Dual Targeting of HER2-Positive Cancer with Trastuzumab Emtansine and Pertuzumab: Critical Role for Neuregulin Blockade in Antitumor Response to Combination Therapy

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Abstract

Purpose: Targeting HER2 with multiple HER2-directed therapies represents a promising area of treatment for HER2-positive cancers. We investigated combining the HER2-directed antibody–drug conjugate trastuzumab emtansine (T-DM1) with the HER2 dimerization inhibitor pertuzumab (Perjeta).

Experimental Design: Drug combination studies with T-DM1 and pertuzumab were performed on cultured tumor cells and in mouse xenograft models of HER2-amplified cancer. In patients with HER2-positive locally advanced or metastatic breast cancer (mBC), T-DM1 was dose-escalated with a fixed standard pertuzumab dose in a 3+3 phase Ib/II study design.

Results: Treatment of HER2-overexpressing tumor cells in vitro with T-DM1 plus pertuzumab resulted in synergistic inhibition of cell proliferation and induction of apoptotic cell death. The presence of the HER3 ligand, heregulin (NRG-1β), reduced the cytotoxic activity of T-DM1 in a subset of breast cancer lines; this effect was reversed by the addition of pertuzumab. Results from mouse xenograft models showed enhanced antitumor efficacy with T-DM1 and pertuzumab resulting from the unique antitumor activities of each agent. In patients with mBC previously treated with trastuzumab, lapatinib, and chemotherapy, T-DM1 could be dosed at the maximum tolerated dose (MTD; 3.6 mg/kg every 3 weeks) with standard dose pertuzumab. Adverse events were mostly grade 1 and 2, with indications of clinical activity.

Conclusions: Dual targeting of HER2 with the combination of T-DM1 and pertuzumab in cell culture and mouse xenograft models resulted in enhanced antitumor activity. In patients, this combination showed an encouraging safety and tolerability profile with preliminary evidence of efficacy.

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Introduction

The ErbB/HER family of receptor tyrosine kinases (RTK) plays critical roles in both development and cancer (1). These receptors include EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. Numerous ligands have been identified that interact with all HER family receptors, with the exception of HER2. Ligand binding activates multiple downstream signal transduction pathways. HER2 is the common coreceptor for the other HER family members. Association of HER2 with HER3 is the most potent signaling complex formed in this receptor family (2, 3). HER3 is considered a pseudo-kinase (4, 5), but can potently activate phosphoinositide 3-kinase (PI3K) signaling upon dimerization with other HER family members (6). In addition to growth factor regulation of cell proliferation and differentiation, recent reports show an important role for multiple RTK ligands in resistance to kinase inhibitors (7–9). The HER3 ligand, neuregulin 1 (NRG-1), was recently shown to mediate resistance to chemotherapeutic agents as well (10). Moreover, HER3 was reported to mediate resistance to EGFR, HER2, and PI3K inhibitors. Treatment resulted in HER3 activation, leading to attenuation of the response to kinase inhibition (11, 12). Trastuzumab (Herceptin), a humanized HER2 antibody that binds domain IV of the HER2 extracellular domain (ECD), is used in combination with chemotherapy for...
The antibody–drug conjugate ado-trastuzumab emtansine (T-DM1; Kadcyla) increases progression-free and overall survival versus lapatinib plus capecitabine in patients with HER2-positive metastatic breast cancer (mBC) who have received prior treatment with trastuzumab (Herceptin) and a taxane chemotherapy, with a better safety profile than the comparator treatments. Dual antibody blockade of HER2 with pertuzumab, trastuzumab plus docetaxel showed increased progression-free and overall survival with the addition of pertuzumab to trastuzumab plus docetaxel. Neoadjuvant treatment with trastuzumab plus pertuzumab (Neosphere trial) or trastuzumab plus lapatinib (NeoALTO trial) in conjunction with taxane-based therapy resulted in better clinical activity than with single-agent HER2-directed therapy. The studies described here show enhanced antitumor activity with T-DM1 plus pertuzumab in preclinical models, with an encouraging safety profile and preliminary evidence of efficacy in a phase Ib study. Moreover, our data show suppression of T-DM1 activity upon ligand activation of HER2/HER3, supporting the rationale for combining pertuzumab with T-DM1.

**Materials and Methods**

**Cell lines and reagents**

Human breast tumor lines BT-474, SK-BR-3, UACC-812, ZR-75-30, HCC1954, and MDA-MB-175-VII (MDA-175), and the non–small cell lung carcinoma line Calu-3 were obtained from the American Type Culture Collection. KPL-4 breast cancer cells were provided by Prof. Junichi Kurebayashi, Kawasaki Medical Hospital, Kurashiki, Okayama, Japan (35). Cells were maintained as previously described (16). T-DM1, pertuzumab, N297A-pertuzumab (36), and E. coli-derived NRG-1β (EGF domain) were produced at Genentech, Inc. Unconjugated DM1 was obtained from ImmunoGen, Inc. and chemotherapeutic drugs from Sigma-Aldrich. Western blot antibodies were from R&D Systems (cleaved PARP), Cell Signaling Technology (phospho-HER3, phospho-Akt, Akt), and Santa Cruz Biotechnology, Inc. (HER3).

**Cell viability and apoptosis assays**

Cell viability and apoptosis assays were performed as described in ref. (16). T-DM1, pertuzumab, or the combination was added to cells; treatments were in duplicate to fit all groups onto one plate and experiments were repeated 5 times. Studies performed without NRG-1β were in medium supplemented with 10% FBS and studies with NRG-1β (2 nmol/L) were in 1% FBS-containing medium. Cell viability was measured at 3 (SK-BR-3, KPL-4) or 5 (BT-474, Calu-3) days after drug addition. Assays to test NRG-1β effects on T-DM1 or chemotherapeutic agent activity were 3 or 5 days (n = 3 per group) and studies were performed three times. Combination Index (CI) values, as described by Chou (37), were generated using CalcuSyn software (Biosoft, Inc). ADCC assays were performed as described in ref. (38) using CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega Corp.). Treatments were T-DM1 alone or combined with pertuzumab or the Fc-mutant N297A-

**Translational Relevance**

The antibody–drug conjugate ado-trastuzumab emtansine (T-DM1; Kadcyla) increases progression-free and overall survival versus lapatinib plus capecitabine in patients with HER2-positive metastatic breast cancer (mBC) who have received prior treatment with trastuzumab (Herceptin) and a taxane chemotherapy, with a better safety profile than the comparator treatments. Dual antibody blockade of HER2 with pertuzumab, trastuzumab plus docetaxel showed increased progression-free and overall survival with the addition of pertuzumab to trastuzumab plus docetaxel. Neoadjuvant treatment with trastuzumab plus pertuzumab (Neosphere trial) or trastuzumab plus lapatinib (NeoALTO trial) in conjunction with taxane-based therapy resulted in better clinical activity than with single-agent HER2-directed therapy. The studies described here show enhanced antitumor activity with T-DM1 plus pertuzumab in preclinical models, with an encouraging safety profile and preliminary evidence of efficacy in a phase Ib study. Moreover, our data show suppression of T-DM1 activity upon ligand activation of HER2/HER3, supporting the rationale for combining pertuzumab with T-DM1.
pertuzumab; trastuzumab and anti-gD isotype-matched antibody were positive and negative controls, respectively.

**Western blot analysis**

For measuring PARP cleavage, MDA-175 cells were seeded and allowed to adhere for 48 hours. T-DM1 (10 μg/mL), pertuzumab (100 μg/mL), or the combination was added and cells were incubated for 48 or 72 hours. For experiments with NRG-1β, SK-BR-3, Calu-3, KPL-4, and MDA-175 were plated and allowed to adhere overnight. Cells were then starved in medium with 0.1% FBS for 24 hours. NRG-1β (1 nmol/L) was added to all cells, except MDA-175, after a 1-hour pretreatment with pertuzumab or N297A-pertuzumab, T-DM1, or the combination. After 15 minutes or 24 hours, cells were lysed in RIPA buffer and Western blotting procedures were performed as described previously (16).

**In vivo efficacy studies**

MDA-175 breast cancer xenograft studies were performed as described previously (21). KPL-4 breast tumor cells were inoculated (3 × 10⁶ per mouse) into mammary fat pads and Calu-3 lung tumor cells were inoculated subcutaneously (5 × 10⁶ per mouse) into flanks of female CB-17 severe combined immunodeficient (SCID) Beige mice (Charles Rivers Laboratories). Animals were randomly assigned into groups with mean tumor volumes of 300 mm³. Animals received a single intravenous injection of T-DM1 (10 mg/kg for MDA-175; 0.3, 1, or 3 mg/kg for KPL-4; and 1, 3, or 7 mg/kg for Calu-3) on study day 0. Mice bearing MDA-175 tumors were administered a single intraperitoneal dose of pertuzumab (3 mg/kg); for studies with KPL-4 or Calu-3 tumors, mice were first injected intraperitoneally with a 2× loading dose of pertuzumab, and received two additional doses once per week. Pertuzumab doses were 30 mg/kg load/15 mg/kg weekly for KPL-4 and 50 mg/kg/load/25 mg/kg weekly for Calu-3. Treatment groups consisted of 8 to 10 animals per group. ANOVA and Tukey–Kramer HSD (honest significant difference) analysis were used for calculation of means, SEs and statistical comparisons of time-to-tumor volume doubling (time-to-progression, TTP). Analyses were performed using JMP Software, version 6.0 (SAS Institute).

**Clinical trial design**

A single-arm, phase Ib/II study (TDM4373g) was designed to investigate the safety and efficacy of T-DM1 combined with pertuzumab in patients with HER2-positive locally advanced or mBC who had been previously treated with a HER2-directed therapy. The study was reviewed and approved by the institutional review board at each site, according to local clinical guidelines. All patients provided informed consent. The phase Ib results are reported herein.

**Study design.** In the 3 + 3 design, patients received pertuzumab (840 mg cycle 1; 420 mg cycle 2, and beyond) with T-DM1 [3.0 mg/kg in Cohort 1 and, in the absence of dose-limiting toxicity (DLT), 3.6 mg/kg in Cohort 2]. Both agents were administered by intravenous infusion. Once dose escalation was complete, additional patients were enrolled into an expansion phase (phase II). Patient inclusion criteria and study assessment guidelines are in Supplementary Methods.

**Results**

**Combination effects of T-DM1 and pertuzumab in cell culture and xenograft models**

**T-DM1 combined with pertuzumab in MDA-175, a model of autocrine heregulin growth.** Growth of MDA-175 breast cancer cells is dependent on autocrine production of γ-heregulin (36) and provides a unique model for studying pertuzumab activity. In addition, MDA-175 cells slightly overexpress HER2 (1 expression) and thus are moderately sensitive to trastuzumab (39) and T-DM1. The combination of pertuzumab with T-DM1 showed enhanced antiproliferative activity compared with either agent alone (Fig. 1A, top left). CI values were determined from these studies. CI values less than 1 denote a synergistic drug interaction, CI values more than 1 denote antagonism, and CI values approximately 1 denote additivity. In MDA-175 cells, T-DM1 combined with pertuzumab resulted in synergy at all drug concentrations tested (CI range, 0.03–0.75). The average CI value for the entire drug-effect range was 0.19 ± 0.05, with the CI values at or near the IC₅₀, markedly less than 1, as depicted in Supplementary Fig. S1A (CI vs. drug effect). The average CI values for five different experiments ranged from 0.10 to 0.50, indicating synergy across multiple studies. No difference was observed between pertuzumab and N297A-pertuzumab on MDA-175 cell proliferation, alone or combined with T-DM1.

Additional studies were performed to determine if T-DM1 plus pertuzumab induced enhanced apoptosis in MDA-175 breast cancer cells. Treatment with pertuzumab resulted in enhanced caspase-3/7 activity as early as 24 hours after treatment (Fig. 1A, top right), whereas T-DM1–induced apoptosis was observed only after prolonged (72 hour) treatment (Fig. 1A, bottom). Maximal caspase activation was observed with the combination of T-DM1 and pertuzumab. Similar to the results shown for caspase activation, maximal PARP cleavage, as indicated by the appearance of the 23 kDa cleavage fragment, was demonstrated in the T-DM1–pertuzumab combination group, compared with single-agent treatment, and was observed at both 48 and 72 hours of treatment (Fig. 1A, bottom right).

The combination of T-DM1 with pertuzumab was tested in vivo in the MDA-175 xenograft model. Treatments were single intravenous injections of 3 mg/kg pertuzumab, 10 mg/kg T-DM1, or the combination (Fig. 1B). Log-rank P values were obtained for differences in time-to-tumor doubling (Supplementary Fig. S2). Single-agent treatment with T-DM1 or pertuzumab resulted in moderate tumor growth inhibition that was statistically different from vehicle-treated controls (P = 0.0247 for pertuzumab vs. control; P = 0.0017 for T-DM1 vs. control). In contrast, tumors in mice treated with T-DM1 combined with pertuzumab regressed for 3 to 4 weeks, with only modest tumor regrowth occurring after...
40 to 50 days. The difference in time-to-tumor doubling for T-DM1 plus pertuzumab compared with single-agent treatment groups or vehicle-treated controls was significant (P < 0.0001 for combination vs. vehicle or pertuzumab; P = 0.0003 for combination vs. T-DM1; Supplementary Fig. S2). Thus, T-DM1 combined with pertuzumab induced apoptotic cell death in the MDA-175 breast cancer line and resulted in significant tumor growth inhibition/regression compared with T-DM1 or pertuzumab alone.

Antitumor mechanisms for combination treatment efficacy, in addition to DM1-mediated cell death, include ADCC and different antisignaling properties of the two antibodies. We therefore investigated the contribution of these different mechanisms to combination activity. The N297A Fc mutation, which prevents binding between the antibody Fc portion and Fc receptors on immune effector cells, was introduced into pertuzumab for ADCC and signal transduction studies. ADCC assays performed in vitro with pertuzumab, N297A-pertuzumab, T-DM1, or the combination of T-DM1 with either wild-type or mutant pertuzumab showed minimal tumor cell lysis (Fig. 1C): 12.8% lysis with control antibody versus 14.7% for pertuzumab, 15.4% for T-DM1, and 18.6% for T-DM1 + pertuzumab. Percentage lysis with the N297A-mutant pertuzumab (10.6%) was similar to the negative control, whereas cell lysis after treatment with T-DM1 + N297A-pertuzumab (17.0%) was similar to T-DM1 + wild-type, ADCC-competent pertuzumab (18.9%).

Constitutive pathway activation from autocrine heregulin production in MDA-175 cells was demonstrated by high basal levels of phospho-HER3 (p-HER3) and phospho-AKT (p-AKT). Both pertuzumab and N297A-pertuzumab suppressed p-HER3 and p-AKT to a similar degree, indicating that the N297A Fc mutation did not interfere with HER2 binding and subsequent inhibition of downstream signaling pathways. T-DM1 treatment resulted in modest inhibition of p-HER3 and p-AKT. Importantly, T-DM1 combined with either wild-type or N297A-pertuzumab markedly suppressed p-HER3 and p-AKT (Fig. 1D). From these studies, we conclude that antitumor activity of combination treatment in vivo is likely due to enhanced antisignaling activities, not increased ADCC.

T-DM1 combined with pertuzumab in HER2-amplified lung and breast cancer cells. Additional studies were performed in tumor cells with 3+ HER2 expression. NRG-1β was included in these experiments as these cells do not express endogenous ligand. In Calu-3, BT-474, and SK-BR-3 cells, T-DM1 was more active than pertuzumab, whereas the combination was more potent than single-agent treatment (Fig. 2A–C, left). The average CI value for the entire drug effect range was 0.24 ± 0.06 for Calu-3 and 0.37 ± 0.17 for BT-474, indicating synergy. (CI values were not determined in SK-BR-3 cells because of minimal pertuzumab activity under these assay conditions). Graphical representations of CI versus fractional effect are in Supplementary Fig. S1B and C. Calu-3 and BT-474 cell growth was stimulated by NRG-1β, whereas SK-BR-3 growth was unaffected under these experimental conditions. Thus, the enhancement by pertuzumab of T-DM1 activity in the presence of NRG-1β was independent of NRG-1β mitogenic activity. In contrast, no combination effect was observed in cells treated in the absence of NRG-1β; i.e., pertuzumab did not enhance the growth-inhibitory effect of T-DM1 under ligand-independent conditions (Fig. 2A–C, right). In KPL-4 breast cancer cells, T-DM1 cytotoxicity was not increased by pertuzumab in ligand-dependent (Fig. 2D, left) or ligand-independent (Fig. 2D, right) conditions.

Inhibition of T-DM1 cytotoxicity by NRG-1β

In the earlier experiments, it was noted that the cytotoxic response to T-DM1 was reduced in the presence of NRG-1β. We therefore investigated NRG-1β inhibition of T-DM1–induced cytotoxicity on a larger panel of HER2-amplified breast cancer cell lines using two experimental formats, comparing a fixed concentration of NRG-1β (2 nmol/L) versus a dose range of T-DM1 and the converse, NRG-1β dose response with a fixed concentration of T-DM1. In BT-474, SK-BR-3, ZR-75-30 (Fig. 3A, left), and UACC812 (data not shown) breast cancer lines, 2 nmol/L NRG-1β suppressed T-DM1 activity. Concentrations as low as 0.01 to 0.2 nmol/L NRG-1β reduced the antiproliferative activity of T-DM1 in all four cell lines (Fig. 3A, right). In contrast, NRG-1β did not block T-DM1 activity in KPL-4 and HCC1954 breast cancer cells (Fig. 3B). Interestingly, these two breast cancer lines harbor activating PIK3CA mutations (H1047R for both; ref. 40), whereas the four cell lines that displayed reduced T-DM1 activity in the presence of NRG-1β do not (40). Finally, the addition of NRG-1β also inhibited T-DM1–induced apoptosis in BT-474 and ZR-75-30 cells, as indicated by reduced caspase activity. Blockade of NRG-1β signaling by the addition of pertuzumab fully restored the apoptotic response to T-DM1 (Fig. 3C and Supplementary Fig. S3, right). Similar to the observations in proliferation assays, T-DM1–induced apoptosis in KPL-4 and HCC1954 cells was not reduced by NRG-1β (Fig. 3C and Supplementary Fig. S3, right). These results provide additional evidence for the rationale of blocking ligand-induced receptor activation with pertuzumab for optimal T-DM1 response in a subset of HER2-amplified cancer cells.

NRG-1β inhibits activity of antimitotic agents

The DM1 component of T-DM1 is a potent antimitotic agent (41). Additional studies were performed to determine if the protective effect of NRG-1β applied to free DM1 and to antimitotic agents used for breast cancer treatment. Similar to the findings with T-DM1, NRG-1β reduced the cytotoxic effects of unconjugated DM1 as well as docetaxel and vinorelbine in BT-474 cells (Supplementary Fig. S4, left). Similar results were obtained with SK-BR-3 (data not shown). In contrast, NRG-1β did not reduce the cytotoxic effects of these agents in KPL-4 cells (Supplementary Fig. S4, right). Overall, these data show NRG-1β inhibition of the cytotoxic activity of T-DM1, docetaxel, and vinorelbine, further validating the importance of blocking NRG-1β activity with pertuzumab.
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Enhanced Antitumor Activity with Trastuzumab Emtansine plus Pertuzumab

Effects of T-DM1, pertuzumab, or the combination on NRG-1β–activated HER2–HER3 signaling

NRG-1β activation of HER2–HER3 signal transduction was investigated in SK-BR-3 and KPL-4 cells to probe the differential effects we observed of NRG-1β on T-DM1 activity. As expected, NRG-1β–stimulated HER3 and AKT phosphorylation was inhibited by pertuzumab, but not T-DM1, in SK-BR-3 cells (Fig. 4, top; ref. 42); this effect was more pronounced after prolonged (24 hours) incubation. Although treatment with T-DM1 alone for 24 hours resulted in reduced p-HER3 and p-AKT, addition of pertuzumab to T-DM1 was required to inhibit AKT activation in the presence of NRG-1β.

In KPL-4 cells, NRG-1β transiently activated HER3, but not AKT, (Fig. 4, bottom), likely due to mutational activation of the PI3K pathway. Interestingly, T-DM1 treatment for 24 hours suppressed both HER3 and AKT phosphorylation, with inhibition of p-AKT sustained even in the presence of NRG-1β. All treatment groups containing T-DM1 (T-DM1 alone, T-DM1 + NRG-1β, T-DM1 + pertuzumab, and T-DM1 + pertuzumab + NRG-1β) showed similar inhibition of AKT phosphorylation, indicating that T-DM1 is sufficient to suppress AKT activation (Fig. 4, right). Because KPL-4 are insensitive to trastuzumab in vitro (42), this effect is mediated by the DM1 component of T-DM1 (unpublished data). Thus, in NRG-1β–sensitive cells such as SK-BR-3, blockade of HER2–HER3 signaling by pertuzumab is essential to achieve the full inhibitory effect of T-DM1 under ligand-dependent conditions, whereas in cells such as KPL-4, which are not stimulated downstream by NRG-1β and therefore not responsive in vitro to pertuzumab, T-DM1 alone potently suppresses HER2–HER3 signaling. We speculate that a contributing factor is hyperactivation of the PI3K pathway in KPL-4 cells due to an activating PIK3CA mutation, versus SK-BR-3 cells that are PIK3CA wild-type. The mechanisms underlying these differing activities are currently under investigation.

T-DM1–pertuzumab combination studies in vivo

To further support our observation that T-DM1 combined with pertuzumab leads to enhanced antitumor effects, we performed in vivo combination studies in two HER2-amplified tumor xenograft models. In the Calu-3 lung cancer model, 25 mg/kg pertuzumab was administered once weekly for 3 weeks; T-DM1 was administered (1, 3, or 7 mg/kg) as a single intravenous injection at the start of the study. Pertuzumab treatment resulted in tumor growth delay (Fig. 5A, right). Only the highest dose of T-DM1 (7 mg/kg) resulted in significant tumor growth inhibition. Enhanced antitumor activity, compared with single-agent treatment, was observed with pertuzumab plus 3 or 7 mg/kg T-DM1. Equivalent efficacy was observed with 7 mg/kg T-DM1 compared with pertuzumab plus 3 mg/kg T-DM1. Pertuzumab with 7 mg/kg T-DM1 was the most active combination tested. Tumors regressed during and after treatment, with no regrowth until several weeks after treatment was stopped. Although more sustained tumor growth inhibition was reported in Calu-3 xenografts treated with pertuzumab and trastuzumab (23), this was likely due to weekly antibody administration for the study duration (45 days). In our studies, limited dosing allows for monitoring of tumor regrowth and calculation of time-to-tumor volume doubling (TTP). Log-rank tests for differences in TTP showed statistically significant differences between single-agent treatment for pertuzumab or T-DM1 versus the corresponding combination group (log-rank P value for pertuzumab vs. pertuzumab + 3 mg/kg T-DM1 = 0.008; log-rank P value < 0.0001 for pertuzumab vs. pertuzumab + 7 mg/kg T-DM1, and for 1 or 3 mg/kg T-DM1 + pertuzumab vs. T-DM1 alone; log-rank P value = 0.0004 for 7 mg/kg T-DM1 vs. pertuzumab + 7 mg/kg T-DM1; Supplementary Fig. S5A).

In KPL-4 breast tumor xenografts, T-DM1 (0.3, 1, 3 mg/kg) or pertuzumab (30 mg/kg loading dose; 15 mg/kg weekly) caused significant tumor growth inhibition compared with vehicle-treated animals (log-rank P value = 0.0204 for 0.3 mg/kg T-DM1 vs. vehicle group; log-rank P value < 0.0001 for all other single-agent treatment groups vs. vehicle control group; Fig. 5B, right; Supplementary Fig. S5B). Administration of pertuzumab with each dose of T-DM1 resulted in pronounced inhibition of tumor growth, reaching statistical significance for each combination group compared with the corresponding single-agent treatment groups (Supplementary Fig. S5B). Sustained tumor regressions were observed in mice treated with 3 mg/kg T-DM1 and with the combination of pertuzumab plus 1 mg/kg T-DM1. After approximately 40 days, tumors in these two groups started to regrow. However, treatment of mice with pertuzumab plus 3 mg/kg T-DM1 resulted in lasting tumor regression for the duration of the study (88 days).

In addition to direct antisignaling activities mediated by T-DM1 and pertuzumab, enhanced ADCC may also...
contribute to combination antitumor efficacy. We therefore performed in vitro ADCC assays comparing T-DM1, pertuzumab, or N-297A-pertuzumab with the corresponding combinations. Trastuzumab was included as a positive control and an isotype-matched antibody as the negative control.

Both T-DM1 and pertuzumab induced immune effector cell-mediated lysis in a dose-dependent manner in Calu-3 and KPL-4 cells similar to trastuzumab (Fig. 5A and B, left). However, T-DM1 combined with pertuzumab or N297A-pertuzumab did not result in greater ADCC activity compared with T-DM1 or wild-type pertuzumab alone. As expected, N297A-pertuzumab and isotype-matched control antibody

Figure 3. The effect of NRG-1β on T-DM1 activity in five HER2-amplified breast cancer lines. A, BT-474, SK-BR-3, and ZR-75-30; inhibitory effect of NRG-1β (2 nmol/L) on a dose titration of T-DM1 (left) with almost complete inhibition of T-DM1 activity. The right panels show a dose-dependent reduction of T-DM1 activity by NRG-1β; the fixed dose of T-DM1 selected for each cell line was the lowest dose giving maximum growth inhibition (1 μg/mL for BT-474 and ZR-75-30; 0.1 μg/mL for SK-BR-3). B, two HER2-amplified breast cancer lines, KPL-4 and HCC1954, are equally sensitive to T-DM1 in the absence or presence of NRG-1β. All treatments were for 3 days.

C, the effect of NRG-1β on T-DM1-induced apoptosis, measured by caspase-3/7 activation, was assessed after a 2-day treatment. NRG-1β completely blocked T-DM1 apoptotic activity in BT-474 cells; this effect was reversed by the addition of pertuzumab. T-DM1-induced apoptosis in KPL-4 cells was unaffected by the addition of NRG-1β.

Figure 2. Influence of NRG-1β on combination activity of T-DM1 plus pertuzumab. Enhanced antiproliferative activity with combination treatment was observed in Calu-3 lung (A) and in BT-474 (B) and SK-BR-3 (C) breast cancer cells only in the presence of NRG-1β, indicating the importance of pertuzumab inhibition of NRG-1β signaling. There was no T-DM1–pertuzumab combination effect in KPL-4 cells (D) despite NRG-1β addition to the culture medium. Cell proliferation in the absence of NRG-1β is denoted by the closed black triangle on the y-axis. Assays were conducted for 5 days for Calu-3 and BT-474, 3 days for SK-BR-3 and KPL-4 cells.
showed no activity. These findings indicate that combination activity in vivo is likely due more to the antisignaling activities of both T-DM1 and pertuzumab and warrant further investigation, in models such as KPL-4, of the role of stromal ligands in the response to pertuzumab in vivo.

Phase Ib study of T-DM1 combined with pertuzumab
A global, single-arm, phase Ib/II study (TDM4373g) was conducted to investigate the safety and efficacy of T-DM1 plus pertuzumab in patients with HER2-positive locally advanced or mBC who had received prior systemic therapy for recurrent locally advanced or metastatic disease and in patients with newly diagnosed or previously untreated mBC (first-line). The primary objectives of this study included characterization of the safety and tolerability of this drug combination and to assess objective response rate (ORR) based on investigator assessment. In the 3+3 study design (Supplementary Fig. S6), patients received the standard pertuzumab dose every 3 weeks (840 mg, cycle 1; 420 mg, cycle 2, and beyond) and T-DM1 at a dose of 3.0 mg/kg every 3 week (Cohort 1). In the absence of DLT, patients then received pertuzumab with 3.6 mg/kg T-DM1 (Cohort 2). In the event of DLTs, the T-DM1 dose was reduced to 2.4 mg/kg (from 3.0 mg/kg, Cohort 1) or to 3.0 mg/kg (from 3.6 mg/kg, Cohort 2). Once dose escalation was completed, patients could then be added to the expansion phase (the phase II part of this study, results to be published in a separate article). Only the phase Ib results are reported here.

In the phase Ib portion of the study, 3 patients received 3.0 mg/kg T-DM1 with the rest receiving the standard 3.6 mg/kg dose, for a total of 9 patients. Patient demographics and baseline characteristics are shown in Supplementary Table S1. The majority (8/9) of patients were White and had Eastern Cooperative Oncology Group (ECOG) performance status of 1 (6/9). Patients had tumors that were either 3+ for HER2 expression by immunohistochemistry (6/9) or positive by FISH (8/9). The median number of previous systemic therapies was eight. All patients had received prior trastuzumab and lapatinib therapy, as well as a taxane and capecitabine (Supplementary Table S2). Most patients (7/9) had also received prior anthracycline therapy.

During dose escalation, no patient treated with 3.0 mg/kg T-DM1 combined with pertuzumab developed a DLT. However, among the first 3 patients treated with 3.6 mg/kg T-DM1, 1 patient developed a DLT (grade 4 thrombocytopenia), and the cohort was expanded to include 3 more patients, none of whom developed a DLT. Therefore, 3.6 mg/kg T-DM1 in combination with standard dose pertuzumab, administered every 3 weeks, was declared the recommended phase II schedule. Table 1 shows adverse events ≥ grade 3 for the 9 patients. The most common ≥ grade 3 adverse effect was fatigue (3/9), with thrombocytopenia and lung complications each occurring in 1 out of 9 patients. All 9 patients experienced at least one adverse event of any grade; these are summarized in Supplementary Table S3 and included gastrointestinal, respiratory, musculoskeletal, and skin disorders, as well as more general disorders such as fatigue, fever, and chills.
About objective response in Cohort 1 (n = 3 patients who received 3.0 mg/kg T-DM1), 2 patients showed a partial response and 1 patient had stable disease. In Cohort 2 (n = 6 patients receiving 3.6 mg/kg T-DM1), 2 patients had a partial response, 3 patients had stable disease, and 1 patient had disease progression as best response. Therefore, for the full phase Ib cohort, the ORR was 44.4% (4/9; Table 2). Disease stability was maintained for at least 6 months for 2 of the 3 patients who had stable disease as best response.

Discussion

With the approval by the FDA in 1998, trastuzumab became widely integrated into standard treatment regimens for HER2-positive mBC (14). Less than a decade later, favorable results were obtained with trastuzumab plus chemotherapy in early breast cancer (43). More recently, trastuzumab in combination with cisplatin and a fluoropyrimidine for treatment of HER2-positive gastric/gastroesophageal cancer was shown to extend survival in these patients (44). Despite advances in the treatment of HER2-positive cancers, a subset of patients will show disease progression through treatment. The antibody–drug conjugate T-DM1 has been tested in preclinical models and in multiple clinical trials as an additional method for targeting HER2-positive cancer. T-DM1 provides intracellular delivery of the cytotoxic agent DM1, resulting in tumor cell death (16), along with the important therapeutic properties of unconjugated trastuzumab (38). Recently, T-DM1 (Kadcyla) was approved for treatment of HER2-positive mBC in patients who had received prior Herceptin and a taxane. Effective targeting of HER2 is also achieved with pertuzumab, a HER2-specific antibody that recognizes an epitope on the HER2 extracellular domain distinct from...
trastuzumab. Pertuzumab was approved by the FDA in 2012 on the basis of the findings of CLEOPATRA, a phase III trial that demonstrated significantly improved progression-free and overall survival with the addition of pertuzumab to trastuzumab plus docetaxel in previously untreated HER2-positive mBC patients (34, 45).

We therefore extended these studies by investigating the efficacy of pertuzumab combined with T-DM1 in preclinical models of HER2-amplified cancers and in an early-phase clinical trial. The combination of pertuzumab and T-DM1 resulted in a synergistic drug effect in multiple tumor cell lines in vitro and was more efficacious than treatment with individual agents in tumor xenograft models. In vitro, NRG-1β suppressed the cytotoxic effects of T-DM1 in a subset of cell lines; the addition of pertuzumab was necessary to overcome this inhibitory effect. As ADCC activity was not increased in vitro compared to treatment with T-DM1 or pertuzumab alone, enhanced antitumor activity is likely due to the multiple and diverse antisignaling properties of pertuzumab and T-DM1, as well as the cytotoxic activity of the DM1 component of T-DM1. Because NRG-1β–mediated resistance was not observed in all cell lines tested, further studies are in progress to investigate the effect of additional growth factors/cytokines on the response not only to T-DM1 but other chemotherapeutic agents as well.

Importantly, these findings add to the emerging body of data describing autocrine or stromal-derived growth factor-mediated resistance to targeted therapies. Several recent reports show that the c-Met receptor ligand HGF (hepatocyte growth factor), as well as fibroblast growth factors (FGF), attenuate the anticaner activity of inhibitors targeting EGFR, B-Raf, and FGF receptors (7–9, 46). NRG-1β was also reported to rescue cells from the inhibitory effects of HER2 and EGFR kinase inhibitors (9, 47). Moreover, the NRG-1β receptor, HER3, mediates inhibitor resistance; upregulation and activation of HER3 was demonstrated in diverse tumor cell types in vitro in response to EGFR, HER2, and PI3K kinase inhibitors (11, 12, 48, 49). Our data showing NRG-1β–mediated resistance to the microtubule-targeted cytotoxic agents T-DM1, docetaxel, and vinorelbine thus expand these observations beyond kinase inhibitors to cytotoxic agents.

Clinical activity of T-DM1 is being assessed in numerous phase II and III trials. Phase II single-arm trials of T-DM1 in patients with HER2-positive mBC who had received prior trastuzumab and/or lapatinib in addition to chemotherapy showed that, in these heavily pretreated patients, the ORR ranged from 25% to 35% (17, 18). Given the small sample size and the specifics of the patient population in the study reported here, it is difficult to compare the response rates for T-DM1 with pertuzumab with response rates for T-DM1 alone. An ongoing phase III, randomized, 3-arm study, MARIANNE (TDM4788g/BO22589), will directly address this question by evaluating the safety and efficacy of T-DM1 with pertuzumab or T-DM1 plus pertuzumab placebo versus trastuzumab plus taxane (docetaxel or paclitaxel) in patients with HER2-positive progressive or recurrent locally advanced or previously untreated mBC. The phase Ib data reported here demonstrate that the combination of T-DM1 with pertuzumab is feasible at full doses with no unexpected safety signals; the majority of adverse events were grade 1 and 2 and were similar to those reported for single-agent T-DM1 (fatigue, thrombocytopenia, and increased serum transaminases; refs. 17–19). Data from the phase II expansion portion of this study will be reported separately.

Results from the EMILIA phase III trial showed that patients treated with T-DM1 had significantly longer progression-free and overall survival with a lower frequency of severe adverse events compared with the control arm (lapatinib plus capecitabine, a standard of care for second-line

| Table 1. National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) grade ≥ 3 adverse events in the phase Ib portion of TDM4373g |
|-----------------|-----------------|
| **Adverse event** | **Number of patients (n = 9)** |
| Fatigue | 3 |
| Thrombocytopenia | 1 |
| Pneumonia | 1 |
| Pleural effusion | 1 |
| Deafness* | 1 |

*Patient had baseline grade 3 hearing loss that worsened but did not increase in grade.

| Table 2. Best clinical response, by cohort, in the phase Ib portion of TDM 4373g |
|-----------------|-----------------|-----------------|
|                  | Cohort 1 (n = 3) | Cohort 2 (n = 6) | Total (n = 9) |
| Complete response | 0               | 0               | 0               |
| Partial response  | 2 (66.7%)       | 2 (33.3%)       | 4 (44.4%)       |
| Stable disease    | 1 (33.3%)       | 3 (50.0%)       | 4 (44.4%)       |
| Progressive disease | 0               | 1 (16.7%)       | 1 (11.1%)       |

NOTE: Pertuzumab dose: 840 mg first cycle, then 420 mg thereafter; all agents administered once every 3 weeks. Cohort 1, 3.0 mg/kg T-DM1 plus pertuzumab; Cohort 2, 3.6 mg/kg T-DM1 plus pertuzumab.
treatment of HER2-positive mBC; ref. 19). Similarly, data from a randomized phase II study, TDM4450g, demonstrated that T-DM1 significantly prolonged progression-free survival with a more favorable safety profile compared to patients treated with trastuzumab plus docetaxel in the first-line setting (50). Overall, these results, together with the CLEOPATRA data, show significantly improved clinical benefit of T-DM1 or trastuzumab/pertuzumab/docetaxel compared with the trastuzumab/docetaxel regimen. From these data, we anticipate the MARIANNE (TDM4788g/BO22589) trial to demonstrate better clinical outcome in the T-DM1 plus pertuzumab arm, further supporting the rationale for using multiple HER2-targeted agents in the treatment of HER2-positive breast cancer.

Disclosure of Potential Conflicts of Interest
K. Miller received commercial research grant from Genentech/Roche and Merrimack. K.S. Albain is consultant/advisory board member of Genentech and Roche. N. Harbeck and V. Dieras have honoraria and are consultant/advisory board members of Roche. L. Fang has ownership interest (including patents) in stock options. S.R. Olsen and M.X. Sliwkowski have ownership interest (including patents) in Roche. No potential conflicts of interest were disclosed by the other authors.

References
3. Graus-Porta D, Beerr RL, 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.

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