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Advances in Brief

The HER-2-Targeting Antibodies Trastuzumab and Pertuzumab Synergistically Inhibit the Survival of Breast Cancer Cells

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Abstract

Trastuzumab (herceptin) and pertuzumab (Omnitarg, 2C4) are recombinant humanized monoclonal antibodies that target different extracellular regions of the HER-2 tyrosine kinase receptor. We explored combination effects of these agents in the HER-2-overexpressing BT474 breast cancer cell line. Trastuzumab and 2C4 synergistically inhibited the survival of BT474 cells, in part, because of increased apoptosis. Trastuzumab increased 2C4-mediated disruption of HER-2 dimerization with the epidermal growth factor receptor and HER-3. Combination drug treatment reduced levels of total and phosphorylated HER-2 protein and blocked receptor signaling through Akt but did not affect mitogen-activated protein kinase. These results suggest that combining HER-2-targeting agents may be a more effective therapeutic strategy in breast cancer rather than treating with a single HER-2 monoclonal antibody.

Introduction

The *her-2* (*erbB-2*, *neu*) gene encodes a M_r 185,000 transmembrane glycoprotein that is a member of the epidermal growth factor receptor (EGFR or erbB) family of receptor tyrosine kinases. As the preferred heterodimerization partner among ligand-bound EGFR family members, HER-2 mediates lateral signal transduction, resulting in mitogenesis, apoptosis, angiogenesis, and cell differentiation (1). The *her-2* gene is amplified and overexpressed in ~20–30% of invasive breast carcinomas, and is associated with increased metastatic potential and decreased overall survival (1, 2).

Trastuzumab (herceptin; Genentech, San Francisco, CA) is a recombinant humanized monoclonal antibody directed against the extracellular domain of the HER-2 tyrosine kinase receptor. Clinical studies established that trastuzumab is active against HER-2-overexpressing metastatic breast cancers, leading to its approval in 1998 by the United States Food and Drug Administration (3). The objective response rates to trastuzumab monotherapy range from 12 to 34% for a median duration of 9 months (4). Current treatment regimens combining trastuzumab with the taxane paclitaxel (5, 6) or docetaxel (7) increase response rates, time to progression, and survival.

Another HER-2-targeted monoclonal antibody, pertuzumab (Omnitarg, 2C4; Genentech), is currently being tested in Phase I clinical trials in cancer patients with different types of solid tumors. In contrast to trastuzumab, pertuzumab sterically blocks HER-2 dimerization with other HER receptors and blocks ligand-activated signaling from HER-2/EGFR and HER-2/HER-3 heterodimers (8). As the majority of breast tumors that initially respond to trastuzumab begin to progress again within ~1 year (4, 6), treatment with combined HER-2-targeting strategies may be beneficial. We demonstrate here that trastuzumab and pertuzumab synergistically block the survival of HER-2-overexpressing BT474 breast cancer cells.

Materials and Methods

Materials. Trastuzumab (Genentech) was dissolved in sterile water at 20 mg/ml. Pertuzumab (Genentech) was provided in sterile water at 25 mg/ml. The MTS CellTiter 96 Aqueous One Solution proliferation reagent (Promega, Madison, WI) was used in accordance with manufacturer guidelines. The caspase inhibitor Z-VAD-FMK was purchased from Promega Corporation (Madison, WI). Annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) were purchased from Becton Dickinson (Franklin Lakes, NJ).

Cell Culture. BT474 breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM supplemented with 10% FCS.

Dose-Response Studies. BT474 cells were seeded at 5×10^4 cells/well in 12-well dishes. After 24 h, cells were treated in triplicate with 2-fold serial dilutions of trastuzumab, pertuzumab, or both drugs simultaneously at a fixed 1:1 ratio at low doses ranging from 0.9 ng/ml to 10 ng/ml. After 5 days, cells were trypsinized and counted by trypan blue exclusion. Growth inhibition was calculated as the percentage of viable cells compared with untreated cultures. Combination indices (C.I.s) were obtained using the method of Chou and Talalay through the commercial software package Calcusyn (Biosoft, Cambridge, United Kingdom; Ref. 9). Results of trypan blue assays were confirmed by MTS assay as directed by the manufacturer. Briefly, BT474 cells were seeded at 1×10^3 cells/well in 96-well dishes. After 24 h, cells were treated in triplicate with 2-fold serial dilutions of trastuzumab, pertuzumab, or both drugs simultaneously at a fixed 1:1 ratio using the same concentrations as in trypan blue assays. In some cases, the caspase inhibitor Z-VAD-FMK was added at a fixed 20-μM concentration. After 5 days, cells were exposed to the MTS reagent, and absorbance was measured in a microplate reader. Growth inhibition was calculated as the percentage of proliferating cells compared with untreated cultures.

Cell Cycle Analysis. BT474 cells were treated in duplicate with control IgG1 (10 μg/ml; Calbiochem, San Diego, CA) or with 0.1, 1, 10, or 100 μg/ml trastuzumab and/or 0.1, 1, 10, or 100 μg/ml pertuzumab. After 5 days of drug treatment, cells were fixed overnight in 70% ethanol and were resuspended in propidium iodide (50 μg/ml) supplemented with RNase A (1 μg/ml). DNA content was measured using a FACScan cytometer (Becton Dickinson). To specifically assess cell death, cells were treated with 0.1, 1, 10, or 100 μg/ml trastuzumab and/or pertuzumab, and were stained with annexin V-PE and 7-AAD after 5 days of drug treatment. Cell death was measured as cells staining positive for annexin V, 7-AAD, or both, as assessed by fluorescence-activated cell sorting analysis.

Immunoblotting. BT474 cells were treated with 0.1, 1, or 10 μg/ml trastuzumab and/or 0.1, 1, or 10 μg/ml pertuzumab. Protein lysates were obtained after 5 days of drug treatment using 1% NP40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 8), and 1% NP40) and immunoblotted (25 μg) for poly(ADP-ribose) polymerase (PARP; polyclonal), HER-2 (monoclonal Ab-3; Oncogene Research Products, La Jolla, CA), phospho-HER-2, phospho-serine 473 Akt (monoclonal), total Akt (polyclonal), phospho-threonine 202/tyrosine 204 p44/p42 mitogen-activated protein kinase (MAPK; polyclonal), and total MAPK (polyclonal). All of the primary antibodies were from Cell Signaling Technology (Beverly, MA), unless otherwise specified, and were used at a 1:1000 dilution in 5% nonfat dried milk in PBS-Tween (PARP, total Akt, and total MAPK) or TRIS-buffered-saline-Tween (phospho-specific antibodies). Secondary antibodies

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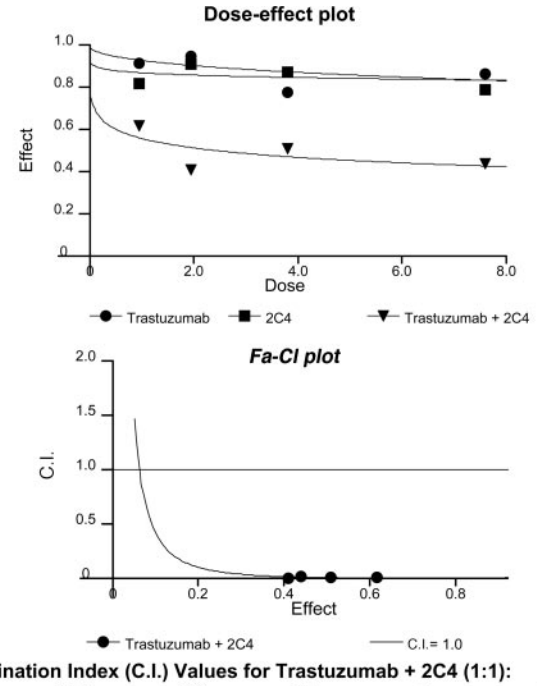
were chosen according to species of origin and were detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitation. BT474 cells were treated with 1 μg/ml trastuzumab and/or 1 μg/ml pertuzumab. Protein lysates were obtained after 24, 48, or 72 h of drug exposure. HER-2 protein (200 μg) was immunoprecipitated from lysates using a monoclonal antibody conjugated to agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates and total protein lysates were immunoblotted using the following antibodies at 1:1000 dilution: EGFR polyclonal (clone 1005; Santa Cruz Biotechnology), HER-3 polyclonal (C-17; Santa Cruz Biotechnology), and β-actin (polyclonal; Santa Cruz Biotechnology). Secondary antibodies were chosen according to species of origin and were detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech).

Results and Discussion

Trastuzumab and Pertuzumab Synergistically Inhibit the Growth of Breast Cancer Cells. HER-2-overexpressing BT474 cells were treated in triplicate with 2-fold serial dilutions of trastuzumab and/or pertuzumab at a fixed 1:1 ng/ml drug concentration ratio. Inhibition of cell survival was examined by trypan blue exclusion after 5 days of treatment, and was calculated as the percentage of viable cells relative to untreated cell cultures. Dose-response experiments were repeated three times for statistical validity. A representative dose-effect plot shows that the combination of trastuzumab and pertuzumab mediates a loss of up to 60% of cells at doses in which individual drugs do not alter cell survival (Fig. 1). Data were analyzed using the method of Chou and Talalay (9) to establish drug C.I. values. Statistically, drug synergy, addition, and antagonism are defined by C.I. values less than 1.0, equal to 1.0, or greater than 1.0, respectively. A plot of the fraction of affected cells *versus* C.I. indicates that trastuzumab and pertuzumab interact synergistically, because C.I. values are below 1.0. Furthermore, C.I. values less than 0.1, as are seen in these experiments, indicate very strong synergism between drugs. Drug synergy was further confirmed by MTS assay (not shown), showing that these antibodies synergistically inhibit the proliferation of BT474 breast cancer cells. These results demonstrate that trastuzumab and pertuzumab are highly synergistic inhibitors of BT474 breast cancer cell survival.



Combination Index (C.I.) Values for Trastuzumab + 2C4 (1:1):

ED_{50}	ED_{75}	ED_{90}	D_m	m	r
0.00983	0.00159	0.00486	2.49063	−0.25841	0.61434

Fig. 1. Trastuzumab and pertuzumab synergistically inhibit the survival of BT474 breast cancer cells. BT474 cells were treated in triplicate with 2-fold serial dilutions of trastuzumab, 2C4 (pertuzumab), or both drugs simultaneously at a fixed 1:1 ratio at low doses ranging from 0.9 to 10 ng/ml. After 5 days, cells were trypsinized and were counted by trypan blue exclusion. Growth inhibition was calculated as the fraction of viable cells compared with untreated cultures. Dose-effect and fraction affected *versus* combination index plots (*Fa-CI plots*) were generated using the method of Chou and Talalay with the commercial software package Calcusyn (Biosoft, Cambridge, UK). Doses on the X axis are stated in ng/ml. C.I. values are listed for effective doses at which 50, 75, or 90% (ED_{50}, ED_{75}, and ED_{90}, respectively) of cells are killed. Statistically drug synergy is defined by C.I. values less than 1.0, and very strong synergy is defined by C.I. values less than 0.1. D_m, the median-effect (ED_{50}) drug concentration as 2.5 ng/ml; $m < 1$ indicates a negative sigmoidal shape to the dose-effect curve; r states the linear correlation coefficient of the median-effect plot.

Trastuzumab and Pertuzumab Inhibit Proliferation and Induce Apoptosis. Flow cytometric cell cycle analysis was performed to determine whether the results of the dose-response assays were a reflection of cytostatic or cytotoxic effects due to cell cycle arrest or apoptosis. BT474 cells were treated in duplicate with a control IgG or with 100 μg/ml trastuzumab and/or pertuzumab. After 5 days of drug treatment, cells were fixed and resuspended in propidium iodide, and DNA content was measured in comparison with untreated cells (Fig. 2*A*). Trastuzumab alone induced a 3-fold increase in the subdiploid cell population, whereas 2C4 had no effect. Together, trastuzumab and 2C4 increased the subdiploid fraction by 12-fold. In addition, the combination of trastuzumab and 2C4 reduced the percentage of proliferating (S-phase) cells by more than 2-fold. Alterations in the percentages of G_1 and G_2-M cells were not significant, because similar changes were noted for cells treated with control IgG. Similar effects on the cell cycle were observed with lower concentrations of drugs at 0.1, 1, and 10 μg/ml. These flow cytometry results are consistent with dose-response experiments, indicating that a combination of trastuzumab and pertuzumab inhibits cell proliferation and survival to a greater degree than does either agent alone.

Cells were treated with 0.1, 1, 10, or 100 μg/ml concentrations of each agent or a control IgG for 5 days and were stained with annexin V-PE and 7-AAD to further assess cell death. Annexin V-PE binds to cells in early apoptosis and the fluorescent dye 7-AAD stains cells in late stages of apoptosis or cells that are already dead (10, 11). The percentages of cells staining positive for annexin V-PE and/or 7-AAD increased up to 5-fold as the concentration of drugs increased (Fig. 2*B*), indicating that trastuzumab and pertuzumab induce apoptotic cell death.

Poly(ADP-ribose) polymerase (PARP), which is cleaved by caspases during apoptosis to produce M_r 89,000 and 24,000 fragments from the full-length M_r 116,000 protein (12), was measured as a marker of apoptosis by immunoblotting. BT474 cells were treated with 0.1, 1, or 10 μg/ml trastuzumab and/or 2C4, and total protein was immunoblotted for PARP after 5 days of drug treatment (Fig. 2*C*). PARP fragments were detected in all drug-treated samples and were most abundant at the highest dose combination used (10 μg/ml concentration of each agent). These data confirm increased apoptosis in cells treated with a combination of trastuzumab and pertuzumab. In addition, the pan-caspase inhibitor Z-VAD-FMK blocked synergy between trastuzumab and pertuzumab, as measured by MTS proliferation assays (not shown), further establishing that the synergistic cytotoxicity achieved by this drug combination is due in part to apoptosis.

Trastuzumab Increases 2C4-Mediated Disruption of HER-2 Dimerization and Downstream Akt Signaling. BT474 cells were treated with 1 μg/ml trastuzumab and/or 2C4 and were lysed for protein after 24, 48, or 72 h. HER-2 was immunoprecipitated from each lysate and was immunoblotted for EGFR and HER-3 to determine effects on receptor dimerization (Fig. 3A). Combination trastuzumab-2C4 reduced HER-2 levels within 24 h to a greater degree than either single agent. Pertuzumab was previously reported to disrupt HER-2 receptor dimerization with EGFR and HER-3 (8). Although trastuzumab alone did not significantly alter levels of EGFR or HER-3 complexed to HER-2, 2C4 reduced the levels of these HER-2 dimers by 72 h. Trastuzumab in-

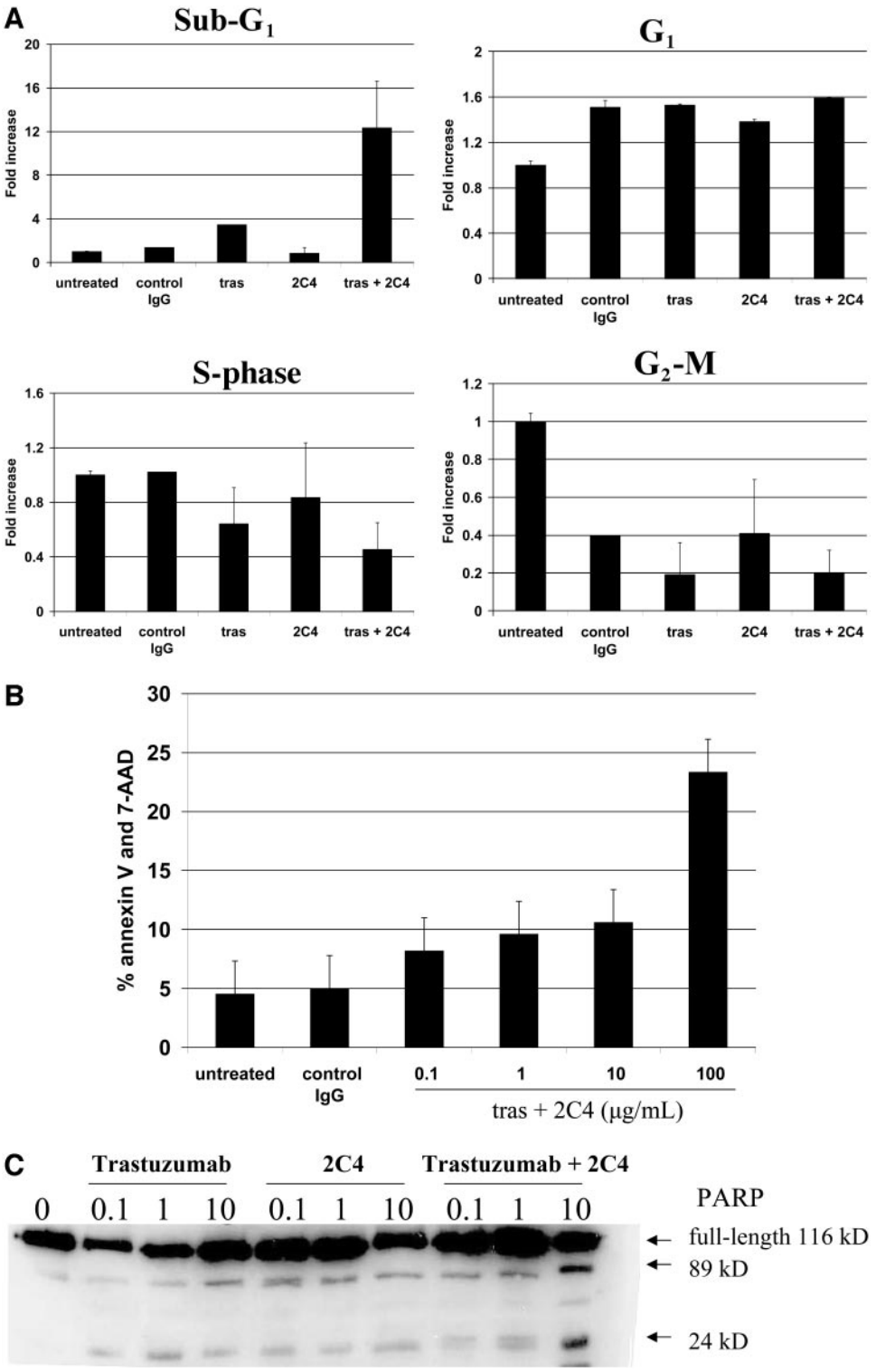


Fig. 2. Trastuzumab and pertuzumab induce apoptosis. *A*, BT474 cells were treated in duplicate with control (*IgG*) or with 100 μg/ml of trastuzumab (*tras*) and/or pertuzumab (*2C4*), cells were fixed, stained with propidium iodide, and measured for DNA content with a FACScan cytometer. The fold-increase in sub-G_1 (subdiploid), G_1, S-phase, and G_2-M cell percentages are shown. *B*, BT474 cells were treated in duplicate with control IgG or with 0.1, 1, 10, or 100 μg/ml of trastuzumab and/or 2C4. After 5 days of drug treatment, cells were stained with annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (*7-AAD*) and were analyzed for cell death on a FACScan cytometer. The percentages of cells staining positive for annexin V-PE and/or 7-AAD are shown. *C*, BT474 cells were treated in duplicate with 0.1, 1, or 10 μg/ml trastuzumab (*tras*) and/or 2C4. Protein lysates were obtained after 5 days of drug treatment and were immunoblotted (25 μg) with a poly(ADP-ribose) polymerase (*PARP*) polyclonal antibody. Cleaved PARP is represented by M_r 89,000 and 24,000 fragments, and full-length PARP is M_r 116,000. *kD*, M_r in thousands.

creased 2C4-mediated disruption of receptor dimers, because little or no EGFR and HER-3 were detectable on immunoblots by 72 h of combination drug treatment. This effect could not be due to down-regulation of HER-2 alone, as HER-2 was still detected in all of the combination drug samples at 72 h. Because total EGFR and HER-3 levels did not change on treatment with the drugs, these data suggest that trastuzumab increases the ability of pertuzumab to disrupt HER-2 receptor dimers. Furthermore, a dose-dependent down-regulation of total and phosphorylated HER-2 receptor levels was achieved by the combination of antibodies, primarily, because of 2C4 (Fig. 3*B*).

Signaling pathways activated by HER-2 include the phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK cascades. The combination of trastuzumab and 2C4 reduced levels of active phospho-serine 473 Akt to a greater degree *versus* either agent alone when administered at doses as low as 0.1 μg/ml for 72 h (Fig. 4). In contrast, signaling from the MAPK cascade was not inhibited, because levels of phosphorylated p44/p42 MAPK were unaltered by the combination of drugs.

Members of the erbB family of receptors are rarely, if ever, expressed alone, and HER-2 is frequently activated because of its role as a common coreceptor (8). The antitumor activity of trastuzumab is

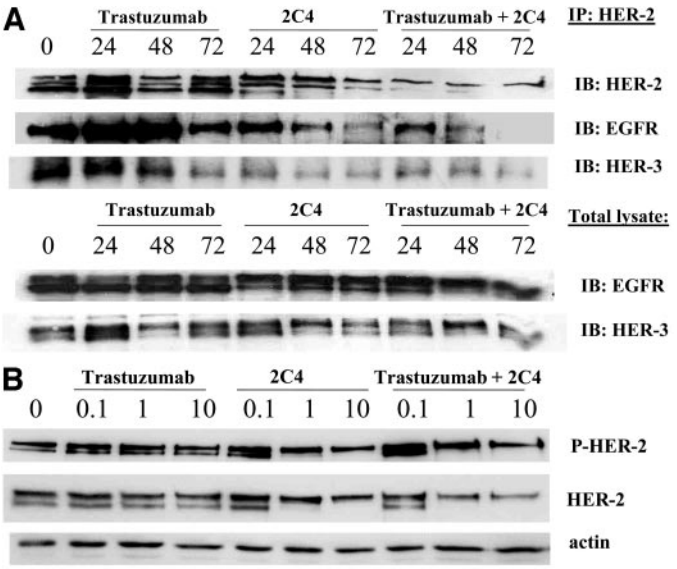


Fig. 3. Trastuzumab increases pertuzumab-mediated disruption of HER-2 dimers. BT474 cells were treated with 1 μg/ml trastuzumab and/or 1 μg/ml pertuzumab (2C4). Protein lysates were obtained after 24, 48, or 72 h of drug exposure. *A*, HER-2 protein (200 μg) was immunoprecipitated from total lysates using an agarose-conjugated monoclonal antibody. Immunoprecipitates (*IP*) were immunoblotted (*IB*) for HER-2, epidermal growth factor receptor (*EGFR*), and HER-3. Total protein lysates were also immunoblotted for total EGFR and HER-3. *B*, BT474 cells were treated with 0.1, 1, or 10 μg/ml trastuzumab and/or 2C4 for 5 days, and total protein lysates (50 μg) were immunoblotted for phosporylated (*P-HER-2*) and total HER-2.

believed to be due in part to HER-2 down-regulation with subsequent inhibition of downstream signaling and antibody-mediated immune function. However, trastuzumab does not affect signaling from other erbB receptors, nor does it inhibit ligand-activated signaling. The HER-2-targeted antibody 2C4, in contrast, inhibited heregulin-activated signaling in breast and prostate cancer models *in vitro* and *in vivo* because of dissociation of HER-2/HER-3 dimers (8, 13). Hence, dual treatment with both of the HER-2-targeted antibodies combines different mechanisms of growth inhibition. The result is synergistic cell death due at least in part to enhanced disruption of receptor dimers and reduced signaling from the Akt cell survival pathway. However, many questions regarding potential efficacy of this drug combination remain unanswered. Most importantly, we must determine what cell populations are most sensitive to combined trastuzumab-2C4 treatment. This may require developing tests to determine the level of HER-2 receptor dimers and the degree of ligand-activated signaling in individual tumors.

Trastuzumab monotherapy offers clinical benefit to a subset of HER-2-overexpressing metastatic breast cancers. However, the majority of breast cancers that initially respond to trastuzumab-containing regimens begin to progress again within 1 year (14). Thus, the potential efficacy of combination trastuzumab-2C4 in trastuzumab-resistant breast cancer cells is the focus of ongoing studies. Additionally, the effect of combining 2C4 with other erbB-targeted agents including tyrosine kinase inhibitors is of interest. Such combinations may prove to be effective not only in HER-2-overexpressing breast cancer cells, but also in cancers that overexpress EGFR or erbB ligands, as both 2C4 and tyrosine kinase inhibitors inhibit EGFR and ligand-activated signaling. Combining 2C4 with other conventional treatments such as chemotherapies may also reveal potential sensitization, as has been seen with other HER-2-targeting approaches (15). Additionally, evaluating combinations of 2C4 with antibodies that target other receptors such as EGFR or insulin-like growth factor-1 receptor (IGF-1R) is important, because these may demonstrate additive or synergistic effects. Other HER-2-targeting antibodies have demonstrated synergy *in vivo* using BT474 breast cancer xenografts (16, 17), directly supporting the findings of our present study. In conclusion, our results suggest that the combination of trastuzumab and pertuzumab may more effectively inhibit breast cancer cell survival *versus* trastuzumab alone.

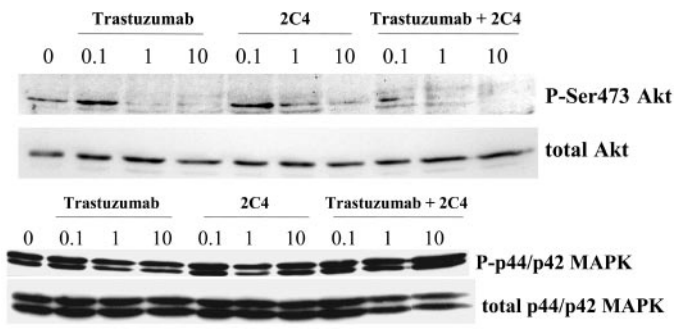


Fig. 4. Trastuzumab and pertuzumab (*2C4*) inhibit Akt signaling. BT474 cells were treated with 0.1, 1, or 10 μg/ml trastuzumab and/or 2C4 for 5 days. Total protein lysates (50 μg) were immunoblotted for phosporylated serine 473 Akt (*P-Ser473 Akt*), total Akt, phosphorylated p44/p42 mitogen-activated protein kinase (*P-p44/p42 MAPK*), and total MAPK (*total p44/p42 MAPK*).

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