Dual targeting of HER2-positive cancer with trastuzumab-emtansine (T-DM1) and pertuzumab: critical role for neuregulin blockade in anti-tumor response to combination therapy

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Running title: Enhanced anti-tumor activity with trastuzumab emtansine (T-DM1) plus pertuzumab

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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, 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 anti-tumor efficacy with T-DM1 and pertuzumab resulting from the unique anti-tumor activities of each agent. In patients with metastatic breast cancer 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 anti-tumor activity. In patients, this combination showed an encouraging safety and tolerability profile with preliminary evidence of efficacy.
Translational relevance

The antibody-drug conjugate ado-trastuzumab emtansine (T-DM1, Kadcyla®) increases progression-free and overall survival vs. lapatinib plus capecitabine in patients with HER2-positive metastatic breast cancer 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 (NeoALTTO 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 anti-tumor 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.

Introduction

The ErbB/HER family of receptor tyrosine kinases (RTK) plays critical roles in development and cancer (1). These receptors include epidermal growth factor receptor (EGFR)/ErbB1, HER2/ErbB2, HER3/ ErbB3 and HER4/ErbB4. Numerous ligands have been identified which interact with all HER family receptors, with the exception of HER2. Ligand binding activates multiple downstream signal transduction pathways. HER2 is the common co-receptor 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 phosphatidylinositol 3-kinase (PI3-K) 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 PI3-K.
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 treatment of HER2-positive breast cancer. Proposed mechanisms of trastuzumab action include inhibition of ECD shedding, disruption of downstream signal transduction pathways, induction of cell cycle arrest, inhibition of DNA repair, decreased angiogenesis, and mediation of antibody-dependent cellular cytotoxicity (ADCC) (13). Clinical benefit from trastuzumab-containing therapy, however, can be limited, especially in the metastatic setting, necessitating development of alternate forms of treatment (14, 15). We developed a cytotoxic drug-conjugate of trastuzumab by covalently linking the anti-mitotic agent DM1 to trastuzumab through a stable MCC linker. Trastuzumab emtansine (T-DM1) potently inhibits growth of trastuzumab-sensitive and -insensitive HER2-amplified cancer cells and shows a favorable safety profile in preclinical toxicity studies (16). T-DM1 activity has been evaluated in Phase II and III clinical trials in patients with HER2-positive locally advanced or metastatic breast cancer (mBC, ref. 17, 18). T-DM1 (Kadcyla®) was recently approved for treatment of patients with HER2-positive metastatic breast cancer who have received prior treatment with Herceptin® and a taxane chemotherapy, based on data showing significantly prolonged progression-free and overall survival and less toxicity compared to standard-of-care treatment, lapatinib (Tykerb®) plus capecitabine (Xeloda®) (19).

In addition to trastuzumab, pertuzumab is a HER2-specific humanized antibody with efficacy demonstrated in mouse xenograft models of breast, lung, prostate and ovarian cancer (20-23). Pertuzumab binds domain II of the HER2 ECD, preventing ligand-dependent association of HER2 with other HER family members (24) and demonstrated clinical activity as a single agent and in combination with chemotherapy in ovarian, non-small cell lung and prostate cancer (25-29). Pertuzumab combined with trastuzumab has anti-tumor activity in cell culture (30) and animal models of HER2-amplified cancer (23, 31), and in
patients with HER2-positive metastatic breast cancer (32, 33). Combining pertuzumab, trastuzumab, and docetaxel significantly improves the pathological complete response rate in the neoadjuvant setting compared to treatment without pertuzumab (33). In June 2012, the US FDA approved Pertuzumab (Perjeta®) in combination with Herceptin® and chemotherapy for treatment of patients with HER2-positive mBC who have not received prior anti-HER2 therapy or chemotherapy for metastatic disease (34). We therefore explored the potential of combining T-DM1 with pertuzumab in models of HER2-amplified breast and lung cancer. Our data underscore the feasibility of using multiple strategies to target HER2-positive cancer.

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; 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 J. Kurebayashi (35). Cells were maintained as previously described (16). Trastuzumab emtansine (T-DM1), pertuzumab, N297A-pertuzumab (38), 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 (16). T-DM1, pertuzumab or the combination was added to cells; treatments were in duplicate in order 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; studies with NRG-1β (2 nM) 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 3 times. Combination Index (C.I.) values, as described by Chou and Talalay (36), were generated using CalcuSyn software (Biosoft, Inc). ADCC assays were performed as described (37) using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega Corp.). Treatments were T-DM1 alone or combined with pertuzumab or the Fc mutant N297A-pertuzumab; trastuzumab and anti-gD isotype-matched antibody were positive and negative controls, respectively.

Western analysis

For measuring PARP (poly (ADP-ribose) polymerase) cleavage, MDA-MB-175-VII cells were seeded and allowed to adhere for 48 h. T-DM1 (10 μg/mL), pertuzumab (100 μg/mL) or the combination was added and cells incubated for
48 or 72 hours. For experiments with NRG-1β, SK-BR-3, Calu-3, KPL-4, and MDA-MB-175-VII were plated and allowed to adhere overnight. Cells were then starved in medium with 0.1% FBS for 24 h. NRG-1β (1 nM) was added to all cells, except MDA-MB-175-VII, after a 1 hour pre-treatment 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 performed as described previously (16).

**In vivo efficacy studies**

MDA-MB-175-VII breast cancer xenograft studies were performed as described previously (21). KPL-4 breast tumor cells were inoculated (3 x 10^6 per mouse) into mammary fat pads and Calu-3 lung tumor cells were inoculated subcutaneously (5 x 10^6 per mouse) into flanks of female CB-17 SCID Beige mice (Charles Rivers Laboratories). Animals were randomly assigned into groups with mean tumor volumes of 300 mm^3. Animals received a single intravenous injection of T-DM1 (10 mg/kg for MDA-MB-175-VII; 0.3, 1, or 3 mg/kg for KPL-4; 1, 3, or 7 mg/kg for Calu-3) on study day 0. Mice bearing MDA-MB-175-VII 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 ip with a 2X loading dose of pertuzumab, and received 2 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 8-10 animals per group. A one-way analysis of variance (ANOVA) and Tukey-Kramer HSD analysis were used for calculation of means, standard errors 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 metastatic breast cancer 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 Supplemental Methods.

**Results**

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

*T-DM1 combined with pertuzumab in MDA-MB-175-VII, a model of autocrine heregulin growth.*

Growth of MDA-MB-175-VII breast cancer cells (hereafter MDA-175) is dependent on autocrine production of gamma (γ)-heregulin (38) and provides a unique model for studying pertuzumab activity. Additionally, MDA-175 cells slightly over-express HER2 (1+ expression) and thus are moderately sensitive to trastuzumab (39) and T-DM1. The combination of pertuzumab with T-DM1 showed enhanced anti-proliferative activity compared to either agent alone (Fig. 1A, upper left). Combination index (C.I.) values were determined from these studies. C.I. values < 1 denote a synergistic drug interaction, C.I. values > 1 denote antagonism, and C.I. values approximately 1 denote additivity. In MDA-175 cells, T-DM1 combined with pertuzumab resulted in synergy at all drug concentrations tested (C.I. range: 0.03-0.75). The average C.I. value for the entire drug effect range was 0.19 +/- 0.05, with the C.I. values at or near the IC_{50} markedly less than 1, as depicted in Supplemental Fig. 1A (C.I. vs. drug effect).
The average C.I. values for 5 different experiments ranged from 0.10 – 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 hr after treatment (Fig. 1A, upper right), whereas T-DM1-induced apoptosis was observed only after prolonged (72 h) treatment (Fig. 1A lower panels). 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 to single agent treatment, and was observed at both 48 and 72 hours of treatment (Fig. 1A lower 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 (Supp. Fig. 2). 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 3-4 weeks, with only modest tumor re-growth occurring after 40-50 days. The difference in time-to-tumor doubling for T-DM1 plus pertuzumab compared to 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, Supp. Fig. 2). 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 to T-DM1 or pertuzumab alone.
Anti-tumor mechanisms for combination treatment efficacy, in addition to DM1-mediated cell death, include ADCC and different anti-signaling 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 vs. 14.7% for pertuzumab, 15.4% for T-DM1 and 18.6% for T-DM1 + pertuzumab. Percent lysis with the N297A-mutant pertuzumab (10.6%) was similar to the negative control; while 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 anti-tumor activity of combination treatment in vivo is likely due to enhanced anti-signaling 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, while the combination was more potent than single agent treatment (Fig. 2A, B, C left panels). The average C.I. 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. (C.I. values weren’t determined in SK-BR-3 cells due to minimal pertuzumab activity under these assay conditions). Graphical representations of C.I. vs. fractional effect are in Supplemental Figures 1B and C. Calu-3 and BT-474 cell growth was stimulated by NRG-1β, while 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, B, C right panels). In KPL-4 breast cancer cells, T-DM1 cytotoxicity was not increased by pertuzumab in ligand-dependent (Fig. 2D, left panel) or ligand-independent (Fig. 2D, right panel) conditions.

**Inhibition of T-DM1 cytotoxicity by NRG-1β**

In the above 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 lines using two experimental formats: comparing a fixed concentration of NRG-1β (2 nM) vs. 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 panels) and UACC812 (data not shown) breast cancer lines, 2 nM NRG-1β suppressed T-DM1 activity. Concentrations as low as 0.01-0.2 nM NRG-1β reduced the anti-proliferative activity of T-DM1 in all 4 cell lines (Fig. 3A, right panels). 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, (40), whereas the 4 cell lines which 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 Supp. Fig. 3 left panels). 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 Supp. Fig. 3 right panels). 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 anti-mitotic agents**

The DM1 component of T-DM1 is a potent anti-mitotic agent (41). Additional studies were performed to determine if the protective effect of NRG-1β applied to free DM1 and to anti-mitotic 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 (Supp. Fig. 4, left panels). 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 (Supp. Fig. 4, right panels). 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.

**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 upper panels) (42); this effect was more pronounced after prolonged (24 h) incubation. Although treatment with T-DM1 alone for 24 h 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 lower panels), likely due to mutational activation of the PI3K pathway. Interestingly, T-DM1 treatment for 24 h 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, T-DM1 + pertuzumab + NRG-1β) showed similar inhibition of AKT phosphorylation, indicating that T-DM1 is sufficient to suppress AKT activation (Fig. 4, right panels). 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, while 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 hyper-activation of the PI3K pathway in KPL-4 cells due to an activating PIK3CA mutation, vs. 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 anti-tumor 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 panel). Only the highest dose of T-DM1 (7mg/kg) resulted in significant tumor growth inhibition. Enhanced anti-tumor activity, compared to 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 re-growth 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 re-growth 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 vs. 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; see Supp. Fig. 5A).

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 to 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 panel, Supp. Fig. 5B). Administration of pertuzumab with each dose of T-DM1 resulted in pronounced inhibition of tumor growth, reaching statistical significance for each combination group compared to the corresponding single agent treatment groups (Supp. Fig. 5B). 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-DM. After approximately 40 days, tumors in these two groups started to re-grow. 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 anti-signaling activities mediated by T-DM1 and pertuzumab, enhanced ADCC may also contribute to combination anti-tumor efficacy. We therefore performed in vitro ADCC assays comparing T-DM1,
pertuzumab, or N-297A-pertuzumab to 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 panels). However, T-DM1 combined with pertuzumab or N297A-pertuzumab did not result in greater ADCC activity compared to T-DM1 or wild-type pertuzumab alone. As expected, N297A-pertuzumab and isotype-matched control antibody showed no activity. These findings indicate that combination activity in vivo is likely due more to the anti-signaling 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 metastatic breast cancer 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, (Supp. Fig. 6), 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 dose-limiting toxicity (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 manuscript). 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 Supplemental Table 1. The majority (8/9) of patients were white and had ECOG performance status of 1 (6/9). Patients had tumors that were either 3+ for HER2 expression by immunohistochemistry (IHC, 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 (see Supp. Table 2). 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, one 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 1A shows adverse events (AEs) ≥ Grade 3 for the 9 patients. The most common ≥ Grade 3 AE 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 Supplemental Table 3 and included gastrointestinal, respiratory, musculoskeletal and skin disorders, as well as more general disorders such as fatigue, fever and chills.

Regarding 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 objective response rate was 44.4% (4/9, Table 1B). 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 US FDA in 1998, trastuzumab became widely integrated into standard treatment regimens for HER2-positive metastatic breast cancer (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/gastro-esophageal 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 trastuzumab emtansine or 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 (37). 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 US FDA in 2012 based on the findings of CLEOPATRA, a Phase III trial which 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 anti-tumor activity is likely due to the multiple and diverse anti-signaling 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 (FGFs), attenuate the anti-cancer 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; up-regulation and activation of HER3 was demonstrated in diverse tumor cell types in vitro in response to EGFR, HER2 and PI3-K 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 pre-treated patients, the ORR ranged from 25-35% (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 to 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 vs. trastuzumab plus taxane (docetaxel or paclitaxel) in patients with HER2-positive progressive or recurrent locally advanced or previously untreated metastatic breast cancer. 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, increased serum transaminases) (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 to the control arm (lapatinib plus capecitabine, a standard of care for 2nd-line treatment of HER2-positive mBC) (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 to 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.
Figure legends

Figure 1. Effects of T-DM1 and pertuzumab, alone and in combination, on MDA-175 cells in vitro and in vivo. A. Growth inhibition and apoptotic effects: treatment of MDA-175 cells for 5 days with T-DM1 and pertuzumab (wild-type or N297A mutant) results in synergistic reduction of cell viability (upper left), as determined by the Chou and Talalay method. Time-course of apoptosis induction by T-DM1 and pertuzumab, as measured by caspase 3/7 activation (24 h upper right, 72 h lower left) and PARP cleavage (lower right). Cell viability and caspase assays were run at full dose-ranges; single doses used for PARP cleavage were 10 μg/mL T-DM1 and 100 μg/mL pertuzumab. B. Anti-tumor activity of T-DM1 and pertuzumab, as single agents or in combination, in MDA-175 cells implanted into mammary fat pads of beige nude XID mice. Mice bearing MDA-175 tumors were treated with a single intravenous injection of 3 mg/kg pertuzumab, 10 mg/kg T-DM1 or the combination of the two agents at the same doses (n=10/group). C. Lack of ADCC activity on MDA-175 cells in vitro: cells were incubated with trastuzumab, pertuzumab, N-297A-pertuzumab, T-DM1, T-DM1 + pertuzumab, T-DM1 + N297A-pertuzumab, or isotype control antibody in the presence of peripheral blood mononuclear cells (PBMC), 25:1 effector:target ratio. Tumor cell lysis was assessed by LDH release. D. Inhibition of HER2-HER3-PI3K signaling in MDA-175 cells (24 h treatment with 10 μg/mL pertuzumab or N-297A-pertuzumab, 10 μg/mL T-DM1 or the respective combinations.

Figure 2. Influence of NRG-1β on combination activity of T-DM1 plus pertuzumab. Enhanced anti-proliferative 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 5 days for Calu-3 and BT-474, 3 days for SK-BR-3 and KPL-4 cells.

Figure 3. The effect of NRG-1β on T-DM1 activity in 5 HER2-amplified breast cancer lines. A, BT-474, SK-BR-3 and ZR-75-30: inhibitory effect of NRG-1β (2 nM) on a dose-titration of T-DM1 (left panels) 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, 2 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 4. Effects of T-DM1 and pertuzumab, alone or in combination, on HER2-HER3-mediated signal transduction in SK-BR-3 and KPL-4 breast cancer cells. T-DM1 (0.1 μg/mL) or pertuzumab (15 μg/mL) were added 1 h prior to stimulation with 1 nM NRG-1β for 15 min or 24 h. Cell lysates were analyzed for total and phospho-HER3 and AKT. The presence of pertuzumab was required for suppression of signaling in NRG-1β-activated SK-BR-3 cells.

Figure 5. Comparison of in vitro ADCC activity (left panels) to in vivo anti-tumor efficacy (right panels) in Calu-3 lung cancer cells (A) and KPL-4 breast cancer cells (B). ADCC assays were performed as described in Figure 1C. No enhanced ADCC activity was observed with combination treatment. Mice bearing Calu-3 subcutaneous tumors were administered 25 mg/kg pertuzumab once per week for 3 weeks; T-DM1 was administered as a single intravenous
injection at doses of 1, 3, or 7 mg/kg (n=9/group). T-DM1 combined with pertuzumab was more efficacious than treatment with single agents. Similarly, \textit{in vivo} tumor growth of KPL-4 breast cancer cells is greatly reduced with T-DM1 and pertuzumab in combination. In this study, mice were treated weekly for 3 weeks with 15 mg/kg pertuzumab or once at treatment initiation with 0.3, 1 or 3 mg/kg T-DM1, n=8/group.

Table 1. A. NCI CTCAE Grade \geq 3 adverse events in the Phase Ib portion of TDM4373g. B. Best clinical response, by cohort, in the Phase Ib portion of TDM4373g. Cohort 1: 3.0 mg/kg T-DM1 plus pertuzumab; Cohort 2: 3.6 mg/kg T-DM1 plus pertuzumab. Pertuzumab dose: 840 mg first cycle, then 420 mg thereafter; all agents administered once every 3 weeks.

References
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Table 1

A. Adverse Events \( \geq \) Grade 3

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Number of Patients (n=9)</th>
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<td>Fatigue</td>
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<td>Thrombocytopenia</td>
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</tr>
<tr>
<td>Pneumonia</td>
<td>1</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>1</td>
</tr>
<tr>
<td>Deafness*</td>
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*Patient had baseline Grade 3 hearing loss which worsened but did not increase in grade

B. Best Clinical Response

<table>
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<td>0</td>
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<td>2 (33.3%)</td>
<td>4 (44.4%)</td>
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<tr>
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<tr>
<td>Progressive disease</td>
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<td>1 (11.1%)</td>
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Dual targeting of HER2-positive cancer with trastuzumab-emtansine (T-DM1) and pertuzumab: critical role for neuregulin blockade in anti-tumor response to combination therapy

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