\( \kappa \), where X and Y represent either trastuzumab (31) or pertuzumab (21). The sequences of the short linkers (SL) are AST (between the two \( V_H \)) and TVA (between the two \( V_L \)). The long linker (LL) sequences are ASTKGPSVF (between the two \( V_H \)) and TVAAPSVF1 (between the two \( V_L \)). We generated six tetravalent antibodies: \( TP_S \) (\( V_{\text{trastuzumab}} \)-SL-\( V_{\text{pertuzumab}} \)-constant), \( TP_L \) (\( V_{\text{trastuzumab}} \)-LL-\( V_{\text{pertuzumab}} \)-constant), \( PT_S \) (\( V_{\text{pertuzumab}} \)-SL-\( V_{\text{trastuzumab}} \)-constant), \( PT_L \) (\( V_{\text{pertuzumab}} \)-LL-\( V_{\text{trastuzumab}} \)-constant), \( TT_L \) (\( V_{\text{trastuzumab}} \)-LL-\( V_{\text{trastuzumab}} \)-constant) and \( PP_L \)
(V_{pertuzumab}-LL-V_{pertuzumab}-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 the similar method as described in our previous report (28). Briefly, the heavy chain and light chain expression vectors were co-transfected 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 $1 \times 10^6$ 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 h at 4°C. Then, the cells were washed and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson). The IC$_{50}$ 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-wk-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 h. Detection was carried out with alkaline phosphatase-conjugated avidin. Pharmacokinetic parameters were calculated using a noncompartmental analysis.

**Immunoprecipitation**

The association of ErbB2 with ErbB3 can not be detected using standard immunoprecipitation methods in the absence of ligand stimulation. To detect ligand-independent ErbB3/ErbB2 heterodimer, Juntila et al. (16) used a reversible chemical crosslinking procedure with 3,3′-dithiobis[sulfosuccinimidyl]propionate (DTSSP). This reagent crosslinks 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 crosslinking procedure described by Junttila et al. (16), with minor modifications. Briefly, cells were incubated with the indicated antibodies for 1 h at 37°C. After washing twice with ice-cold HEPES/NaCl buffer (50 mM HEPES (pH 7.2), 150 mM NaCl), the cells were incubated with 2 mM DTSSP (Thermo Scientific) dissolved in HEPES/NaCl buffer for 1 h at 4°C. The cells were then washed three times with ice-cold 25 mM Tris (pH 7.1), 150 mM NaCl and lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors. For the determination of the expression levels of 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) and ErbB3 (sc-285; Santa Cruz Biotechnology). For coimmunoprecipitation experiments, we incubated the total cell lysate with an agarose-conjugated anti-ErbB2 monoclonal antibody (sc-7301 AC; Santa Cruz Biotechnology) overnight at 4°C. The precipitated proteins were subjected to SDS-PAGE followed by Western blot analysis with antibodies specific for EGFR (sc-03; Santa Cruz Biotechnology), ErbB2 (sc-7301; Santa Cruz Biotechnology) or ErbB3 (sc-285; Santa Cruz Biotechnology).

The formation of ligand-induced ErbB2-containing heterodimers was assayed by the method described previously (20), with slight modifications. Briefly, the cells were starved overnight in growth medium without serum and then incubated with the indicated antibodies for 1 h at 37°C. Recombinant human EGF (R&D Systems) and HRG (R&D Systems) were added at a final concentration of 5 and 1 nM, respectively. EGF is a ligand for EGFR and HRG is a ligand for ErbB3 and ErbB4. After an
additional 10 min incubation, the cells were washed three times and lysed in NP-40 lysis buffer. For the determination of the expression levels of 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) and ErbB3 (sc-285; Santa Cruz Biotechnology). For coimmunoprecipitation experiments, we incubated the total cell lysate with an agarose-conjugated anti-ErbB2 monoclonal antibody (sc-7301 AC; Santa Cruz Biotechnology) overnight at 4°C. The precipitated proteins were subjected to SDS-PAGE followed by Western blot analysis with antibodies specific for EGFR (sc-03; Santa Cruz Biotechnology), ErbB2 (sc-7301; Santa Cruz Biotechnology) or ErbB3 (sc-285; Santa Cruz Biotechnology).

**Immunoblotting**

Cells were incubated with the indicated antibodies in serum-free medium for 1 h at 37°C. The cells were then treated with EGF (5 nM) or HRG (1 nM) or not treated for 15 min. After washing, the cells were lysed in SDS lysis buffer and the cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies against EGFR (sc-03; Santa Cruz Biotechnology), phospho-EGFR-Tyr1068 (2236; Cell Signaling), ErbB2 (sc-7301; Santa Cruz Biotechnology), phospho-ErbB2-Tyr1221/1222 (2243; Cell Signaling), ErbB3 (sc-285; Santa Cruz Biotechnology), phospho-ErbB3-Tyr1289 (4791; Cell Signaling), AKT (9272; Cell Signaling), phospho-AKT-Ser473 (4060; Cell Signaling), p44/42 MAPK (9102; Cell Signaling) or phospho-p44/2 MAPK-Thr202/Tyr204 (9106; Cell Signaling).

**Small interfering RNA (siRNA) transfection**
Cells were transfected with 100 pmol siRNA using DharmaFECT 4 transfection reagent (Dharmacon). Sequences for interfering RNAs were: 5´-CTA CTT 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 h, followed by the addition of ErbB ligands or not. Recombinant human EGF and HRG were added at a final concentration of 5 and 1 nM, 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 about 100 mm³, the mice were randomly divided into groups of 10 mice each. Treatments consisted of twice weekly intravenous injection of different anti-ErbB2 mAbs for four 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 × (width)^2/2.

**Real-time quantitative PCR**

Total RNA was isolated using the TRIzol reagent (Invitrogen) and reverse transcribed with PrimeScript RT reagent kit (Takara, Dalian, China). The real-time quantitative PCR was carried out on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the SYBR Premix Ex Tag 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 TCC 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 GTG 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´-GGT TCA CGC TTC CCA GAG T-3´; BTC (betacellulin): (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´-GTG ATT CCA TCA TGT ATC CCA GG-3´, (R)5´-TGT CTG AAC TAA AGC TGT GCA G-3´; HRG: (F) 5´-TGG CTG ACA GCA GGA CTA AC-3´, (R) 5´-CTG GCC TGG ATT TCT TC-3´; β-actin: (F) 5´-CTC GAC ACC AGG GCG TTA TG-3´, (R) 5´-TCT CCC ACG TAG CAG TCC TTC-3´.

**Statistical analysis**

Statistical analysis was performed by Student's 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 (TT_l and PP_l) and four bispecific tetravalent antibodies (TP_S, TP_L, 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_S, TP_L, PT_S, PT_L TT_l and PP_l) yielded two protein bands with a molecular mass of ~65 kDa (heavy chain) and ~35 kDa (light chain), respectively (Fig. 1B). The SDS-PAGE analysis under non-reducing conditions showed a single band at ~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 performed to examine the relative binding affinity of TP_S, TP_L, 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} ± 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 chose 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 enzyme-linked immunosorbent assay...
The data shown in Supplementary Table S3 indicated that TP$_L$ had an ErbB2-binding affinity comparable to that of trastuzumab and much higher than that of pertuzumab. Moreover, the affinity constant ($K_d$) of TT$_L$ or PP$_L$ for ErbB2-ECD was also 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, TT$_L$, PP$_L$ and TP$_L$ 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 TT$_L$ and PP$_L$ in mice were very close to those of their respective parental mAbs, trastuzumab and pertuzumab. The serum concentrations of TP$_L$ 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 TP$_L$ 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 TP$_L$ 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, PP, TTl plus PP, and TPL to disrupt ErbB2 heterodimerization in BT-474 and MCF-7 cell lines. In both cell lines, trastuzumab 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 to 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). TTl and PP, either alone or in combination, was as effective as the parental mAbs, trastuzumab and pertuzumab (Fig. 2B and C). Remarkably, TPL 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\textsubscript{L} (Fig. 2B).

**TP\textsubscript{L} 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 (Fig. 2 and 3). In the absence of ligand stimulation, treatment of trastuzumab-sensitive BT474 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). Of note, TP\textsubscript{L} 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\textsubscript{L} (Fig. 3A). Moreover, in trastuzumab-resistant MDA-MB-231 and HCC-1954 cell lines, only TP\textsubscript{L} 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\textsubscript{L} 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 demonstrated 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 to pertuzumab alone (Fig. 3C). Of note, TP_L 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_L-Fab is as effective as TP_L in inhibiting ErbB2 heterodimerization and signaling in breast cancer cell lines**

To determine whether TP_L must be tetravalent in order to prevent ErbB2 heterodimerization and block ErbB2 activation, we assessed the properties of a Fab version of TP_L, TP_L-Fab (Fig. 4A). TP_L-Fab, which is bivalent and bispecific, was obtained by papain digestion of TP_L and purified as described previously (33). In the absence of ErbB ligand stimulation, TP_L-Fab was as effective as TP_L in disrupting the association of ErbB2 with either EGFR or ErbB3 (Fig. 4B). Both TP_L 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_L-Fab showed a similar ability as TP_L to inhibit both ligand-independent and ligand-induced phosphorylation of MAPK and AKT (Fig. 4D and E). We next compared the effects of TP_L and its Fab version on breast cancer cell proliferation. The results indicated that in the absence or present of ErbB ligand, TP_L and TP_L-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_L to block ErbB2 heterodimerization and signaling is independent of tetravalency.

**TP_L has superior in vitro antitumor activity compared with the combination of trastuzumab and pertuzumab**

We evaluated the ability of trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TT_L, PP_L, TT_L plus PP_L, and TP_L 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_L and PP_L, either alone or in combination, were similar to those of trastuzumab and pertuzumab (Fig. 5A). Remarkably, TP_L demonstrated 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 L, 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 TP L is associated with an ErbB2-dependent mechanism.

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

The therapeutic efficacy of trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TT L plus PP L, and TP L was examined in nude mice bearing established BT-474, HCC-1954, MDA-MB-231, or MCF-7 xenograft tumors. Trastuzumab suppressed tumor growth better than pertuzumab in the BT-474 xenograft model (Fig. 5B). In contrast, pertuzumab, but not trastuzumab, significantly delayed the MDA-MB-231 and MCF-7 tumor progression (Fig. 5B). Both trastuzumab and pertuzumab did not inhibit the HCC-1954 tumor growth (Fig. 5B). Trastuzumab plus pertuzumab was more efficient in inhibition of the BT-474 and HCC-1954 tumors than either mAb alone (Fig. 5B). However, combinatorial treatment with these two mAbs did not result in a significant benefit over single-agent pertuzumab treatment in the MDA-MB-231 and MCF-7 xenograft mouse models (Fig. 5B). The antitumor activity of TT L plus PP L 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, TP_L 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 TP_L inhibited breast cancer cell growth in a dose-dependent manner both in vitro and in vivo (Supplementary Fig.S4).

**TP_L 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 nine months to obtain 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). We also 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. Trasfection of EGFR siRNA and ErbB3 siRNA dramatically downregulated the amount of EGFR and ErbB3 in BT-474TraR cells, respectively (Supplementary Fig.S5A and B). Treatment with EGFR siRNA inhibited
ligand-independent EGFR signaling (Supplementary Fig.S5C, left panel) and considerably resensitized BT-474TraR cells to trastuzumab treatment (Supplementary Fig.S5D, left panel). Further data indicated that EGFR siRNA effectively inhibited EGF-induced EGFR signaling (Supplementary Fig.S5C, middle panel) and cell growth (Supplementary Fig.S5D, middle panel). Our data also demonstrated that treatment with ErbB3 siRNA resulted in effective inhibition of HRG-mediated ErbB3 signaling (Supplementary Fig.S5C, right panel) and cell growth (Supplementary Fig.S5D, right panel) 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 demonstrated to be able to block both ligand-independent and ligand-induced ErbB 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 demonstrated 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).
Discussion

Here, we constructed the bispecific antibody TP_L 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 TP_L 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 TP_L 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 et al., who developed an anti-VEGFR-2 bispecific antibody (34). The extracellular domain of VEGFR-2 consists of seven immunoglobulin-like domains, and VEGF binds to the second and third immunoglobulin-like domains (35). Lu et al. engineered a bispecific diabody using two anti-VEGFR2 single chain antibodies (scFvs) (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, Lu et al.’s and our results 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. Our results also indicate that signaling from ligand-independent ErbB2/EGFR heterodimer and ligand-mediated ErbB2-containing heterodimers may contribute to resistance to trastuzumab, suggesting that simultaneous targeting of multiple ErbB2-containing heterodimers may be a promising therapeutic avenue for patients with trastuzumab-resistant tumors.

ErbB2 remains a valid therapeutic target even after cancers have progressed on multiple ErbB2-directed therapies (26, 27, 36, 37). 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 combine 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 II 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 monotherapy or pertuzumab monotherapy \(26, 27\). In this study, TP\(L\) has been demonstrated 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, TP\(L\) treatment may lead to a better therapeutic outcome for ErbB2-positive breast cancer patients 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, TP\(L\),
demonstrates 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.

**Authors' Contributions**

**Conception and design**: B. Li, Y. Guo


**Analysis and interpretation of data**: B. Li, Y. Meng, L. Zheng, S. Hou, Y. Guo

**Writing, review, and/or revision of the manuscript**: B. Li, Y. Guo

**Grant Support**

This work was supported by National Natural Science Foundation of China (30973468), Ministry of Science & Technology of China (973 program projects), National Key project for New Drug Development and Manufacture, Shanghai Commission of Science & Technology, and Shanghai Leading Academic Discipline Project (B905).

**References**


13. 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.


15. Johannessen LE, Haugen KE, østvold AC, Stang E, Madshus IH. Heterodimerization of the epidermal-growth-factor (EGF) receptor and ErbB2 and the affinity of EGF binding are regulated by different mechanisms. Biochem


FIGURE LEGENDS

Figure 1. Characterization of anti-ErbB2 tetravalent antibodies. A, Schematic representation of anti-ErbB2 tetravalent antibodies. B, SDS-PAGE analysis of purified anti-ErbB2 antibodies under non-reducing and reducing conditions. Lane 1, protein marker; lane 2, trastuzumab; lane 3, pertuzumab; lane 4, TT_L; lane 5, PP_L; lane 6, TP_L; lane 7, PT_L; lane 8, TP_S; lane 9, PT_S. C, Competitive binding assay. Trastuzumab, pertuzumab, TT_L, PP_L, TP_L, PT_L, TP_S and PT_S were evaluated for their ability to compete with Alexa Fluor 488-labeled trastuzumab or Alexa Fluor 488-labeled pertuzumab for binding to BT-474 cells.

Figure 2. TP_L blocks ErbB2 heterodimerization in the absence and presence of ErbB ligand. A, Trastuzumab response in breast cancer cell lines. Cells were incubated with 100 nM of trastuzumab for four days. Cell proliferation was determined by MTS assay. Sensitive was defined as a growth inhibition rate of > 20%. B, Coimmunoprecipitation assay was performed to evaluate the ability of 100 nM of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TT_L, PP_L, TT_L plus PP_L, or TP_L 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 nM of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TT_L, PP_L, TT_L plus PP_L, or TP_L pretreatment on EGF-induced ErbB2/EGFR and HRG-induced ErbB2/ErbB3 heterodimerization in BT-474 and MCF-7 cell lines.

Figure 3. TP_L inhibits ErbB2 signaling in both trastuzumab-sensitive and -resistant
breast cancer cell lines. A, Immunoblots examining the ability of 100 nM of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TT\textsubscript{L}, PP\textsubscript{L}, TT\textsubscript{L} plus PP\textsubscript{L}, or TP\textsubscript{L} to inhibit the phosphorylation of EGFR, ErbB2, ErbB3, AKT and MAPK in BT-474 cells in the absence of ErbB ligand. B, Immunoblots examining the ability of 100 nM of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TT\textsubscript{L}, PP\textsubscript{L}, TT\textsubscript{L} plus PP\textsubscript{L}, or TP\textsubscript{L} 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 nM of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TT\textsubscript{L}, PP\textsubscript{L}, TT\textsubscript{L} plus PP\textsubscript{L}, or TP\textsubscript{L} 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 nM of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TT\textsubscript{L}, PP\textsubscript{L}, TT\textsubscript{L} plus PP\textsubscript{L}, or TP\textsubscript{L}.

**Figure 4.** TP\textsubscript{L} displayed an ability similar to intact TP\textsubscript{L} to block ErbB2 heterodimerization and activation and suppress breast cancer cell proliferation. A, Schematic representation of TP\textsubscript{L}-Fab. B, Coimmunoprecipitation assay evaluating the ability of 100 nM of control IgG, trastuzumab, pertuzumab, TP\textsubscript{L} or TP\textsubscript{L}-Fab to disrupt the formation of ligand-independent ErbB2-containing heterodimers in BT-474 cells. C, Coimmunoprecipitation assay detecting EGF-induced ErbB2/EGFR and HRG-induced ErbB2/ErbB3 heterodimerization in BT-474 cells pretreated with 100 nM of control IgG, trastuzumab, pertuzumab, TP\textsubscript{L} or TP\textsubscript{L}-Fab. D, Immunoblots
assessing the phosphorylation of AKT and MAPK in BT-474 cells upon treatment with 100 nM of control IgG, trastuzumab, pertuzumab, TP_L or TP_L-Fab in the absence of ErbB ligand. E, Immunoblots evaluating the effects of 100 nM of control IgG, trastuzumab, pertuzumab, TP_L or TP_L-Fab pretreatment on EGF- or HRG-activated ErbB2 signaling in BT-474 cells. F, MTS assay examining the effects of 100 nM of control IgG, trastuzumab, pertuzumab, TP_L or TP_L-Fab 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.

**Figure 5.** TP_L 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 nM of control IgG, trastuzumab, pertuzumab, trastuzumab plus pertuzumab, TT_L, PP_L, TT_L plus PP_L, or TP_L 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), TT_L plus PP_L (5 mg/kg each), or TP_L (10 mg/kg). Data are shown as means ± SEM. *P < 0.05, **P < 0.001, ***P < 0.0001, Mann-Whitney test.

**Figure 6.** TP_L treatment overcomes acquired resistance to trastuzumab. A, MTS assay evaluating cell proliferation of indicated parental breast cancer cell lines and TraR sublines upon treatment with 100 nM of trastuzumab or TP_L. Error bars, SD. *P <
B, Tumor volume of BT-474 and BT-474TraR breast tumor xenografts after treatment with 10 mg/kg of control IgG, trastuzumab, or TP\textsubscript{L}. Data are shown as means ± SEM. **P < 0.001, ***P < 0.0001. C, Immunoblots comparing major cell signaling changes between the indicated parental breast cancer cell lines and their corresponding TraR sublines. D, Coimmunoprecipitation assay detecting ligand-independent ErbB2/EGFR and ErbB2/ErbB3 heterodimerization in the indicated parental breast cancer cell lines and TraR sublines. E, Real-time quantitative PCR analysis of expression of ErbB ligands. Data are shown as means ± SD. F, Coimmunoprecipitation assay examining the ability of 100 nM of control IgG, trastuzumab, or TP\textsubscript{L} to disrupt the ligand-independent association of ErbB2 with EGFR or ErbB3 in BT-474 and BT-474TraR cell lines. G, Coimmunoprecipitation assay evaluating the effects of 100 nM of control IgG, trastuzumab, or TP\textsubscript{L} pretreatment on EGF-induced ErbB2/EGFR and HRG-induced ErbB2/ErbB3 heterodimerization in BT-474 and BT-474TraR cell lines. H, Immunoblots assessing ErbB signaling in BT-474 and BT-474TraR cell lines upon treatment with 100 nM of control IgG, trastuzumab or TP\textsubscript{L} in the absence of ErbB ligand. I, Immunoblots evaluating the effects of 100 nM of control IgG, trastuzumab or TP\textsubscript{L} pretreatment on EGF- or HRG-activated ErbB signaling in BT-474 and BT-474TraR cell lines. J, MTS assay evaluating the in vitro proliferation of BT-474TraR cell line upon treatment with 100 nM of trastuzumab, pertuzumab, trastuzumab plus pertuzumab, or TP\textsubscript{L}. Data are shown as means ± SD. *P < 0.05. K, Tumor volume of BT-474TraR breast tumor xenografts after treatment with control IgG (10 mg/kg), trastuzumab plus pertuzumab.
(5 mg/kg each), or TP_L (10 mg/kg). Data are shown as means ± SEM.
Figure 1
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Figure 5
Bispecific antibody to ErbB2 overcomes trastuzumab resistance through comprehensive blockade of ErbB2 heterodimerization

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Cancer Res  Published OnlineFirst September 17, 2013.