

# Strongly Enhanced Antitumor Activity of Trastuzumab and Pertuzumab Combination Treatment on HER2-Positive Human Xenograft Tumor Models

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## Abstract

The human epidermal growth factor receptor (HER) family plays an important role in cell survival and proliferation, and is implicated in oncogenesis. Overexpression of HER2 is associated with aggressive disease and poor prognosis. Trastuzumab is a humanized monoclonal antibody targeting HER2 and has proven survival benefit for women with HER2-positive early and metastatic breast cancer. Pertuzumab, another monoclonal antibody, is a HER2 dimerization inhibitor that binds to a different epitope on HER2 than trastuzumab and inhibits HER2 dimer formation with other HER family members such as HER3 and HER1. We investigated the antitumor activity of these agents alone and in combination in HER2-positive breast and non-small cell lung cancer xenografts. Our data show that the combination of trastuzumab and pertuzumab has a strongly enhanced antitumor effect and induces tumor regression in both xenograft models, something that cannot be achieved by either monotherapy. The enhanced efficacy of the combination was also observed after tumor progression during trastuzumab monotherapy. Near-IR fluorescence imaging experiments confirm that pertuzumab binding to tumors is not impaired by trastuzumab pretreatment. Furthermore, we show by *in vitro* assay that both trastuzumab and pertuzumab potently activate antibody-dependent cellular cytotoxicity. However, our data suggest that the strongly enhanced antitumor activity is mainly due to the differing but complementary mechanisms of action of trastuzumab and pertuzumab, namely inhibition of HER2 dimerization and prevention of p95HER2 formation. [Cancer Res 2009;69(24):9330–6]

## Introduction

The human epidermal growth factor receptor (HER) family comprises HER1, HER2, HER3, and HER4. These proteins are capable of forming heterodimers and homodimers, which activate signal transduction pathways that regulate many cellular processes, including growth, proliferation, and survival (1). As such, the HER proteins have been implicated in oncogenesis (2); in particular, tumors that overexpress the HER2 protein or have amplification of the HER2 gene are associated with aggressive disease and poor prognosis (3–6). HER2 overexpression has been identified in

numerous tumor types, including 20% to 25% of breast cancers (7–10), 4% to 6% of non-small cell lung cancers (NSCLC; refs. 3, 11), and 20% to 24% of gastric cancers (5, 12).

Trastuzumab (Herceptin) is a humanized monoclonal antibody that binds to the extracellular domain of HER2, inhibiting HER2-mediated proliferation by activating antibody-dependent cellular cytotoxicity (ADCC; refs. 13, 14), preventing formation of p95HER2 (a truncated and constitutively active form of HER2; ref. 15), blocking ligand-independent HER2 signaling (16), and inhibiting HER2-mediated angiogenesis (17). Trastuzumab has a proven survival benefit for women with HER2-positive breast cancer both in advanced metastatic disease and in early disease when administered for 1 year as adjuvant therapy (18–22). A number of phase II clinical trials have also been carried out in patients with HER2-positive NSCLC with some success, although the number of patients in these trials has been small (23–25).

Pertuzumab, a humanized monoclonal antibody, is the first in a new class of HER2 dimerization inhibitors that block HER2 dimerization with other HER family members, thus inhibiting the downstream signaling processes that are associated with tumor growth and progression (26–28). Trastuzumab and pertuzumab bind to distinct epitopes on the HER2 extracellular domain, and it has been hypothesized that a combination of the two agents might provide a more effective inhibition of tumor growth than either agent alone (27, 29). Initial *in vitro* data suggest that this hypothesis is correct, although *in vivo* data have been lacking (30). The aim of this study was to investigate the antitumor effect of the combination of trastuzumab and pertuzumab in HER2-overexpressing NSCLC (Calu-3) and breast cancer (KPL-4) xenograft models.

## Materials and Methods

**Cell lines and culture.** Calu-3 cells were obtained from Chugai Pharmaceuticals Co., Ltd. KPL-4 cells were provided by Professor J. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan). The cells were cultured as previously described (31).

**Tumor xenografts.** To initiate tumor xenografts,  $5 \times 10^6$  Calu-3 cells were injected s.c. into BALB/c nu/nu female mice and  $3 \times 10^6$  KPL-4 cells were implanted orthotopically into the right penultimate inguinal mammary fat pad of female severe combined immunodeficient (SCID) beige mice. Tumors were allowed to establish growth (21 d for Calu-3 and 20 d for KPL-4) after implantation before initiation of treatment.

**Treatment of animals.** Calu-3 or KPL-4 tumors (100 mm<sup>3</sup>) were treated with trastuzumab (30 mg/kg loading dose, then 15 mg/kg weekly), pertuzumab (30 mg/kg loading dose, then 15 mg/kg weekly), or both, administered i.p. for the duration of the study. Tumor volumes and body weights were measured twice weekly. For the acute study, advanced Calu-3 tumors of ~400 mm<sup>3</sup> were treated once with trastuzumab and/or pertuzumab at a dose of 30 mg/kg. Samples were harvested 7 d later for immunohistochemistry (IHC) and Western blot analysis.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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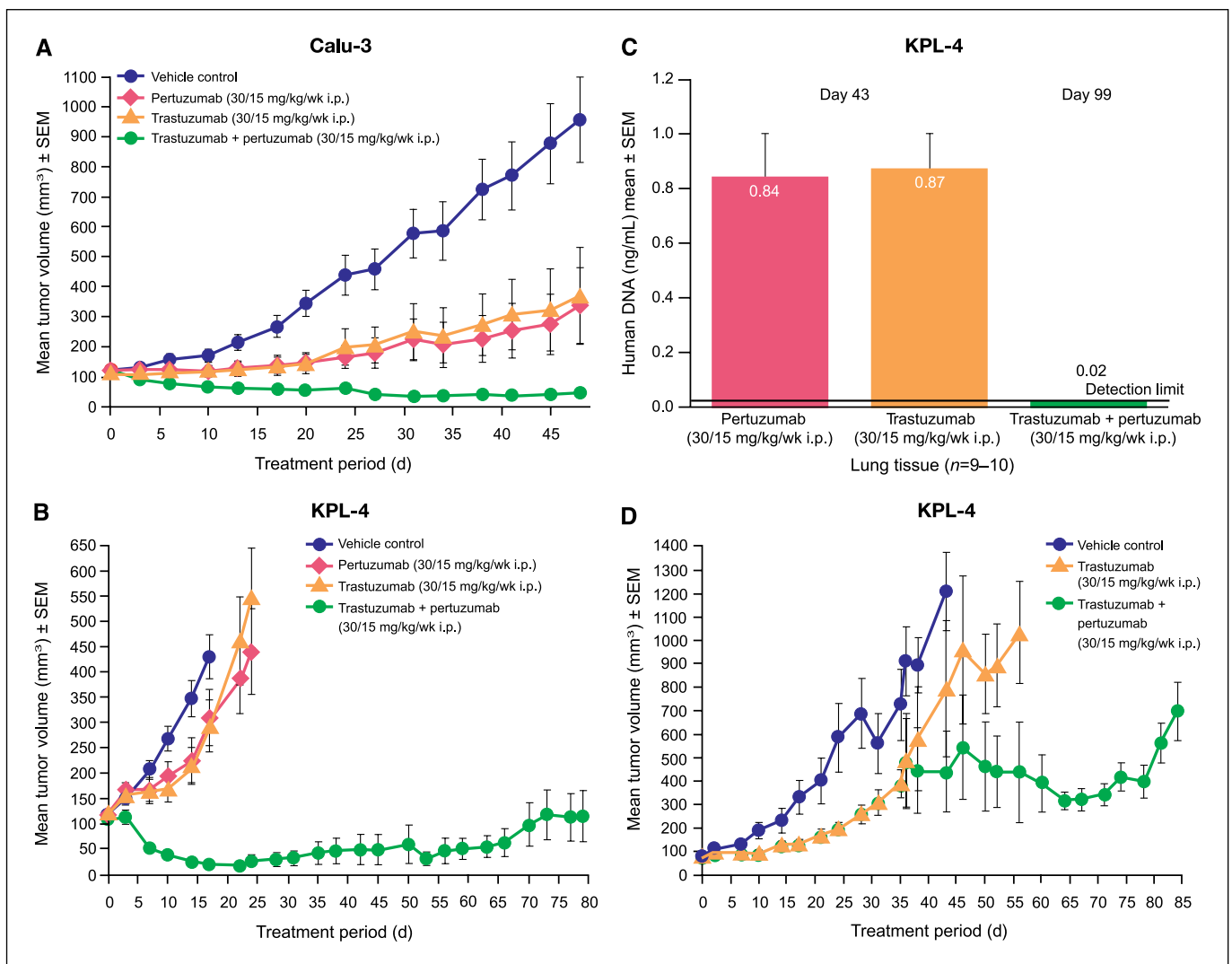
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**Western blot analysis.** For Western blot analysis, protein lysates (20  $\mu$ g protein/lane) were electrophoresed on TRIS NuPage Ready-Gel (1.0 mm; Invitrogen) for 1 h at 60 V, transferred to nitrocellulose membrane (Hybond enhanced chemiluminescence, Amersham) and incubated with a polyclonal rabbit anti-human c-erbB2 antibody (Dako) at 1  $\mu$ g/mL. HER2 protein was detected using a labeled secondary antibody diluted at 1:5,000 (goat anti-rabbit POD labeled; Bio-Rad Laboratories GmbH) and Lumi-Light Western Blotting Substrate (Roche Diagnostics GmbH). The Odyssey detection system (LI-COR Bioscience) was used for quantification.

For control of siRNA experiments (Fig. 5), 60  $\mu$ g protein/lane was electrophoresed and blotted onto a polyvinylidene difluoride membrane (Invitrogen). Polyclonal rabbit anti-human ErbB-3 antibody (C-17, sc-285, Santa Cruz) at 1  $\mu$ g/mL and polyclonal rabbit anti-human epidermal growth factor receptor (EGFR) antibody (06-847, Upstate) at 1  $\mu$ g/mL were used. Anti-rabbit IgG-HRP-linked antibody (7074, Cell Signaling) was used as secondary antibody at 1:2,000 dilution. Luminescence was detected with Lumi-Light Western Blotting Substrate on the Lumilager Instrument (Roche Diagnostics GmbH).

**Assessment of metastases by quantification of human DNA.** Genomic DNA was isolated using the High Pure PCR Template Preparation kit (Roche Diagnostics GmbH) and quantified using the PicoGreen Quantification kit (Molecular Probes). Primers for human Alu repeats were synthesized by TIB MOLBIOL GmbH and quantitative PCR (qPCR) was performed using the Light-Cycler System (Roche Diagnostics GmbH), as previously described (32).

**Near IR fluorescence imaging *in vivo*.** Trastuzumab and pertuzumab were labeled with the fluorescent dye Cy5 (General Electric) according to the manufacturer's instructions. The labeling ratio was 5:1 (Cy5/antibody). When KPL-4 tumors reached a volume of 200 mm<sup>3</sup>, mice were injected i.v. with a single 50- $\mu$ g dose of either trastuzumab-Cy5 or pertuzumab-Cy5. Near IR fluorescence (NIRF) measurements were performed using the BonSAI Imaging System (Siemens Medizintechnik) equipped with an inhalation mask for anesthesia. Fluorescence acquisition time was 4 s for all experiments. For the evaluation of binding competition, mice were injected i.v. with a single dose of trastuzumab (30 mg/kg). After 48 h, Cy5-labeled trastuzumab or pertuzumab (50  $\mu$ g/mouse) was injected i.v. NIRF signals were measured as described above.



**Figure 1.** Antitumor activity of trastuzumab and/or pertuzumab in NSCLC (Calu-3) and breast cancer (KPL-4) xenograft tumor models. Mice with (A) Calu-3 and (B) KPL-4 xenograft tumors were treated for the duration of the study with vehicle (violet circle), pertuzumab (pink diamond), trastuzumab (orange triangle), trastuzumab + pertuzumab (green circle). Points, mean tumor volume (mm<sup>3</sup>) (n = 10 mice/group); bars, SEM. C, investigation of KPL-4 metastatic tumor spread to the lungs of mice. Genomic DNA from lung samples of mice with KPL-4 tumors that had been treated with trastuzumab, pertuzumab, or trastuzumab with pertuzumab was extracted and qPCR performed to investigate any KPL-4 tumor infiltrate into the lung. Data are the mean of 9 to 10 mice per group. D, mice with KPL-4 xenograft tumors were treated for the duration of the study with vehicle (violet circle), trastuzumab (orange triangle), and trastuzumab followed by pertuzumab (trastuzumab then pertuzumab added on day 35; green circle).

**Table 1.** Synergistic antitumor activity by trastuzumab and pertuzumab combination therapy

Xenograft	Treatment	Response, TCR	Confidence interval	TGI,%	Tumor-free animals, <i>n</i>
Calu-3	P	0.23	0.08–0.53	85	0/10
	T	0.27	0.06–0.57	82	0/10
	P + T	0.05	0.02–0.11	>100*	3/10
KPL-4	P	0.67	0.45–0.94	38	0/10
	T	0.65	0.43–0.91	45	0/10
	P + T	0.04	0.00–0.25	>100*	6/10

Abbreviations: P, pertuzumab; T, trastuzumab; TCR, treatment:control ratio; TGI, tumor growth inhibition.  
\*Tumor regression.

**ADCC assays.** Twenty-four hours before the assay, effector cells [peripheral blood mononuclear cells (PBMC)] were prepared from a healthy blood donor by Ficoll gradient centrifugation. PBMCs were diluted to a final concentration of  $2.5 \times 10^6$  cells/mL in RPMI/10% fetal bovine serum (FBS) and cultured overnight. The following day, the target KPL-4 and Calu-3 cells grown in RPMI/10% FBS were detached using Accutase (PAA Laboratories), sedimented by centrifugation, and resuspended in RPMI/10% FBS at a final concentration of  $1 \times 10^6$  cells/mL. Cells were labeled by adding 1  $\mu$ L bis (acetoxymethyl) 2,2':6,2'-terpyridine-6,6'-dicarboxylic acid (Perkin-Elmer LAS GmbH) per milliliter cell suspension, mixing carefully and incubating for 25 min at 37°C and 5% CO<sub>2</sub>. Cells were washed thrice with 10 mL cell culture medium and diluted to  $1.0 \times 10^5$  cells/mL.

To perform the assay, 50  $\mu$ L of serial dilutions of either trastuzumab or pertuzumab was added to each well of a 96-well plate (Greiner Bio-One GmbH). To test the combination of trastuzumab and pertuzumab, 25  $\mu$ L of each antibody were added instead. Thus, the final total concentration of the antibody mixture was the same as in the single antibody experiments. Fifty microliters of target cells and PBMCs were added, resulting in a final antibody concentration that ranged from 0.4 to 50 ng/mL and an effector/target ratio of 25:1. Controls included effector cells alone, only target cells and effector cells, and target cells with 3% Triton X-100. Plates were incubated for 2 h at 37°C and 5% CO<sub>2</sub>, and centrifuged at 200 $\times$  g for 5 min, then 20  $\mu$ L of each supernatant was transferred into an OptiPlate-96 F (Perkin-Elmer LAS GmbH). After addition of 200  $\mu$ L Europium solution (Perkin-Elmer LAS GmbH) to each well, the plates were incubated for 15 min on a shaker. Fluorescence was measured using the Victor2 plate reader (Perkin-Elmer LAS GmbH) according to the Eu-TDA protocol (excitation, 340 nm; emission, 615 nm; delay, 400  $\mu$ s). The percentage of specific release was determined using the following formula: (experimental release – spontaneous release)/(maximum release – spontaneous release)  $\times$  100.

**siRNA experiments.** Calu-3 cells were cultured in RPMI 1640 (Invitrogen) and KPL-4 cells were grown in DMEM (Invitrogen), both were supplemented with 10% FBS. Cells were transfected according to the DharmaFECT 2 reverse transfection protocol (Dharmacon). siRNAs for HER3 and EGFR were purchased from Dharmacon and used at a concentration of 50 nmol/L per well. siRNAs against Eg5 and luciferase were used as positive and negative controls, respectively.

For checking cell proliferation,  $1 \times 10^4$  cells were seeded in normal (Calu-3) or polyhema-coated (KPL-4) 96-well microtiter plates. After 4 d, cells were incubated with CellTiter-Glo reagent (Promega). Luminescence was measured in a Tecan Reader (Tecan Deutschland GmbH). Mock-treated cells were taken as 100%.

For Western blot and reverse transcription-PCR (RT-PCR),  $1 \times 10^6$  cells were seeded in normal (Calu-3) or polyhema-coated 6-well microtiter plates.

For quantitative real-time PCR, cells were harvested at 24 h after transfection. RNA was isolated using an RNeasy Mini kit (Qiagen) with on-column DNase treatment. First-Strand cDNA was produced with Transcriptor First-Strand cDNA Synthesis kit (Roche Diagnostics GmbH). Primers and probes were designed with the Universal Probe Library Assay

design center (Roche Diagnostics GmbH). Quantitative RT-PCR was performed using LightCycler 480 Probes Master in duplex PCR with the recommended housekeeping genes (UPL #50 with  $\beta$ -actin for EGFR, UPL #37 with hypoxanthine phosphoribosyltransferase for HER3) on a Light-Cycler 480 II System (Roche Diagnostics GmbH).

Cells for Western blot analysis were harvested at 48 h after transfection.

**Statistical analyses.** Statistical analyses were performed using SAS software version 8.1 (SAS, Inc.) and the TUMGRO module according to Fieller (33), and Munzel and Hothorn (34). Relative tumor growth inhibition was calculated according to the formula  $(1 - [(T - T_0)/(C - C_0)]) \times 100$ .

## Results

### Antitumor Activity of Trastuzumab and Pertuzumab Both Alone and in Combination

**Trastuzumab in combination with pertuzumab has strongly enhanced antitumor activity.** We investigated the efficacy of trastuzumab and pertuzumab in Calu-3 and KPL-4 tumor xenografts. Mice were treated with either: vehicle control, pertuzumab or trastuzumab alone, or trastuzumab and pertuzumab in combination. In Calu-3 NSCLC xenografts, monotherapy with pertuzumab or trastuzumab was able to significantly inhibit tumor growth, with treatment-to-control ratios (TCR) of 0.23 and 0.27, respectively. The combination of trastuzumab and pertuzumab produced a dramatically enhanced antitumor activity compared with single-agent treatments (TCR 0.05; Table 1; Fig. 1A), resulting in tumor regression and, in 3 of 10 animals, complete tumor remission (Supplementary Fig. S1; Table 1).

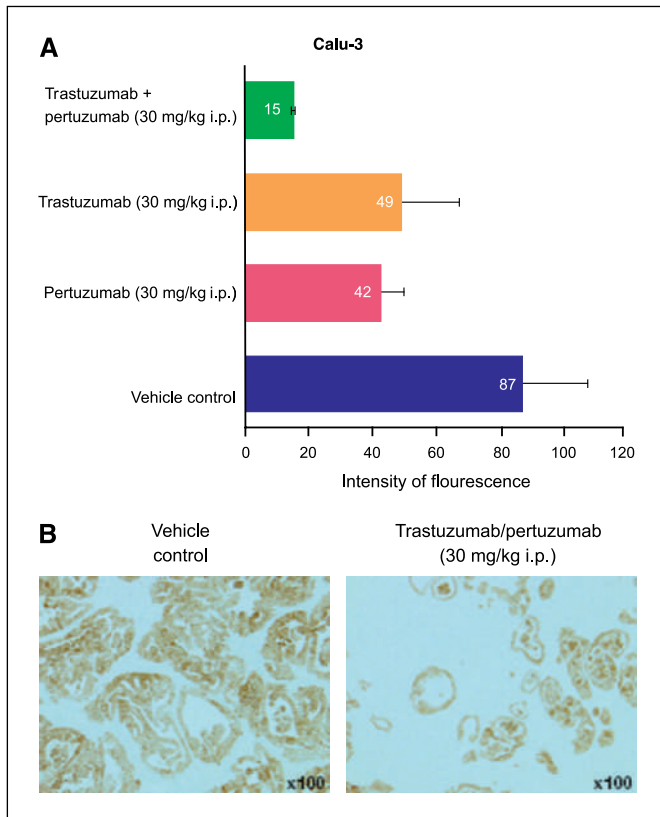
Treatment of KPL-4 breast cancer xenografts with either trastuzumab or pertuzumab inhibited tumor growth with TCRs of 0.67 and 0.65, respectively. Again, combining trastuzumab and pertuzumab induced strongly enhanced antitumor activity compared with either agent alone (TCR 0.04; Table 1; Fig. 1B), resulting in tumor regression and, in 6 of 10 animals, complete tumor remission (Table 1).

**Combination of trastuzumab and pertuzumab can inhibit metastatic tumor spread.** Genomic DNA from lung samples of host animals with KPL-4 xenografts was analyzed by qPCR to investigate if the tumor had metastasized to the lung. Monotherapy with either trastuzumab or pertuzumab did not prevent metastases forming in the lungs of the mice by day 43; however, no human DNA was detected in the lungs of mice treated with the combination of both trastuzumab and pertuzumab, even at day 99, suggesting that this combination completely prevented metastatic tumor spread to the lungs of these animals (Fig. 1C). Findings for the liver were similar (data not shown).

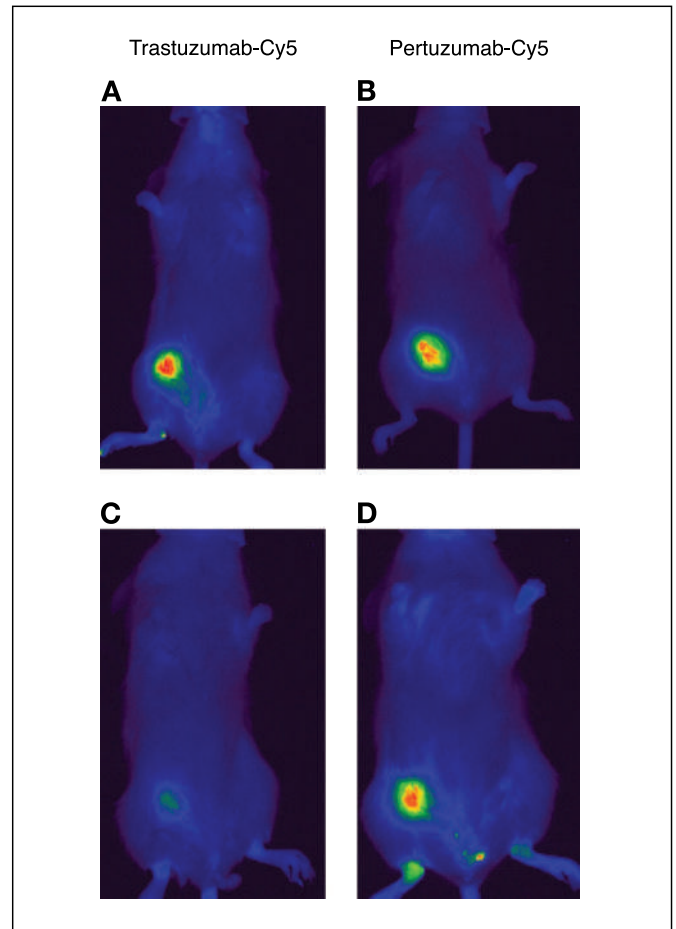


**Pertuzumab maintains antitumor activity after progression on trastuzumab.** To investigate the combination of trastuzumab and pertuzumab after progression on trastuzumab, SCID mice bearing KPL-4 xenograft tumors were treated with trastuzumab (30 mg/kg loading dose followed by 15 mg/kg weekly) until progression (day 35). At this point, mice either continued treatment with trastuzumab alone or with trastuzumab in combination with pertuzumab. The combination of the two agents was sufficient to inhibit tumor growth and, indeed, reduce tumor mass for a further 45 days (Fig. 1D). A similar result was obtained using the Calu-3 xenograft model: addition of pertuzumab to continued trastuzumab treatment-induced tumor shrinkage despite progressive growth on trastuzumab single-agent treatment (Supplementary Fig. S2).

**Trastuzumab with pertuzumab induces tumor cell loss.** To determine how trastuzumab and pertuzumab were inhibiting tumor growth, an acute study, using just one dose of either trastuzumab and pertuzumab monotherapy or combination therapy, was performed in mice with advanced Calu-3 xenograft tumors. Western blot analysis of HER2 protein levels in tumor lysates showed that both trastuzumab and pertuzumab as single agents reduced HER2 protein levels significantly (TCR, 0.48 and 0.56, respectively). However, combination of trastuzumab with pertuzumab was clearly superior [TCR, 0.17 (confidence interval, 0.00–0.39); Fig. 2A]. Similar protein decreases were found for HER1, HER3, phosphoHER2, and downstream signaling components (data not shown). A single treatment with combined trastuzumab



**Figure 2.** Investigation of the synergistic antitumor effects of trastuzumab and pertuzumab. Mice with Calu-3 tumors were treated with a single dose of antibody, and tumors were explanted 1 wk after antibody treatment and analyzed by A, Western blot and B, IHC for HER2 protein expression.



**Figure 3.** *In vivo* imaging of KPL-4 tumor xenografts, with labeled trastuzumab and pertuzumab using NIRF technology. Comparable NIRF signals were obtained at the tumor site when KPL-4 xenograft-carrying mice were treated with either (A) trastuzumab-Cy5 or (B) pertuzumab-Cy5. When mice were pretreated with unlabeled trastuzumab, no tumor staining was seen with (C) trastuzumab-Cy5, whereas (D) pertuzumab-Cy5 treatment showed similar staining intensity to tumors not pretreated with trastuzumab.

and pertuzumab resulted in a more than additive effect on the level of HER2 protein (Fig. 2A). IHC analysis of tumor tissue confirmed that the reduction in HER2 was due to tumor cell loss and not downregulation of HER2 from the cell surface, as the remaining Calu-3 cells were still HER2 positive (Fig. 2B). IHC analysis also revealed that the space generated by loss of tumor cells was partially filled by mouse stromal cells, which explains the relative loss of HER2 protein with respect to total protein levels shown by Western blotting (Fig. 2A).

***In vivo* visualization of trastuzumab and pertuzumab binding to xenograft tumors.** Using NIRF optical imaging, we showed that single-agent trastuzumab-Cy5 and pertuzumab-Cy5 were able to bind to KPL-4 tumors *in vivo* with equal intensity (Fig. 3A and B, respectively). To investigate if trastuzumab and pertuzumab could bind simultaneously to xenograft tumors *in vivo*, mice were pretreated with unlabeled trastuzumab then with either trastuzumab-Cy5 or pertuzumab-Cy5. Pretreatment with unlabeled trastuzumab inhibited trastuzumab-Cy5 NIRF, indicating receptor saturation (Fig. 3C). Treatment with pertuzumab-Cy5 resulted in a similar staining intensity as in tumors not pretreated with unlabeled trastuzumab (Fig. 3D), indicating that binding of pertuzumab is not impaired by trastuzumab.

**ADCC assays with trastuzumab and/or pertuzumab.** To identify the mechanism of action for the strongly enhanced activity of trastuzumab and pertuzumab, we performed *in vitro* ADCC assays using human PBMCs (effector cells) and either cultured Calu-3 or KPL-4 cells (target cells). Both trastuzumab and pertuzumab applied as single agents effectively activated ADCC against Calu-3 (Fig. 4A) and KPL-4 cells (Fig. 4B) with equal potency. However, there was no increased ADCC efficiency observed when both agents were used in combination.

**HER1, HER2, and HER3 expression in human NSCLC and breast cancer cell lines used for xenograft models and siRNA downregulation of HER1 and HER3.** The HER2-positive cell lines KPL-4 and Calu-3 were analyzed for HER-family receptor expression by Western blotting. Whereas Calu-3 showed strong HER1 and moderate HER3 coexpression, KPL-4 cell lysates produced a very strong HER3 and strong HER1 band (see Fig. 5B and D).

To confirm the HER2 status according to clinical standards, IHC was performed on Calu-3 and KPL-4 xenograft tumors. Both Calu-3 and KPL-4 xenograft tumors were strongly positive for HER2 staining (IHC 3+ using the HercepTest scoring recommendations).

Because HER2 dimerization with either HER1 or HER3 can promote cell survival and proliferation, and act as a potential escape mechanism from trastuzumab inhibition, we performed a knock-down of HER1 and HER3, respectively, and investigated the effect on proliferation of Calu-3 and KPL-4 cells. Calu-3 and KPL-4 cells were transfected with siRNAs targeting either HER1 or HER3, and proliferation was measured. HER1 and HER3 targeting siRNAs effectively downregulated expression of the respective mRNAs roughly by about 60% to 80% in both cell lines (Fig. 5A and C). Western blot data confirmed that HER1 and HER3 protein expression was effectively reduced after siRNA treatments (Fig. 5B and D). In Calu-3 cells, HER1 siRNA treatment inhibited cell growth by ~30%, whereas downregulation of HER3 reduced growth only by ~7%, an effect that was no longer statistically significant (Fig. 5A). Both results on Calu-3 cell growth are consistent with the relative coexpression of HER1 and HER3 in this HER2-positive

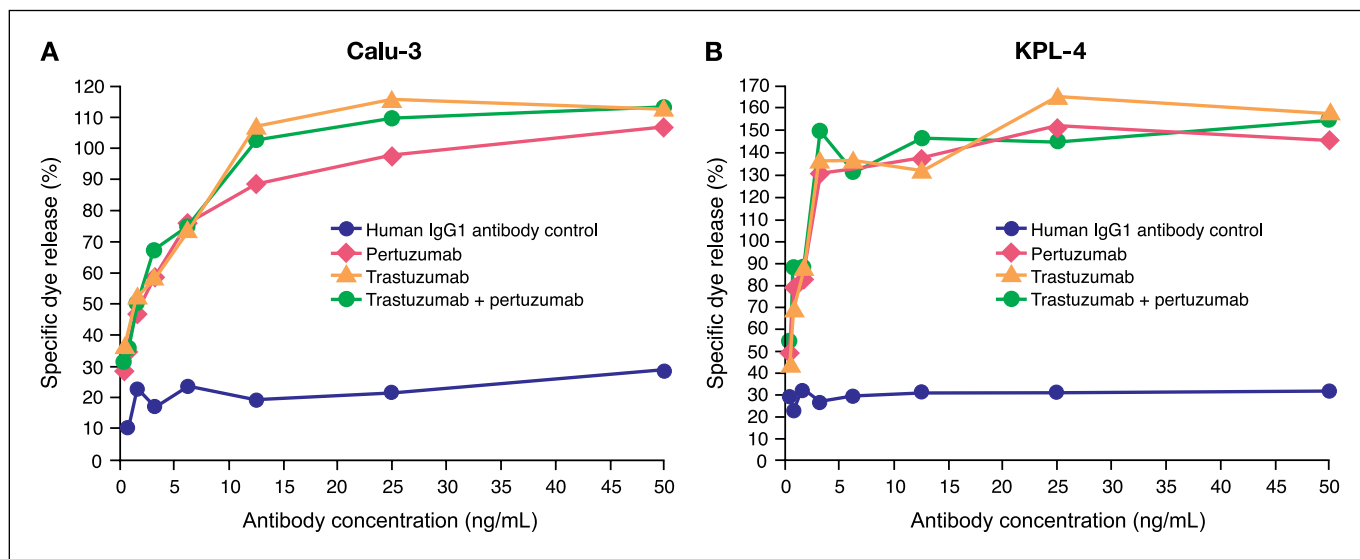
cell line. In KPL-4 cells, HER3 expression turned out to be more important for cell growth than HER1: whereas HER1-specific siRNA reduced cell growth by ~10%, HER3 siRNA treatment entailed ~25% growth inhibition (Fig. 5C). Again, this result is consistent with the relative coexpression of HER1 and HER3 in the HER2-positive cell line KPL-4.

## Discussion

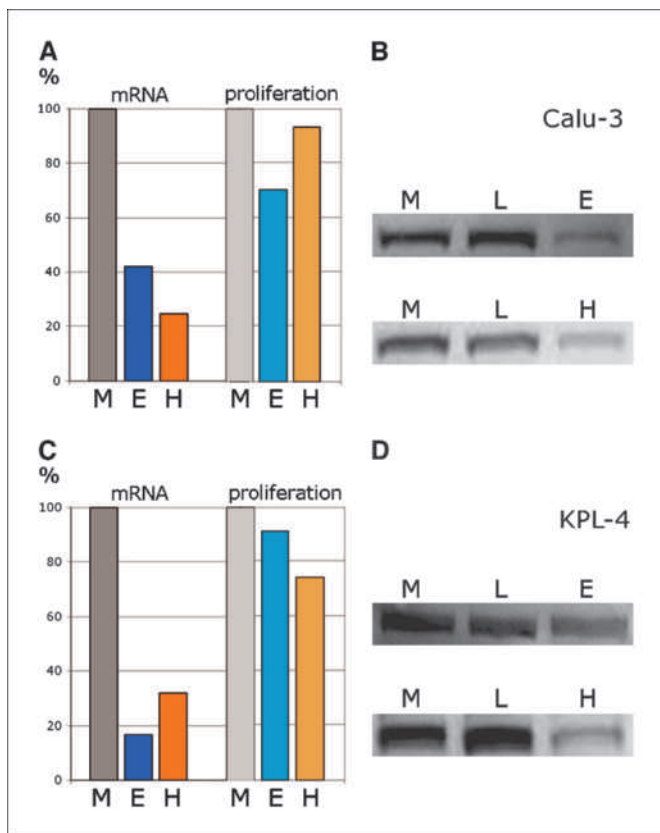
This study has shown that monotherapy with either trastuzumab or pertuzumab has a moderate growth inhibitory effect on NSCLC (Calu-3) and breast (KPL-4) xenograft tumors. The combination treatment with trastuzumab and pertuzumab induced a strongly enhanced antitumor activity in both Calu-3 and KPL-4 xenograft models, resulting in tumor regression and a complete inhibition of metastatic tumor spread in host animals. Moreover, addition of pertuzumab to trastuzumab therapy enhanced antitumor activity in KPL-4 and Calu-3 xenograft tumors that had progressed on trastuzumab. Reduction in the levels of HER2 protein following treatment with trastuzumab and/or pertuzumab was due to a loss of cells expressing HER2 rather than a downregulation of HER2 protein at the cell surface.

The synergistic action of trastuzumab and pertuzumab combination treatment was previously shown *in vitro* (30) using the calculation of the combination index as outlined by Chou and Talalay (35, 36). In the present work, combination treatment with trastuzumab and pertuzumab dramatically enhanced the antitumor effect compared with each drug alone: whereas single agent treatments could only delay tumor growth (transiently in KPL-4), the combination treatment induced effective tumor regressions and cures in both xenograft models. We believe that this qualitative difference in antitumor efficacy is a consequence of the synergistic action of both antibodies described *in vitro*.

*In vitro* studies investigating the mechanism of action of trastuzumab and pertuzumab have shown that these agents bind to different epitopes on the HER2 protein (27, 37); however, concomitant



**Figure 4.** *In vitro* ADCC analysis of trastuzumab, pertuzumab, or trastuzumab in combination with pertuzumab. PBMCs (effector cells) were added with either (A) Calu-3 or (B) KPL-4 cells (target cells) into 96-well plates containing control human IgG1 (violet circle), pertuzumab (pink diamond), trastuzumab (orange triangle), or trastuzumab and pertuzumab (green circle) at different concentrations. All experiments were performed in triplicate.



**Figure 5.** Effects of siRNA-mediated knockdown of EGFR and HER3 mRNA in Calu-3 (A and B) and KPL-4 cells (C and D). A and C, % mRNA and proliferation (compared with mock treatment); B and D, Western blot of EGFR-protein (top row) or HER3-protein (bottom row). M, mock treatment with transfection agent only; L, cells transfected with siRNA against luciferase; E and H, cells transfected with siRNAs specifically downmodulating EGFR and HER3, respectively.

binding to HER2-positive tumors has not been shown *in vivo*. Using NIRF imaging, we show for the first time that *in vivo* binding of trastuzumab and pertuzumab is not mutually exclusive. Similar amounts of pertuzumab and trastuzumab, respectively, can bind to HER2-positive tumors when used either in monotherapy or in combination.

Activation of ADCC has been described as a major mechanism of action of trastuzumab (13, 14, 38) and the present study confirms that this is also a property of pertuzumab. Since trastuzumab and pertuzumab are able to bind simultaneously to tumors *in vivo*, we investigated if the strongly enhanced antitumor effect of these two agents was due to enhanced ADCC. Each antibody alone mediated a strong activation of ADCC, reaching comparable levels of maximal specific target cell lysis. The combination of trastuzumab and pertuzumab (final total antibody concentration identical to those in the single antibody assays) resulted in dose-response curves indistinguishable from those obtained with the single antibodies. Thus, the simultaneous binding of trastuzumab and pertuzumab onto target cells mediates an additive but not synergistic effect on ADCC. The combination of both agents at receptor-saturating concentrations did not enhance this immune effect. This is likely explained by the high receptor density in HER2-overexpressing target cells, which leads to an antibody load that is more than sufficient to elicit the maximum activation of immune

effector cells. In this respect, the situation is expected to be similar *in vivo* because plasma concentrations obtained by therapeutic dosages are above the 20- $\mu\text{g}/\text{mL}$  threshold, which ensures HER2 binding saturation with trastuzumab. These data suggest that enhanced ADCC is unlikely to be the mechanism by which the strongly enhanced activity of trastuzumab and pertuzumab, observed *in vivo*, is mediated. However, as HER2 overexpression is not lost after treatment with either trastuzumab or pertuzumab, ADCC may still provide a mechanism by which both trastuzumab and pertuzumab can continue to provide an antitumor effect even after progression on trastuzumab, as observed in recent clinical trials (39–41). These trials and our data suggest that patients who progress on trastuzumab therapy may still benefit from continued trastuzumab and that acquired resistance to trastuzumab is not equivalent to traditional mechanisms of chemotherapy resistance.

The strongly enhanced activity of trastuzumab and pertuzumab is likely attributable to their alternative, differing mechanisms of action. Previous studies have reported that trastuzumab can sterically inhibit access for the proteases that cleave the extracellular domain and, therefore, prevent the formation of p95HER2, the truncated and very active form of HER2 (15, 42). However, trastuzumab is unable to prevent ligand-activated HER2/HER3 or HER2/HER1 heterodimerization, which may provide tumor cells with an escape mechanism from the inhibitory effects of trastuzumab. In the current study, downregulation of HER3 and HER1 mRNA and protein reduced the proliferation of both Calu-3 and KPL-4 cells. This illustrates the importance of these pathways in the propagation of HER2-positive tumors and they may even become more important when ligand-independent HER2 activation is inhibited by trastuzumab. Pertuzumab prevents the HER2 protein from dimerizing with HER3 and/or HER1 (26, 27, 29, 43), thus blocking these potential escape pathways. Alternatively, pertuzumab monotherapy can obstruct ligand-activated HER2 dimerization and signaling but would be unable to prevent formation of p95HER2, which is readily achieved by trastuzumab.

Thus, the differing mechanisms of action of trastuzumab and pertuzumab can act in a complementary fashion to provide a more complete blockade of HER2-mediated signal transduction than either agent alone.

In the clinical setting, dramatically enhanced antitumor activity of trastuzumab and pertuzumab has been shown in a phase II clinical trial that investigated the efficacy and safety of a trastuzumab/pertuzumab combination in 66 patients with HER2-positive metastatic breast cancer whose disease had progressed during trastuzumab therapy. Of these patients, 8% had a complete response, with an overall response rate of 24.2% and a clinical benefit rate of 50% (39). Data from the current study also indicate a potential role for pertuzumab and trastuzumab in HER2-positive NSCLC, although it should be noted that HER2 overexpression is rare in NSCLC.

## Conclusions

Trastuzumab and pertuzumab in combination have a strongly enhanced antitumor effect and promote tumor regression in xenograft models of HER2-positive breast cancer and NSCLC. Although both agents potentially activate ADCC, this is unlikely to be the main mechanism by which the strongly enhanced effects of this combination are mediated. Instead, the differing but complementary mechanisms of action of trastuzumab and pertuzumab may explain the significantly enhanced antitumor activity produced by

combined treatment with these agents. The combination of these agents may prove to be of substantial benefit to patients who currently respond suboptimally to trastuzumab therapy; further trials are warranted.

## Disclosure of Potential Conflicts of Interest

All authors are employees of Roche Diagnostics GmbH.

## References

- Yarden Y, Slivkowsky MX. Untangling the ErbB signaling network. *Nat Rev Mol Cell Biol* 2001;2:127–37.
- Yarden Y. The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities. *Eur J Cancer* 2001;37:S3–8.
- Hirsch FR, Franklin WA, Veve R, Varella-Garcia M, Bunn PA, Jr. HER2/neu expression in malignant lung tumors. *Semin Oncol* 2002;29:51–8.
- Onn A, Correa AM, Gilcrease M, et al. Synchronous overexpression of epidermal growth factor receptor and HER2-neu protein is a predictor of poor outcome in patients with stage I non-small cell lung cancer. *Clin Cancer Res* 2004;10:136–43.
- Tanner M, Hollmén M, Junttila TT, et al. Amplification of HER-2 in gastric carcinoma: association with Topoisomerase II $\alpha$  gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. *Ann Oncol* 2005;16:273–8.
- Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987;235:177–82.
- Ross JS, Fletcher JA, Bloom KJ, et al. Targeted therapy in breast cancer. The HER-2/neu gene and protein. *Mol Cell Proteomics* 2004;3:379–98.
- Wolf J, Hammond MEH, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007;25:118–45.
- Yaziji H, Goldstein LC, Barry TS, et al. HER-2 testing in breast cancer using parallel tissue-based methods. *JAMA* 2004;291:1972–7.
- Owens MA, Horten BC, Da Silva MM. HER2 amplification ratios by fluorescence *in situ* hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. *Clin Breast Cancer* 2004;5:63–9.
- Cox G, Vyberg M, Melgaard B, Askaa J, Oster A, O'Byrne KJ. HercepTest: HER2 expression and gene amplification in non-small cell lung cancer. *Int J Cancer* 2001;92:480–3.
- Hofmann M, Stoss O, Shi D, et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. *Histopathology* 2008;52:797–805.
- Barok M, Isola J, Pálvi-Krekke Z, et al. Trastuzumab causes antibody-dependent cellular cytotoxicity-mediated growth inhibition of submacroscopic JIMT-1 breast cancer xenografts despite intrinsic drug resistance. *Mol Cancer Ther* 2007;6:2065–72.
- Musolino A, Naldi N, Bortesi B, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol* 2008;26:1789–96.
- Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J. Trastuzumab (Herceptin), a humanized anti-HER2 receptor monoclonal antibody, inhibits basal and activated HER2 ectodomain cleavage in breast cancer cells. *Cancer Res* 2001;61:4744–9.
- Junttila TT, Akita RW, Parsons K, et al. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer Cell* 2009;15:429–40.
- Izumi Y, Xu L, di Tomaso E, Fukumura D, Jain RK. Tumour biology: herceptin acts as an anti-angiogenic cocktail. *Nature* 2002;416:279–80.
- Buzdar AU, Valero V, Ibrahim NK, et al. Neoadjuvant therapy with paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide chemotherapy and concurrent trastuzumab in human epidermal growth factor receptor 2-positive operable breast cancer: an update of the initial randomized study population and data of additional patients treated with the same regimen. *Clin Cancer Res* 2007;13:228–33.
- Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 2005;353:1673–84.
- Slamon D, Eiermann W, Robert N, et al. BCIRG 006: 2nd interim analysis phase III randomized trial comparing doxorubicin and cyclophosphamide followed by docetaxel (AC $\rightarrow$ T) with doxorubicin and cyclophosphamide followed by docetaxel and trastuzumab (AC $\rightarrow$ TH) with docetaxel, carboplatin and trastuzumab (TCH) in Her2neu positive early breast cancer patients. 2006. Available at: [http://www.abstracts2view.com/sabcs06/view.php?nu=SABCS06L\\_78](http://www.abstracts2view.com/sabcs06/view.php?nu=SABCS06L_78). Accessed October 10, 2009.
- Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;344:783–92.
- Smith I, Procter M, Gelber RD, et al. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet* 2007;369:29–36.
- Ferrone M, Motl SE. Trastuzumab for the treatment of non-small-cell lung cancer. *Ann Pharmacother* 2003;37:1904–8.
- Gatzemeier U, Groth G, Butts C, et al. Randomized phase II trial of gemcitabine-cisplatin with or without trastuzumab in HER2-positive non-small-cell lung cancer. *Ann Oncol* 2004;15:19–27.
- Lara PN, Jr., Laptalo L, Longmate J, et al. Trastuzumab plus docetaxel in HER2/neu-positive non-small-cell lung cancer: a California Cancer Consortium screening and phase II trial. *Clin Lung Cancer* 2004;5:231–6.
- Adams CW, Allison DE, Flagella K, et al. Humanization of a recombinant monoclonal antibody to produce a therapeutic HER dimerization inhibitor, pertuzumab. *Cancer Immunol Immunother* 2006;55:717–27.
- Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Slivkowsky MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* 2004;5:317–28.
- Diermeier-Daucher S, Hasmann M, Brockhoff G. Flow cytometric FRET analysis of erbB receptor interaction on a cell-by-cell basis. *Ann N Y Acad Sci* 2008;1130:280–6.
- Hubbard SR. EGF receptor inhibition: attacks on multiple fronts. *Cancer Cell* 2005;7:287–8.
- Nahta R, Hung M-C, Esteva FJ. The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. *Cancer Res* 2004;64:2343–6.
- Frless T, Scheuer W, Hasmann M. Combination treatment with erlotinib and pertuzumab against human tumor xenografts is superior to monotherapy. *Clin Cancer Res* 2005;11:5300–9.
- Schneider T, Osl F, Frless T, Stockinger H, Scheuer WV. Quantification of human Alu sequences by real-time PCR - an improved method to measure therapeutic efficacy of anti-metastatic drugs in human xenotransplants. *Clin Exp Metastasis* 2002;19:571–82.
- Fieller EC. Some problems in interval estimation. *J R Stat Soc Ser B* 1954;16:175–85.
- Munzel U, Hothorn LA. A unified approach to simultaneous rank test procedures in the unbalanced one-way layout. *Biom J* 2001;43:553–69.
- Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984;22:27–55.
- Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 2006;58:621–81.
- Cho H-S, Mason K, Ramyar KX, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab [letter]. *Nature* 2003;421:756–60.
- Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. *Nat Med* 2000;6:443–6.
- Gelmon KA, Fumoleau P, Verma S, et al. Results of a phase II trial of trastuzumab (H) and pertuzumab (P) in patients (pts) with HER2-positive metastatic breast cancer (MBC) who had progressed during trastuzumab therapy. *J Clin Oncol (Meeting Abstracts)* 2008;26:47s. abs 1026.
- O'Shaughnessy J, Blackwell KL, Burstein H, et al. A randomized study of lapatinib alone or in combination with trastuzumab in heavily pretreated HER2+ metastatic breast cancer progressing on trastuzumab therapy. *J Clin Oncol (Meeting Abstracts)* 2008;26:44s. abs 1015.
- von Minckwitz G, Zielinski C, Maartense E, et al. Capecitabine vs. capecitabine + trastuzumab in patients with HER2-positive metastatic breast cancer progressing during trastuzumab treatment: the TBP phase III study (GBG 26/BIG 3-05). *J Clin Oncol (Meeting Abstracts)* 2008;26:abs 1025.
- Nahta R, Esteva FJ. Herceptin: mechanisms of action and resistance. *Cancer Lett* 2006;232:123–38.
- Agus DB, Akita RW, Fox WD, et al. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* 2002;2:127–37.