Insulin-like Growth Factor-I Receptor/Human Epidermal Growth Factor Receptor 2 Heterodimerization Contributes to Trastuzumab Resistance of Breast Cancer Cells

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Abstract
The majority of breast cancer patients who achieve an initial therapeutic response to the human epidermal growth factor receptor 2 (HER-2)–targeted antibody trastuzumab will show disease progression within 1 year. We previously reported the characterization of SKBR3-derived trastuzumab-resistant pools. In the current study, we show that HER-2 interacts with insulin-like growth factor-1 receptor (IGF-IR) uniquely in these resistant cells and not in parental trastuzumab-sensitive cells. The occurrence of cross talk between IGF-IR and HER-2 exclusively in resistant cells is evidenced by the IGF-I stimulation resulting in increased phosphorylation of HER-2 in resistant cells, but not in parental cells, and by the inhibition of IGF-IR tyrosine kinase activity leading to decreased HER-2 phosphorylation only in resistant cells. In addition, inhibition of IGF-IR tyrosine kinase activity by I-Ome-AG538 increased sensitivity of resistant cells to trastuzumab. HER-2/IGF-IR interaction was disrupted on exposure of resistant cells to the anti-IGF-IR antibody α-IR3 and, to a lesser extent, when exposed to the anti-HER-2 antibody pertuzumab. Heterodimer disruption by α-IR3 dramatically restored sensitivity to trastuzumab and resistant cells showed a slightly increased sensitivity to pertuzumab versus parental cells. Neither α-IR3 nor pertuzumab decreased HER-2 phosphorylation, suggesting that additional sources of phosphorylation other than IGF-IR exist when HER-2 and IGF-IR are not physically bound. Our data support a unique interaction between HER-2 and IGF-IR in trastuzumab-resistant cells such that cross talk occurs between IGF-IR and HER-2. These data suggest that the IGF-IR/HER-2 heterodimer contributes to trastuzumab resistance and justify the need for further studies examining this complex as a potential therapeutic target in breast cancers that have progressed while on trastuzumab. (Cancer Res 2005; 65(23): 11118-28)

Introduction
The her-2 (erbB2/neu) gene is amplified and/or overexpressed in ~20% to 30% of invasive breast carcinomas and is associated with increased metastatic potential and decreased overall survival (1). Trastuzumab (Herceptin; Genentech, South San Francisco, CA) is a recombinant humanized monoclonal antibody directed against the extracellular domain of the human epidermal growth factor (EGF) receptor 2 (HER-2) tyrosine kinase receptor (2). Clinical studies established that trastuzumab is active against HER-2-overexpressing metastatic breast cancers (3–5), leading to its approval in 1998 by the U.S. Food and Drug Administration. However, the objective response rates to trastuzumab monotherapy are quite low, ranging from 12% to 34% for a median duration of 9 months (4, 5). The majority of patients who achieve an initial response to trastuzumab-based therapy acquire resistance within 1 year of treatment initiation (6, 7). Elucidating the mechanisms by which tumors escape the cytotoxic properties of trastuzumab is critical to improving the survival of metastatic breast cancer patients whose tumors overexpress HER-2.

Trastuzumab resistance has been associated with overexpression of the insulin-like growth factor-I receptor (IGF-IR; ref. 8). We previously reported the characterization of a pair of trastuzumab-resistant pools derived from the SKBR3 HER-2-overexpressing breast cancer cell line (9). In the current study, we extend this characterization by showing that (a) IGF-IR interacts with HER-2 exclusively in trastuzumab-resistant cells; (b) IGF-IR induces phosphorylation of HER-2 in resistant cells, but not parental cells; and (c) The IGF-IR tyrosine kinase inhibitor I-Ome-AG538 reduces HER-2 phosphorylation only in the resistant cells. These data suggest that cross talk occurs between IGF-IR and HER-2 in trastuzumab-resistant breast cancers that show HER-2/IGF-IR heterodimerization. Disruption of the IGF-IR/HER-2 heterodimer by the anti-IGF-IR antibody α-IR3 or the anti-HER-2 antibody pertuzumab did not affect HER-2 phosphorylation, suggesting that additional sources of HER-2 phosphorylation exist in the absence of dimerization with IGF-IR. Importantly, IGF-IR–targeted agents restored trastuzumab sensitivity to resistant cells, supporting further study of such agents for use in the context of trastuzumab-resistant breast cancers.

Materials and Methods

Materials. Trastuzumab (Genentech) was dissolved in sterile water at a stock concentration of 20 mg/mL. IGF-I (Sigma-Aldrich, St. Louis, MO) was dissolved at 100 μg/mL in PBS and used at 100 ng/mL. The IGF-IR tyrosine kinase inhibitor tyrphostin I-Ome-AG538 (Calbiochem, San Diego, CA) was dissolved in DMSO at 10 mmol/L and used at a working concentration of 1 or 10 μmol/L. The anti–IGF-IR antibody clone α-IR3 was purchased from Calbiochem. Genentech provided the anti-HER-2 antibody pertuzumab (2C4), which was dissolved in sterile water at a final concentration of 25 mg/mL.

Cell culture. SKBR3 parental and trastuzumab-resistant breast cancer cells were maintained in DMEM supplemented with 10% FCS. Trastuzumab-resistant SKBR3 pools were developed as previously described (9). Pools were maintained in 4 μg/mL trastuzumab.

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**Immunoprecipitation.** Parental and trastuzumab-resistant cells were lysed in buffer containing 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and protease and phosphatase inhibitor cocktails (Sigma Chemical Co., St. Louis, MO). IGF-IR was immunoprecipitated (polyclonal; Cell Signaling, Beverly, MA) from total protein extracts (200 µg) overnight, washed in PBS-0.1% Tween 20 buffer, and immunoblotted to detect phosphotyrosine (monoclonal PT66; Sigma Chemical); IGF-IR (polyclonal; Cell Signaling), or HER-2 (monoclonal Ab-3: Oncogene Research Products, EMD Biosciences, Inc., San Diego, CA).

**Immunoblotting.** Cells were lysed in buffer containing 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and protease and phosphatase inhibitor cocktails (Sigma Chemical). Total protein extracts (50 µg) were immunoblotted using the following antibodies at the indicated dilutions: polyclonal antibodies for EGF receptor (EGFR, integrin β3, and platelet-derived growth factor receptor (PDGFR) at 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal p-Tyr1162/1163 IR/IGF-IR at 1:500 (BioSource); HER-2 monoclonal Ab-3 at 1:1000 (Oncogene Research Products); p-Ser789-IRS-1 (monoclonal 587F11) and total and phosphorylated IRS-1 antibodies against IGF-IR, insulin receptor substrate 1 (IRS-1), Akt, p-Thr202/Tyr204 p42/p44 MAPK at 1:1,000; and total and phosphorylated EGFR, integrin β3, and PDGFR at 1:500 (Biosciences, Lincoln, NE).

**Mass spectrometry.** The silver-stained gel bands were manually cut, destained, and digested with sequencing-grade trypsin (Promega, Madison, WI). Peptides were extracted with 100 µL of 50% acetonitrile/0.01% trifluoroacetic acid (TFA) and total p42/p44 MAPK at 1:1,000 (Cell Signaling); polyclonal against p-Ser789-IRS-1 and polyclonal against p-Tyr1248-HER-2 at 1:200 (Upstate Biotechnology, Lake Placid, NY); pT27 polyclonal at 1:250 and β-actin monoclonal at 1:5,000 (Santa Cruz Biotechnology). Secondary antibodies were chosen according to the species of origin of the primary antibody and detected by enhanced chemiluminescence (Amersham-Pharmacia Biotech, Piscataway, NJ) or by using the Odyssey Imaging System (LICor Biosciences, Lincoln, NE).

**Results**

**Heterodimerization between insulin-like growth factor-1 receptor and human epidermal growth factor receptor 2 occurs uniquely in trastuzumab-resistant cells.** Reduced sensitivity to trastuzumab was previously associated with increased expression of IGF-IR in the SKBR3 breast cancer cell line (8). Thus, we examined our SKBR3-derived trastuzumab-resistant pools for expression and phosphorylation of IGF-IR. SKBR3 parental cells express low levels of IGF-IR (8) and these levels were unchanged in resistant cells (Fig. 1A). Phospho-IGF-IR levels were also similar among parental and resistant cells as shown by immunoprecipitation of IGF-IR followed by immunoblotting with an anti-phosphotyrosine antibody (monoclonal PT66), which detected phospho-IGF-IR at the expected molecular weight of 90 kDa (Fig. 1B). In addition to the 90-kDa band, a phosphoprotein migrating at ~185 kDa (Fig. 1B, asterisk) was detected in the IGF-IR immunoprecipitates. Interestingly, this protein was detected exclusively in the resistant cells. Peptide analysis identified this 185-kDa protein as HER-2 (Fig. 1C). The identity was confirmed by immunoprecipitating IGF-IR and immunoblotting for HER-2 (Fig. 1D). Again, HER-2 interacted with IGF-IR only in resistant cells, not in parental cells. Conversely, IGF-IR was detected in HER-2 immunoprecipitates from resistant cells (Fig. 1D). To determine the specificity of IGF-IR interaction with HER-2, IGF-IR immunoprecipitates were immunoblotted for EGFR, integrin β3, and PDGFR (Fig. 1E). Faint bands were observed in resistant cell lysates blotted for EGFR, which likely represents cross-reactivity of the EGFR antibody with HER-2. IGF-IR interaction with integrin β3 was similar between parental and pool 1 cells and less in pool 2 cells. PDGFR did not interact with IGF-IR in any of the lysates. These data show that IGF-IR specifically interacts with HER-2 in resistant cells as association of IGF-IR with a non-tyrosine kinase receptor transmembrane protein (integrin β3) or non-EGFR tyrosine kinase receptor (PDGFR) was not increased in resistant cells relative to parental cells. In addition, total levels of proteins with phosphotyrosine (monoclonal PT66) total and phosphorylated HER-2 were similar among parental cells and pools (Fig. 1F). These data show that IGF-IR and HER-2 interact exclusively in trastuzumab-resistant cells.

**Evidence of cross talk between insulin-like growth factor-1 receptor and human epidermal growth factor receptor 2 in trastuzumab-resistant cells.** Next, we examined the effect of IGF-IR on HER-2 phosphorylation in SKBR3 parental and resistant cells. Cells were serum starved overnight and stimulated with IGF-I (100 ng/mL) for the indicated time points. Within 5 minutes, IGF-IR phosphorylation increased in parental cells (Fig. 2A). Phosphorylation of HER-2 on Tyr1248, a major phosphorylation site involved in the oncogenic function of HER-2, was slightly decreased in IGF-1-stimulated parental cells, possibly due to feedback mechanisms because IGF-IR and HER-2 share common signaling pathways. Downstream of IGF-IR, IRS-1, phosphatidylinositol 3-kinase/Akt, and MAPK signaling were activated as shown by increased phosphorylation of Ser789 on IRS-1, Ser473 on Akt, and Thr202 and Tyr204 on p42 MAPK and p44 MAPK. In addition, IGF-1 stimulation of SKBR3 cells resulted in decreased levels of p27kip1, consistent with data reported by Lu et al. (10).

IGF-1 stimulation of resistant cells increased phosphorylation of IGF-IR within 1 minute (Fig. 2B). In contrast to parental cells, HER-2 phosphorylation on Tyr1248 increased in trastuzumab-resistant cells within 15 minutes of IGF-1 exposure. Total levels of IGF-IR and HER-2 were unaffected. Similar to parental cells, IRS-1, Akt, and MAPK...
Figure 1. Heterodimerization between IGF-IR and HER-2 occurs uniquely in trastuzumab-resistant cells. A, relative levels of total IGF-IR were assessed in parental cells and resistant pools by immunoblotting. B, IGF-IR was immunoprecipitated (200 μg) from whole-cell lysates of parental and resistant cells and immunoblotted for phosphotyrosine (monoclonal PT66). A 90-kDa band corresponding to phospho-IGF-IR (p-IGF-IR) was detected in all immunoprecipitates. A 185-kDa phosphoprotein (asterisk) was also observed and was found exclusively in trastuzumab-resistant cells (pool 1 and pool 2). Immunoblotting for IGF-IR confirmed equal levels of IGF-IR in all immunoprecipitates. C, the 185-kDa band found exclusively in the resistant cells was manually cut from a silver-stained gel. After trypsin digestion, peptides were extracted and analyzed by LC-MS/MS. Tryptic peptide coverage map; boldfaced sequences, identified by MS and matched HER-2 (gi|4758298|). D, to confirm the HER-2 identity, IGF-IR was immunoprecipitated from whole-cell lysates and immunoblotted for HER-2. Conversely, immunoprecipitation of HER-2 with immunoblotting for IGF-IR reveals the same exclusivity of the IGF-IR/HER-2 interaction in resistant cells. E, to determine the specificity of IGF-IR interaction with HER-2 in resistant cells, IGF-IR was immunoprecipitated from parental and resistant cells and immunoblotted for EGFR (a related family member of HER-2), integrin β3 (a nontyrosine kinase receptor membrane protein), or PDGFR (a non-EGFR tyrosine kinase receptor). Interaction between IGF-IR and EGFR was not observed in any of the immunoprecipitates. (Faint bands observed in resistant cell immunoprecipitates likely represent cross-reactivity of the EGFR antibody with HER-2.) As a control for PDGFR western blotting, HER-2 was immunoprecipitated and immunoblotted for PDGFR. F, whole-cell lysates from parental and resistant cells were immunoblotted for total phosphotyrosine (monoclonal PT66), HER-2 phosphorylated on tyrosine (Y) 1248, and total HER-2, all of which were expressed at similar levels in parental and resistant cells.
signaling increased in resistant cells on IGF-I stimulation. However, in contrast to parental cells, these downstream signaling pathways were stimulated more rapidly, within 1 minute versus 5 minutes in parental cells. These data suggest that cross talk occurs between IGF-IR and HER-2 in trastuzumab-resistant cells.

Reduced levels of p27kip1 were previously reported to contribute to trastuzumab resistance in these cells (9). Stimulation of serum-starved resistant cells with IGF-I resulted in down-regulation of p27kip1 (Fig. 2B), similar to parental cells (Fig. 2A). Hence, increased IGF-IR signaling is a potential upstream mechanism by which p27kip1 levels are reduced in resistant cells.

Interaction between HER-2 and IGF-IR in resistant cells was unaffected by IGF-I stimulation (Fig. 2C). Interestingly, in parental cells, IGF-I stimulation seemed to promote heterodimerization to a small degree as low levels of HER-2 were detected in IGF-IR immunoprecipitates from IGF-IR-stimulated cells. These data suggest that IGF-IR signaling may promote interaction between IGF-IR and HER-2.

Additional evidence supporting cross talk between IGF-IR and HER-2 was obtained by inhibiting IGF-IR tyrosine kinase activity and examining HER-2 phosphorylation. Parental and resistant cells were treated overnight with 1 or 10 μmol/L of the IGF-IR tyrosine kinase inhibitor I-OMe-AG538. Inhibition of IGF-IR phosphorylation was observed in both parental and resistant cells but was more pronounced in resistant cells (Fig. 3A). Total IGF-IR levels were unchanged. Phosphorylation of HER-2 was unaffected by I-OMe-AG538 in parental, trastuzumab-sensitive cells. In contrast, I-OMe-AG538 inhibited phosphorylation of HER-2 in resistant cells whereas total levels of HER-2 remained unchanged. Although it is feasible that reduced phosphorylation of HER-2 by I-OMe-AG538 is due to inhibition of other tyrosine kinases including autophosphorylation by HER-2, I-OMe-AG538 did not change phospho-HER-2 levels in parental cells, showing that this inhibitor does not directly inhibit HER-2 phosphorylation. Consistent with the finding that I-OMe-AG538 inhibited IGF-IR to a greater degree in resistant cells versus parental cells,
Figure 3. Evidence of receptor cross talk: IGF-IR tyrosine kinase inhibition reduces HER-2 phosphorylation in trastuzumab-resistant cells. A, SKBR3 parental and resistant pool 2 cells were treated with the IGF-IR tyrosine kinase inhibitor I-OMe-AG538 overnight at 0, 1, or 10 μmol/L. Western blot analysis was done for p-IGF-IR (Y1163), total IGF-IR, p-HER-2 (Y1248), and total HER-2. I-OMe-AG538 inhibited IGF-IR phosphorylation in parental and resistant cells although resistant cells showed a more significant inhibition of IGF-IR phosphorylation. IGF-IR inhibition was associated with reduced HER-2 phosphorylation in resistant cells. Downstream signaling was studied by immunoblotting protein lysates (50 μg) for p-IRS-1 (S789), total IRS-1, p-Akt (S473), total Akt, p-MAPK (Thr202/Tyr204), total MAPK (p42/p44), p27kip1, and actin. Consistent with the finding that resistant cells showed a greater decrease in IGF-IR phosphorylation on I-OMe-AG538 treatment, resistant cells showed a greater decrease in signaling through IRS-1 and Akt. MAPK signaling was unaffected. P27kip1 was modestly increased after I-OMe-AG538 treatment. B, resistant cells were treated overnight with 0, 1, or 10 μmol/L I-OMe-AG538. IGF-IR was immunoprecipitated (200 μg) and immunoblotted for HER-2 or IGF-IR. IGF-IR inhibition by I-OMe-AG538 did not affect the HER-2/IGF-IR heterodimer. C, pool 2 cells were serum starved overnight and then stimulated with 100 ng/mL IGF-I for 15 or 20 minutes, treated with 10 μmol/L I-OMe-AG538 for 6 hours, or pretreated with 10 μmol/L I-OMe-AG538 for 6 hours followed by 15 or 20 minutes of 100 ng/mL IGF-I stimulation. Lysates were immunoblotted for p-HER-2 (Y1248), total HER-2, p-IGF-IR (Y1163), and total IGF-IR. IGF-I stimulated phosphorylation of HER-2 and IGF-IR. I-OMe-AG538 at 6 hours partially blocked IGF-I-mediated IGF-IR phosphorylation. HER-2 phosphorylation was blocked in response to IGF-I when cells were pretreated with the IGF-IR kinase inhibitor. D, parental and resistant cells were pretreated with I-OMe-AG538 overnight at 0, 1, 5, or 10 μmol/L. Medium was then replaced with medium alone or medium containing trastuzumab at 0, 1, 5, or 20 μg/mL for 72 hours. Cell viability was measured by trypan blue exclusion assay. Cell viability is given as a percentage of untreated cells. All experiments were repeated at least twice. Points, mean of three iterations; bars, SD. Trastuzumab-resistant cells were highly sensitive to the IGF-IR tyrosine kinase inhibitor I-OMe-AG538 and this sensitivity was increased even further when combined with trastuzumab. Statistical significance between cell viability of trastuzumab alone versus trastuzumab combined with I-OMe-AG538 was P = 0.0355 for parental and P = 0.0023 for pool 2 cells (Student’s t test).
we observed decreased phosphorylation of IRS-1 and Akt downstream of IGF-IR in resistant cells (Fig. 3A). In addition, p27kip1 was modestly increased in resistant cells treated with I-Ome-AG538 in contrast to parental cells, supporting the concept that IGF-IR signaling may contribute to p27kip1 down-regulation in trastuzumab-resistant cells and that inhibition of IGF-IR may increase p27kip1 levels. Immunoprecipitation of IGF-IR from resistant cells showed that dimerization between HER-2 and IGF-IR was unaffected by I-Ome-AG538 (Fig. 3B).

We next examined the effect of IGF-IR kinase inhibition on IGF-I-stimulated cells. Resistant pool 2 cells were serum starved overnight and then either stimulated with IGF-I, treated with I-Ome-AG538, or pretreated with I-Ome-AG538 for 6 hours, followed by IGF-I stimulation. Immunoblotting showed that IGF-I stimulated phosphorylation of both HER-2 and IGF-IR within 15 and 20 minutes (Fig. 3C). Cells were exposed to I-Ome-AG538 for 6 hours, which only partially blocked IGF-I-mediated IGF-IR phosphorylation, in contrast to overnight I-Ome-AG538 treatment (Fig. 3A). IGF-I-mediated phosphorylation of HER-2 was blocked in resistant cells when pretreated with the IGF-IR kinase inhibitor.

We next determined if inhibition of IGF-IR tyrosine kinase activity by I-Ome-AG538 could restore trastuzumab sensitivity to resistant cells. Parental and resistant cells were treated with I-Ome-AG538 at 0, 1, 5, or 10 μmol/L for 24 hours, at which point medium was aspirated and replaced with medium containing trastuzumab at 0, 1, 5, or 20 μg/mL, doses at which these cells were previously shown to be resistant (9). Cell viability was measured after 72 hours by trypan blue exclusion assay. Parental cells showed similar sensitivity to trastuzumab and I-Ome-AG538 and pretreatment with I-Ome-AG538 slightly increased trastuzumab sensitivity at the higher doses (Fig. 3D). Resistant cells were highly sensitive to I-Ome-AG538 and sensitivity to trastuzumab was increased when cells were pretreated with I-Ome-AG538. These data suggest that the IGF-IR tyrosine kinase may be an important molecular target in trastuzumab-resistant breast cancer cells.

Thus, evidence supporting cross talk between IGF-IR and HER-2 in trastuzumab-resistant cells, but not in the parental trastuzumab-sensitive cells, includes the following: (a) IGF-I stimulated HER-2 phosphorylation in resistant cells, (b) inhibition of IGF-IR tyrosine kinase decreased HER-2 phosphorylation in resistant cells, and (c) IGF-IR inhibition suppressed IGF-I-mediated effects on HER-2 phosphorylation.

Blockade of insulin-like growth factor-1 receptor disrupts insulin-like growth factor-1 receptor/human epidermal growth factor receptor 2 heterodimerization and restores trastuzumab sensitivity. The anti–IGF-IR monoclonal antibody α-IR3 blocks the receptor-binding domain of IGF-IR and inhibits serum-dependent growth of breast cancer cells (11). Resistant cells were treated with 0.5 μg/mL α-IR3 for 30 or 60 minutes, followed by immunoprecipitation of IGF-IR and immunoblotting for HER-2 (Fig. 4A). Levels of HER-2 bound to IGF-IR were reduced as early as 30 minutes after α-IR3 treatment whereas total IGF-IR and HER-2 levels were unchanged (Fig. 4B), indicating that IGF-IR blockade by α-IR3 partially disrupts the IGF-IR/HER-2 heterodimer. Phosphorylation of IGF-IR was decreased within 60 minutes in both pools. However, α-IR3 did not decrease phosphorylation of HER-2 on Tyr1248 (Fig. 4B) or on other phosphorylation sites of HER-2 (not shown) when cells were treated up to 60 minutes. Thus, although direct inhibition of the IGF-IR tyrosine kinase by I-Ome-AG538 correlated with decreased HER-2 phosphorylation, IGF-IR antibody-mediated disruption of the IGF-IR/HER-2 heterodimer was not sufficient to inhibit phosphorylation of HER-2. These results suggest that HER-2 phosphorylation can occur independent of heterodimer formation with IGF-IR, most likely due to phosphorylation by other tyrosine kinases. However, when HER-2 is in complex with IGF-IR, IGF-IR seems to be a major contributor of HER-2 phosphorylation as stimulation or inhibition of IGF-IR signaling was associated with increased or decreased HER-2 phosphorylation, respectively.

Treatment of trastuzumab-resistant cells with α-IR3 resulted in reduced signaling downstream of IGF-IR. Within 60 minutes of exposure to α-IR3, there was reduced phosphorylation of Ser789 on IRS-1, Ser473 on Akt, and Thr202 and Tyr204 on p42 MAPK and p44 MAPK (Fig. 4B). However, expression of p27kip1 was unaffected within 60 minutes of treatment with α-IR3. These results indicate that IGF-IR signaling can be inhibited in resistant cells using an IGF-IR–targeted antibody.

We next sought to determine the biological importance of the IGF-IR/HER-2 heterodimer in the context of trastuzumab resistance. The biological effect of blocking IGF-IR with α-IR3 was studied by trypan blue exclusion cell viability assay. Pool 1 (Fig. 4C) and pool 2 (Fig. 4D) resistant cells were treated with 0.25, 0.5, or 1 μg/mL α-IR3 for 24 hours, at which point medium was replaced with medium containing trastuzumab at 1, 5, or 20 μg/mL. Trypan blue cell viability assays were done after 72 hours. Pretreatment of cells with α-IR3 dramatically restored trastuzumab sensitivity to resistant cells whereas α-IR3 alone did not significantly decrease cell survival. Hence, blockade of IGF-IR, such that it can no longer bind HER-2, facilitated trastuzumab-mediated cytotoxicity in resistant cells. These results suggest that the IGF-IR/HER-2 heterodimer contributes to trastuzumab resistance and that disruption of this complex may be a valid therapeutic approach in trastuzumab-resistant cells.

The human epidermal growth factor receptor 2–targeted antibody pertuzumab partially disrupts the insulin-like growth factor-1 receptor/human epidermal growth factor receptor 2 heterodimer and decreases viability of resistant cells. In addition to examining the response of resistant cells to IGF-IR blockade, we also examined HER-2 blockade as a potential strategy for disrupting heterodimerization and reverting trastuzumab resistance. The HER-2-targeted monoclonal antibody pertuzumab partially disrupted the interaction between IGF-IR and HER-2 (Fig. 5A) without affecting total levels of IGF-IR and HER-2 (Fig. 5B). Pertuzumab did not inhibit phosphorylation of HER-2 on Tyr1248 (Fig. 5B), again suggesting that additional sources of HER-2 phosphorylation exist when HER-2 is not bound to IGF-IR and that these sources are still active at these doses of pertuzumab. Phosphorylation of IGF-IR (Fig. 5B) and the IGF-IR downstream molecule IRS-1 (Fig. 5C) was not significantly altered by pertuzumab. Phosphorylation of Akt, which lies downstream of HER-2, was reduced in resistant cells whereas MAPK phosphorylation was unaffected, which is consistent with results observed in another HER-2-overexpressing breast cancer cell line (12). In addition, p27kip1 was slightly induced by pertuzumab in resistant cells. The biological effect of pertuzumab was studied by trypan blue viability assay in trastuzumab-resistant cells (Fig. 5D). Although not statistically significant, resistant cells showed a trend of increased sensitivity to pertuzumab relative to parental cells, particularly at lower doses. Thus, trastuzumab-resistant cells may retain the ability to respond to different HER-2-targeted therapies.

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The following novel findings are described in this study: (a) IGF-IR and HER-2 heterodimerize uniquely in trastuzumab-resistant cells. (b) IGF-I stimulates phosphorylation of HER-2 exclusively in the resistant cells. (c) IGF-IR tyrosine kinase inhibition blocks HER-2 phosphorylation only in the resistant cells. (d) Antibody-mediated blockade of IGF-IR disrupts IGF-IR interaction with HER-2 and restores trastuzumab sensitivity. To a lesser degree, HER-2 blockade also disrupts heterodimerization and decreases viability of resistant cells. In addition, resistant cells are highly sensitive to the IGF-IR tyrosine kinase inhibitor. These results suggest that cross talk exists between IGF-IR and HER-2 when these receptors are physically bound and that IGF-IR may be a suitable molecular target in breast cancers that have progressed while on trastuzumab.

Figure 4. Blockade of IGF-IR by anti–IGF-IR monoclonal antibody α-IR3 disrupts IGF-IR/HER-2 interaction and reduces downstream signaling. SKBR3-derived pools 1 and 2 were treated for 0, 30, or 60 minutes with 0.5 μg/mL of α-IR3. A, IGF-IR was immunoprecipitated from protein lysates (200 μg) and immunoblotted for HER-2 and IGF-IR. HER-2/IGF-IR heterodimerization was disrupted in cells treated with α-IR3. B, total lysates (50 μg) were immunoblotted for p-Tyr1248-HER-2, total HER-2, p-Tyr1163-IGF-IR, and total IGF-IR. Phosphorylation of IGF-IR was reduced by 60 minutes whereas total levels of IGF-IR and HER-2 were unaffected. Phosphorylation of HER-2 was unaffected by α-IR3-mediated heterodimer disruption. Total lysates (50 μg) were also immunoblotted for p-IRS-1 (Ser789), total IRS-1, p-Akt (Ser473), total Akt, p-MAPK (Thr202/Tyr204 p42/p44 MAPK), total MAPK (p42/p44), p27kip1, and actin. Reduced IGF-IR signaling was observed within 60 minutes with decreased p-IRS-1, p-Akt, and p-MAPK. p27kip1 levels were unaffected by 60 minutes of α-IR3 treatment. Pool 1 (C) and pool 2 (D) cells were pretreated with 0.25, 0.5, or 1 μg/mL of α-IR3 for 24 hours. Medium was then replaced with medium alone or medium containing 0, 1, 5, or 20 μg/mL of trastuzumab for 72 hours. Cell viability was measured by trypan blue exclusion assay and is given as a percentage of untreated cells. All experiments were repeated at least twice. Points, mean of three iterations; bars, SD. Trastuzumab sensitivity was dramatically increased when resistant cells were pretreated with α-IR3. Statistical significance between cell viability of trastuzumab alone versus trastuzumab combined with α-IR3 was P = 0.0213 for pool 1 and P = 0.0219 for pool 2 cells (Student’s t test).
than IGF-IR whereas in trastuzumab-resistant cells, HER-2 and IGF-IR interact, facilitating cross talk between IGF-IR and HER-2. Hence, IGF-I stimulation does not affect HER-2 phosphorylation in sensitive parental cells but increases HER-2 phosphorylation in resistant cells (Fig. 6A) whereas IGF-IR inhibition blocks HER-2 phosphorylation in resistant cells with no effect in sensitive cells (Fig. 6B). Importantly, IGF-I signaling may promote the interaction between HER-2 and IGF-IR as parental cells stimulated with IGF-I showed low levels of heterodimerization.

Physical interaction between IGF-IR and HER-2 was previously reported (13). However, the association of this receptor heterodimer with trastuzumab resistance is a novel and potentially clinically important finding. In contrast to Lu et al. (8), we did not find an association between total IGF-IR levels and resistance. Nonetheless, our studies support the concept that the IGF-IR may be an important molecular target in trastuzumab-resistant disease as the anti–IGF-IR antibody α-IR3 disrupted HER-2/IGF-IR interaction, blocked IGF-IR signaling, and restored trastuzumab sensitivity in resistant cells. Treatment of cells with α-IR3 for 24 hours was previously shown to down-regulate IGF-IR (14). However, when used over a shorter time course, we show that total IGF-IR levels are unaffected by α-IR3, indicating that the decreased levels of HER-2 in IGF-IR immunoprecipitates are truly due to disrupted heterodimerization and not caused by IGF-IR down-regulation. Although α-IR3 blocked IGF-IR signaling and disrupted dimerization with HER-2 in resistant cells, HER-2 phosphorylation was unaffected by 60 minutes of α-IR3 treatment. Hence, we propose that on heterodimer disruption, HER-2 phosphorylation can occur independent of IGF-IR, most likely due to phosphorylation by other tyrosine kinases, such as autophosphorylation by HER-2 and phosphorylation by EGFR (Fig. 6C and data not shown). However, when HER-2 is in complex with IGF-IR, IGF-IR seems to be a major contributor of HER-2 phosphorylation as IGF-I-mediated stimulation of IGF-IR signaling was associated with increased HER-2 phosphorylation and IGF-IR kinase inhibition was associated with decreased HER-2 phosphorylation.

Blockade of IGF-IR increased trastuzumab sensitivity to a greater degree than did HER-2 blockade. Although not statistically significant, resistant cells treated with pertuzumab did show a trend toward decreased cell viability versus parental cells, which was associated with slightly decreased phosphorylation of Akt downstream of HER-2. We previously reported synergistic cytotoxicity between trastuzumab and pertuzumab in another breast cancer cell line (12). Our current results extend these findings by suggesting that pertuzumab may remain an effective mediator of cell death once trastuzumab resistance develops. Pertuzumab has been shown to block dimerization between HER-2 and EGFR or HER-3 (15). The current study provides the first description of pertuzumab-mediated inhibition of HER-2 dimerization with a non-erbB family member (IGF-IR). Thus, alternate forms of anti-HER-2 therapy may still be beneficial in patients resistant to trastuzumab. However, as molecular profiles will vary among different tumors, it is unlikely that all trastuzumab-resistant tumors will benefit from another HER-2-targeted therapy. Some tumors may show cross-resistance, as suggested by Tanner et al. (16), who did not observe any benefit from pertuzumab in their trastuzumab-resistant JIMT-1 cell model. Interestingly, however, their cell model showed decreased binding of trastuzumab to HER-2 whereas pertuzumab was still able to bind to HER-2 (17). Thus, different HER-2-targeted agents seem to have different effects on trastuzumab-resistant cells.
Several molecular mechanisms have been proposed to contribute to trastuzumab resistance. The JIMT-1 model mentioned above showed increased expression of the cell surface mucin MUC-4 (17). The MUC-4 level was inversely correlated with the trastuzumab-binding capacity of single cells, and knockdown of MUC-4 increased trastuzumab sensitivity of JIMT-1 cells. Thus, the authors proposed that elevated MUC-4 expression masks the trastuzumab-binding epitopes of HER-2, which decreases the interaction between this antibody and its therapeutic target, resulting in drug resistance. Interestingly, the authors also state that HER-2 is unable to interact with other proteins, such as EGFR or HER-3, due to masking by MUC-4. This is in contrast to

Figure 6. Proposed model for IGF-IR signaling and cross talk in trastuzumab resistance. A, in trastuzumab-sensitive cells, IGF-I stimulates IGF-IR signaling, resulting in phosphorylation of IGF-IR, IRS, and Akt. HER-2 is phosphorylated by other membrane receptor tyrosine kinases such as EGFR (arrows) and by autophosphorylation. In trastuzumab-resistant cells, IGF-I stimulates IGF-IR signaling with increased phosphorylation of IGF-IR, IRS, and Akt. Interaction between IGF-IR and HER-2 facilitates IGF-IR signaling to HER-2, resulting in increased HER-2 phosphorylation. B, in trastuzumab-sensitive cells, IGF-IR tyrosine kinase inhibition partially blocks phosphorylation and activation of IGF-IR signaling. HER-2 phosphorylation is still regulated by other membrane receptor tyrosine kinases and by autophosphorylation. Trastuzumab-resistant cells are more sensitive to IGF-IR tyrosine kinase inhibition, which results in a dramatic decrease in IGF-IR downstream signaling as well as significantly decreased cross-signaling to HER-2 even in the presence of IGF-I. This inhibition of IGF-IR activity results in increased trastuzumab sensitivity. C, antibody-mediated blockade of IGF-IR disrupts receptor interaction and diminishes IGF-IR signaling in trastuzumab-resistant cells. HER-2 is maintained in a phosphorylated and active state perhaps due to activity of other membrane receptor tyrosine kinases and autophosphorylation (Nahta and Esteva, unpublished results). IGF-IR blockade results in a dramatic restoration of trastuzumab sensitivity, supporting the concept that IGF-IR is a promising molecular target in this subset of breast cancers.
the mechanism we describe here in which HER-2 shows increased interaction with a non-EGFR tyrosine kinase receptor (IGF-IR). Increased coexpression and interaction of HER-2 with EGFR family members has also been shown to affect trastuzumab response in SKBR3 cells (18). In addition, increased levels of erbB ligands herigelin and EGF blocked trastuzumab-mediated growth inhibition of SKBR3 (18) and BT474 cells (19). The erbB kinase inhibitor RALT/MIG-6 was shown to counteract the ability of erbB ligands to promote trastuzumab resistance (20). Thus, molecular profiling of tumor cells to determine the relative levels of other EGFR family members and their ligands may be useful to predict sensitivity to trastuzumab. In addition, blocking multiple growth factor receptors simultaneously may increase sensitivity.

Another very important and interesting potential mechanism of trastuzumab resistance, described by Nagata et al. (21), is decreased expression of the phosphatase and tensin homologue (PTEN). The authors show that when trastuzumab is effective, it causes dissociation of Src from HER-2, freeing PTEN to inhibit Akt and induce growth arrest. However, when PTEN levels are low, Akt remains active and trastuzumab response is reduced. Importantly, they validated their cell culture model in tumor tissues and showed that PTEN deficiency is associated with poor response to trastuzumab. The trastuzumab-resistant model used in our current study did not show any changes in Akt activity relative to parental cells nor any difference in sensitivity to Akt inhibiting agents (not shown). Thus, the mechanism contributing to resistance in our SKBR3-derived model is clearly different.

Our proposed mechanism supports the findings of Lu et al. (10) in which IGF-1 is shown to increase p27kip1 degradation and decrease sensitivity to trastuzumab. The authors also showed in a prior publication that overexpression of IGF-IR was associated with trastuzumab resistance (8). Although total IGF-IR levels were not elevated in our resistant model versus the parental cells, we show that IGF-IR signals to HER-2 as part of a heterodimer in resistant cells and that blockade of IGF-IR signaling is a potentially important therapeutic strategy in these cells. Similar to Lu et al. (10), we observed decreased p27kip1 levels in response to IGF-1 in parental SKBR3 cells. Resistant cells also showed p27kip1 down-regulation on IGF-1 stimulation. We previously showed that p27kip1 down-regulation contributes to trastuzumab resistance in these cells (9). Thus, our current results suggest that a potential molecular mechanism by which IGF-IR/HER-2 contributes to trastuzumab resistance may be increased signaling such that p27kip1, a critical mediator of responsiveness to trastuzumab (22, 23), is down-regulated.

Future studies will examine the IGF-IR/HER-2 heterodimer as a potential target in trastuzumab-resistant breast cancers. We showed here that blockade of IGF-IR/HER-2 heterodimerization using an anti–IGF-IR antibody resulted in increased trastuzumab sensitivity and that resistant cells are highly sensitive to the IGF-IR tyrosine kinase inhibitor. These results are important as they suggest potential experimental therapeutics that can be further developed preclinically for testing against trastuzumab-resistant disease. Combination regimens targeting both HER-2 and IGF-IR should also be tested preclinically given our research findings. Therapies that target multiple growth factor receptors including IGF-IR and HER-2 have been described and seem to produce benefit in trastuzumab-resistant cells (24). Our current studies seek to validate the heterodimer as a marker of resistance to trastuzumab-based therapy in primary breast cancer tissue. Additionally, preclinical studies using anti–IGF-IR agents being developed for clinical use are being done to establish the IGF-IR/HER-2 heterodimer as a valid therapeutic target in trastuzumab-resistant breast cancers. Elucidating molecular markers of trastuzumab resistance and validating novel therapeutic targets such as the IGF-IR/HER-2 heterodimer are critical for increasing survival rates in this subset of breast cancer patients.

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References


Correction: IGF-IR/HER-2 Contributes to Trastuzumab Resistance

In the article on how IGF-IR/HER-2 contributes to trastuzumab resistance in the December 1, 2005 issue of Cancer Research (1), Fig. 2A is incorrect. The corrected panel appears below.

![Corrected Panel](image)

Insulin-like Growth Factor-I Receptor/Human Epidermal Growth Factor Receptor 2 Heterodimerization Contributes to Trastuzumab Resistance of Breast Cancer Cells

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