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Mechanisms of Adipocytokine-Mediated Trastuzumab Resistance in HER2-Positive Breast Cancer Cell Lines

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Abstract

Acquired resistance to trastuzumab is a clinical problem in the treatment of HER2-over-expressing metastatic breast cancer. Importantly, an earlier report suggested that high body mass index was associated with reduced overall survival and reduced time to progression in patients with early stage or metastatic HER2-positive breast cancer treated with trastuzumab. Adipocyte-secreted factors may stimulate growth of HER2-positive cancers, blocking the growth inhibitory action of trastuzumab. Leptin and growth differentiation factor 15 (GDF15) are two adipocytokines that have been reported to stimulate HER2-PI3K signaling. We previously showed that cells with acquired trastuzumab resistance express increased levels of GDF15, and that GDF15 knockdown restores sensitivity to trastuzumab. The objective of the current study was to identify potential molecular mechanisms by which adipocytes stimulate resistance to trastuzumab in HER2-over-expressing breast cancer cell lines. Cells were cultured in complete media or conditioned media from differentiated adipocytes (CM). Cell viability of trastuzumab-treated cells was examined under anchorage-dependent and -independent conditions. Phosphorylation of Akt was assessed by Western blotting, and response to trastuzumab was reassessed upon treatment with the PI3K inhibitor LY294002 or after transfection with kinase-dead Akt. We report that CM significantly reduced trastuzumab-mediated growth inhibition of HER2-positive cells, and stimulated rapid phosphorylation of Akt. Pharmacologic or genetic inhibition of PI3K overcame CM-mediated trastuzumab resistance. Leptin and GDF15 were both measured in CM, but only GDF15 conferred resistance to trastuzumab. Leptin, on the other hand, abrogated sensitivity to lapatinib but not trastuzumab. Our observations suggest that adipocyte-secreted factors such as GDF15 stimulate PI3K signaling, resulting in reduced response to trastuzumab. The utility of adipocytokines as predictors of drug resistance and approaches to mitigate the cancer-promoting effects of adipocyte-secreted factors should be further examined. Our work supports additional investigation into GDF15 as a potential biomarker of trastuzumab resistance, and development of approaches to therapeutically target GDF15 in HER2-positive breast cancers that have progressed on trastuzumab.

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CONFLICTS OF INTEREST

None declared/applicable.

Keywords

Cancer therapeutics; GDF15; drug resistance; erbB2; Herceptin; lapatinib; pharmacogenetics; trastuzumab

INTRODUCTION

HER2 receptor tyrosine kinase over-expression and activation is found in approximately one-third of metastatic breast cancers, and is associated with reduced disease-free survival [1]. Trastuzumab (Herceptin; Genentech, South San Francisco, CA), a humanized anti-HER2 monoclonal antibody, is the major first-line treatment for HER2-positive breast cancer. Most patients show an initial response to trastuzumab plus chemotherapy, but many eventually develop disease progression [2–7]. While multiple mechanisms of acquired trastuzumab resistance have been proposed in the literature, few have translated into clinical application. The most widely studied mechanism of resistance is hyper-activated PI3K signaling, generally due to *PIK3CA* mutation, PTEN down-regulation, or increased growth factor signaling.

Recent data suggests that obesity, defined as a body mass index (BMI) greater than 30 kg/m², may affect progression of HER2-positive breast cancer and response to trastuzumab. In one study, a high fat diet, which caused a small, but statistically significant weight gain, also resulted in a tumor-promoting effect in MMTV-*HER2* transgenic mice which was not linked to insulin resistance [8]. However, there have been studies that suggest that there is not a correlation, or possibly even an inverse relationship, between BMI and HER2-positive breast cancer [9, 10]. Interestingly, in a multivariate analysis of response to neoadjuvant chemotherapy, BMI was not predictive of pathologic complete response (pCR) in the total population of 256 breast cancer patients [11]. However, in the HER2-positive subgroup, BMI was an independent predictive factor of pCR ($p=0.001$), with approximately half of the patients receiving trastuzumab. Amongst luminal A, luminal B, and triple negative tumors, BMI alone was not predictive of pCR. In addition, amongst 103 patients with HER2-positive breast cancer treated with adjuvant trastuzumab, high BMI was associated with reduced overall survival and time to progression for patients who had relapsed [12]. Further, in the N9831 phase III trial of adjuvant trastuzumab in 3,017 high risk patients with early stage HER2-positive breast cancer, obesity was associated with worse clinical outcome, although trastuzumab improved clinical outcome in all groups regardless of BMI [13]. These results suggest a potential link between obesity and resistance to chemotherapy and trastuzumab.

Although the mechanistic basis for the potential correlation between obesity and trastuzumab remains unknown, the increased secretion of adipocytokines and their signaling effects on breast cancer cells may contribute to drug resistance. Leptin is one of the best-studied adipocyte-secreted factors in cancer research. Previous work showed that the leptin receptor and HER2 physically interact [14], and that leptin induces rapid phosphorylation of HER2 in breast cancer cells that express normal levels of HER2 [14] and in HER2-over-expressing breast cancer cells [15]. We recently reported that the cytokine growth differentiation factor 15 (GDF15, MIC-1) stimulates phosphorylation of HER2 and downstream PI3K/MAPK signaling [16]. Cells with acquired or primary resistance to trastuzumab expressed significantly higher levels of endogenous and secreted GDF15 [16]. In addition to being secreted by trastuzumab-resistant breast cancer cells, GDF15 has been reported to be secreted by adipocytes [17]. Further, adipocyte culture media has been shown to stimulate secretion of GDF15 from breast cancer cells in association with increased cancer cell invasiveness [18].

In the current study, we used HER2-over-expressing breast cancer cell lines to determine the effects of adipocyte-secreted factors on sensitivity to trastuzumab.

MATERIAL AND METHODS

Materials

Trastuzumab was purchased from the Winship Cancer Institute pharmacy and dissolved with sterile water provided by the manufacturer at a stock concentration of 20 mg/mL. LY294002 (Cell Signaling; Danvers, MA) was dissolved in DMSO at 10 mM stock concentration. Lapatinib (Santa Cruz, Biotech, Santa Cruz, CA) was dissolved in DMSO at a stock concentration of 10 mM. Recombinant human leptin (EMD Biosciences, San Diego, CA) was dissolved at 1 mg/mL in PBS. Recombinant human GDF15 (rhGDF15; R&D Systems, Minneapolis, MN) was dissolved to a final stock concentration of 200 µg/mL in 4mM HCl containing 0.1% BSA vehicle.

Cancer Cell Culture

BT474 and SKBR3 HER2-over-expressing breast cancer cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were maintained in DMEM + 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), and cultured in humidified incubators at 37°C with 5% CO₂.

Adipocyte Cell Culture and Differentiation

3T3-L1 murine pre-adipocytes and human visceral omental pre-adipocytes obtained from a patient with a BMI > 30 kg/m² (obese) were purchased from Zen-Bio (Research Triangle Park, NC). 3T3-L1 cells were maintained in high-glucose DMEM + 10% FBS + 1% P/S for days 0 through 6 (Fig. (1A)). During days 6 to 9, cells were cultured in Differentiation Medium (DM-2-L1; Zen-Bio), which contains isobutylmethylxanthine and a PPARγ agonist. Differentiation was confirmed visually by the presence of lipid droplets (Fig. (1B)). Differentiated cells were then cultured in Maintenance Medium (AM-1; Zen-Bio). To obtain adipocyte conditioned media (CM), cells were incubated in serum-free Maintenance Medium (AM-1-SF; Zen-Bio) for 2 days prior to collecting media from cells. Human visceral pre-adipocytes were maintained in visceral pre-adipocyte media (OM-PM; Zen-Bio); differentiation was stimulated by culturing in visceral differentiation media (OM-DM; Zen-Bio). To obtain adipocyte conditioned media, cells were incubated in serum-free media for 2 days prior to collecting media from cells.

Trypan Blue Exclusion Assay

For growth inhibition assays, breast cancer cells were plated in complete DMEM at 3×10^4 per well in 12-well plate format. The next day, media was aspirated and replaced with complete DMEM, 3T3-L1 CM, Omental CM, or adipocyte serum starvation media. Cells were then treated with trastuzumab, LY294002, lapatinib, GDF15, and/or leptin for an additional 48 or 72 hours. Control cultures were treated with appropriate vehicle (solvent) for each drug or cytokine. Viable cells were counted under a light microscope by trypan blue exclusion assay.

Matrigel Growth Assay

BT474 cells were plated in matrigel (1:1) with complete DMEM or 3T3-L1 CM. Cells were untreated or treated with 20 µg/mL trastuzumab. Media plus drug was changed twice a week for 3–4 weeks. Matrigel was then dissolved using dispase (Sigma), and viable cells were counted by trypan blue exclusion.

Western Blotting

Cells were lysed in RIPA buffer (Cell Signaling; Danvers, MA) supplemented with protease and phosphatase inhibitors (Cell Signaling). Total protein extracts were run on SDS-PAGE and blotted onto nitrocellulose. Blots were probed overnight using p-S473 Akt-XP (Cell Signaling; 1:1000), polyclonal antibody against Akt (Cell Signaling; 1:1000), or α -actin monoclonal AC-15 (Sigma-Aldrich; 1:10,000). Protein bands were detected using the Odyssey Imaging System (Li-Cor Biosciences; Lincoln, NE).

Transfection

Cells were plated in antibiotic-free media. The next day, cells were transfected using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) with 1 μ g of one of the following plasmids: dominant negative kinase dead Akt1 mutant (pcDNA3-Akt1-K179A), constitutively active Akt1 mutant (pcDNA3-Akt1-T308D/S473D), or pcDNA3 empty vector control (plasmids generously provided by Dr. Keqiang Ye, Emory). Media was changed after 6 hours.

ELISA

To quantify the amount of GDF15 and leptin released into the media from cells, human GDF15 or leptin immunoassay (R&D Systems, Minneapolis, MN) was used according to the manufacturer's directions. Briefly, sample media was incubated in GDF15/leptin antibody-coated microplate for 2 hours, which was then washed 4X and incubated with GDF15/leptin antibody conjugated to horseradish peroxidase for 1 hour. After washing 4X the wells were incubated with color reagent (hydrogen peroxide-chromogen mix) for 30 minutes, at which point the stop solution was added. Optical density of each well was determined using a microplate reader set to 450nm. The concentrations were calculated according to the standards supplied with the kit by creating a four parameter logistic curve-fit.

Statistical Analysis

Significance of differences was determined using the Student's t-test in Microsoft Excel 2007 for each experimental group versus control group. A two sided p-value of less than 0.05 was considered significant.

RESULTS

Adipocyte-Conditioned Media Confers Resistance to Trastuzumab

Growth of BT474 HER2-over-expressing breast cancer cells was inhibited by trastuzumab as expected (Fig. (2A)). However, when BT474 cells were cultured in CM collected from differentiated 3T3-L1 adipocytes, sensitivity to trastuzumab was lost. The adipocyte serum starvation media alone did not affect trastuzumab sensitivity, indicating that CM-mediated resistance to trastuzumab is due to factors secreted into the adipocyte media within the 48 hour starvation period. In addition to using 3T3-L1 murine adipocytes, we also differentiated human omental pre-adipocytes, which were originally collected from an obese subject. Trastuzumab-mediated growth inhibition was significantly reduced when BT474 cells were cultured in omental adipocyte CM (Fig. (2B)).

Next, BT474 cells were grown in matrigel to determine if CM mediates trastuzumab resistance under anchorage-independent conditions. Quantification of colony growth showed that the presence of 3T3-L1 CM significantly abrogated trastuzumab-mediated growth inhibition (Fig. (3)).

Increased PI3K Signaling Contributes to Trastuzumab Resistance Stimulated by Adipocyte Conditioned Media

Stimulation of BT474 cells with adipocyte CM resulted in rapid phosphorylation of Akt, in contrast to incubation in serum starvation media (Fig. (4)). Further, while 3T3-L1 CM abrogated trastuzumab-mediated growth inhibition, the PI3K inhibitor LY294002 partially blocked this effect (Fig. (5A)). Transfection of BT474 cells with constitutively active Akt did not reduce response to trastuzumab or affect CM-mediated trastuzumab resistance. However, kinase-dead Akt rescued the trastuzumab-resistant phenotype conferred by adipocyte CM (Fig. (5B)). Both pharmacologic and genetic inhibition of Akt suppressed the ability of adipocyte CM to confer resistance to trastuzumab, indicating that PI3K activation may be one molecular mechanism mediating adipocyte-stimulated trastuzumab resistance.

Differential Effects of Adipocytokines Leptin and GDF15 on Response to HER2-Targeted Agents

ELISA experiments showed that the adipocytokines leptin and GDF15 are found in human omental adipocyte CM (Fig. (6)). Treatment of SKBR3 HER2-over-expressing breast cancer cells with leptin suppressed the growth inhibitory effects of the dual EGFR/HER2 tyrosine kinase inhibitor lapatinib (Fig. (7A)). However, leptin did not significantly reduce trastuzumab-mediated growth inhibition of SKBR3 cells. In contrast, the adipocytokine GDF15 significantly reduced trastuzumab sensitivity in BT474 and SKBR3 cells, without affecting lapatinib-mediated growth inhibition (Fig. (7B)).

DISCUSSION

Our results indicate that adipocyte conditioned media reduces response of HER2-positive breast cancer cells to the HER2-targeted drug trastuzumab (Fig. (8)). Obesity has been associated with worse prognosis in several subtypes of breast cancer. Recent studies indicated that patients with HER2-positive breast cancer may have a worse clinical outcome if they have a BMI greater than 30 kg/m², indicating obesity. Our results support the concept that an adipogenic state may affect drug sensitivity in this specific breast cancer subtype.

In our experiments, we mimicked the endocrine state of obesity by culturing HER2-positive breast cancer cells in conditioned media collected from differentiated murine adipocytes or human omental adipocytes collected from the visceral omentum of an obese patient. Both sources of adipocyte CM suppressed trastuzumab-mediated growth inhibition, as measured by trypan blue exclusion assays. A limitation of trypan blue assays is that cells undergoing early apoptosis may not become stained, resulting in an artificially lower measurement of cell death. Matrigel assays provided further confirmation that CM reduced growth inhibition by trastuzumab. Overall, these results suggest that obesity may limit sensitivity of HER2-positive cells to trastuzumab, and that therapeutic or lifestyle management strategies to reduce BMI may improve therapeutic response.

Our data suggest that adipocyte CM activates PI3K signaling, which then contributes to trastuzumab resistance. PI3K inhibition using pharmacologic inhibitor LY294002 or kinase-dead Akt increased sensitivity to trastuzumab in the presence of adipocyte CM. PI3K hyperactivity due to *PIK3CA* mutation, PTEN down-regulation, or increased upstream growth factor signaling has been correlated with trastuzumab resistance in pre-clinical models and in clinical studies. Unexpectedly, transfection of constitutively active Akt alone did not reduce responsiveness of BT474 cells to trastuzumab. This may be due to baseline endogenous hyper-activation of Akt present in the BT474 cell line. Although BT474 express PTEN and the *PIK3CA* mutation (K111N) present in these cells is only weakly oncogenic compared to H1047R, BT474 cells still express high baseline levels of phosphorylated T308

and S473 Akt [19]. Thus, transfection of CA-Akt may have increased phosphorylation of Akt only mildly relative to the endogenous phospho-Akt level. However, our data are consistent with previous studies showing that PI3K/Akt/mTOR inhibition can overcome trastuzumab resistance [20–22]. Thus, increased adipocytokine signaling may be another mechanism by which PI3K is activated in resistant cells. Multiple PI3K inhibitors are being developed for clinical use in patients with breast cancer. Our work suggests a new therapeutic context for these inhibitors- in patients with high BMI and HER2-positive breast cancer.

Obesity is considered an endocrine disorder, with increased concentrations of growth factors and cytokines circulating in the serum of obese patients. The signaling effect of these cytokines is thought to contribute to the putative link between adiposity and cancer progression. Two adipocytokines linked to trastuzumab resistance are leptin and GDF15. Leptin and GDF15 have both been shown to activate HER2 signaling in breast cancer cells [14–16]. In addition, we showed that HER2-positive breast cancer cells that have primary or acquired resistance to trastuzumab express elevated levels of GDF15 [16]. Knockdown of GDF15 improved sensitivity to trastuzumab in resistant cells [16]. The work presented here further confirms that GDF15 reduces trastuzumab-mediated growth inhibition, but does not appear to inhibit lapatinib-mediated cytotoxicity.

Similarly, previous reports showed that leptin stimulated phosphorylation of HER2 in MCF7 [14] and SKBR3 [15] breast cancer cells. Co-immunoprecipitation and immunofluorescence experiments showed that leptin receptor and HER2 interact and co-localize in MCF7 breast cancer cells [14]. These results support the concept of cross-talk and interaction between leptin receptor and HER2, similar to the concept of cross-talk to HER2 being mediated by GDF15. In a small IHC correlative study, HER2 over-expression did not correlate with leptin receptor co-expression in breast tumor tissues [14]. However, the clinical significance between HER2 phosphorylation and levels of secreted leptin has not yet been determined in human samples.

Similar to HER2, IGF-IR has also been shown to interact with leptin receptor and to become phosphorylated upon stimulation with leptin [23]. IGF-IR signaling has been implicated in trastuzumab resistance. Thus, it is possible that co-expression of multiple adipocytokines (leptin, IGF-I, GDF15) may activate HER2 as well as additional cell surface receptors with which HER2 interacts (i.e. leptin receptor and IGF-IR). Conversely, in our experiments, leptin abrogated lapatinib-mediated growth inhibition of BT474 cells, but did not reduce trastuzumab-mediated growth inhibition. Thus, differential signaling from individual adipocyte-secreted factors may elicit distinct responses depending on the treatment. A similar trend was shown for IGF-IR signaling, which led to trastuzumab resistance [24, 25], but not resistance to lapatinib [26]. Further analysis of whether increased circulating levels of GDF15 predict for poor response to trastuzumab, or increased leptin predicts for reduced response to lapatinib may be warranted.

In addition, leptin and IGF-I have been shown to induce invasiveness of breast cancer cells in part by transactivating EGFR [27]. We recently showed that GDF15 promotes invasion and epithelial-to-mesenchymal transition (EMT) in ovarian cancer cells [28]. Thus, adipocytokines IGF-I, leptin, and GDF15 should be studied for their effects on motility and metastasis of breast cancer cells, and whether increased levels of circulating adipokines correlates with progression and metastatic disease.

A limitation of our study is that the results require further verification *in vivo* using animal models or human tumor samples. We showed that conditioned media from human omental adipocytes collected from an obese patient also conferred trastuzumab resistance and had

measurable levels of secreted GDF15. Future studies should examine activation of PI3K in HER2-positive cells stimulated with human omental CM and determine whether PI3K inhibition rescues omental CM-mediated resistance. Further, xenografts of HER2-positive cells maintained long-term in CM may be developed to determine if they are resistant to trastuzumab *in vivo*, and whether PI3K inhibition restores trastuzumab sensitivity to CM-maintained cells. Finally, future studies will examine the role of ER and IGF-IR signaling in CM-stimulated resistance. Obesity has been associated with an increased risk of ER-positive breast cancer [29, 30]. Our data suggests that while adipocyte CM confers resistance to trastuzumab in ER-positive, HER2-positive breast cancer cells, ER-negative cells do not become resistant when stimulated with adipocyte conditioned media (Griner and Nahta, unpublished), suggesting that ER signaling may contribute to CM-mediated trastuzumab resistance. Higher circulating levels of IGF-I and downstream activation of mTOR signaling have also been documented in patients with high BMI and in animal models of obesity [29]. Inhibition of mTOR has been shown to suppress obesity-driven mammary tumors [31] and trastuzumab resistance [20]. Similarly, we have shown that inhibition of IGF-IR overcomes acquired resistance to trastuzumab [25, 32].

Our data suggest that adipocyte signaling and GDF15 stimulation contribute to trastuzumab resistance. The implications of these new findings are several fold. (1) GDF15 expression levels and/or BMI may be used to predict resistance to trastuzumab, (2) GDF15 may serve as a novel molecular target for improving response to trastuzumab. (3) Adipocytokines may stimulate resistance via PI3K activation. Published work suggests that increased BMI may be associated with reduced response to trastuzumab. Our studies support the hypothesis that adipocytokines stimulate PI3K signaling and trastuzumab resistance in HER2-positive breast cancer cell lines. Adipocyte CM-mediated resistance was associated with increased expression of adipocytokine GDF15. Combined analysis of BMI, PI3K hyper-activation, and serum levels of GDF15 may serve as a predictor of resistance to trastuzumab, facilitating a personalized therapeutic approach for HER2-over-expressing breast cancers. Since GDF15-over-expressing cells appeared to maintain sensitivity to lapatinib, individualized therapy in the setting of GDF15 over-expression may include HER2 kinase inhibition.

In summary, we showed that conditioned media from human omental or mouse adipocytes confers acquired resistance to trastuzumab in HER2-positive breast cancer cells. Adipocyte CM stimulated phosphorylation of Akt, and PI3K inhibition rescued CM-mediated resistance. Further, GDF15 and leptin were secreted in CM, but only GDF15 appeared to confer resistance to trastuzumab. Ongoing studies continue to examine the molecular mechanisms by which GDF15 confers resistance, whether GDF15 inhibition improves trastuzumab response, and additional mechanisms by which adipocytes confer resistance. These studies are necessary for understanding how obesity affects response to treatment in HER2-positive breast cancers. Once clinical correlations have been validated between high BMI, high serum levels of GDF15, and poor response to trastuzumab, these markers may collectively be established as novel predictors of trastuzumab resistance. Such studies can also usefully inform the selection and prioritization of new lead compounds for targeted therapeutics in the broad field of cancer clinical pharmacology [33, 34].

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of data; drafting the article or revising it critically for important intellectual content; and approved the final version to be published. The views expressed are entirely the personal opinions of the authors.

ABBREVIATIONS

GDF15	growth differentiation factor 15
CM	adipocyte conditioned media
pCR	pathologic complete response
BMI	body mass index
EGFR	epidermal growth factor receptor
ER	estrogen receptor
HER2	human epidermal growth factor receptor 2
IGF-IR	insulin-like growth factor-I receptor
PI3K	phosphatidylinositol 3 kinase
PTEN	phosphatase and tensin homolog

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Fig. 1.

A) A schematic of the individual cell culture stages required to stimulate pre-adipocyte differentiation is shown. **B)** Representative photos of stages 1–3 are shown at 5X, with inserts magnified at 20X to demonstrate the formation of lipid droplets within the cells after differentiation.

Fig. 2.

A) BT474 HER2-over-expressing cells were cultured in complete DMEM media containing 10% FBS, conditioned media (CM) from differentiated 3T3-L1 mouse adipocytes, or 3T3-L1 serum starvation media (SS). **B)** BT474 cells were cultured in complete DMEM media containing 10% FBS or conditioned media from differentiated human omental adipocytes. For both (A) and (B), cells were untreated (white bars) or treated with 20 μ g/mL trastuzumab (black bars) for 72 h, at which point cells were counted by trypan blue exclusion. The percentage of viable cells is shown relative to the untreated control per group. All groups were run in triplicate, and error bars represent standard deviation between triplicates. Experiments were repeated at least twice with reproducible results; p-values were determined by two-tailed student's t-test. Adipocyte conditioned media from 3T3 or human

omental cells reduced trastuzumab-mediated growth inhibition of BT474 cells, suggesting that adipocyte-secreted factors may confer resistance to trastuzumab.

Fig. 3.

BT474 breast cancer cells were plated in matrigel, and maintained in 3T3 CM or complete DMEM, and untreated or treated with 20 $\mu\text{g}/\text{mL}$ trastuzumab. Media plus drug was changed twice a week for 3–4 weeks. Matrigel was dissolved using dispase and cells were counted by trypan blue exclusion. Each group was run in triplicate; error bars represent standard deviation between triplicates; p-values were determined by two-tailed student's t-test. The experiment was performed twice with reproducible results. Adipocyte conditioned media abrogated trastuzumab-mediated growth inhibition of BT474 cells in matrigel, again supporting the concept that adipocyte-secreted factors reduce trastuzumab sensitivity.

Fig. 4.

BT474 cells were serum starved overnight, and then stimulated with adipocyte serum starvation medium (SS) or conditioned media (CM) from differentiated 3T3 adipocytes for 5, 15, or 30 minutes. Total protein lysates were Western blotted for p-S473 Akt and total Akt. The experiment was performed three times; representative blots are shown. 3T3 CM stimulated phosphorylation of Akt.

Fig. (5A)

Fig. (5B)

Fig. 5.

A) BT474 cells were cultured in complete DMEM or conditioned media (CM) from differentiated 3T3 adipocytes, and untreated, treated with 20 $\mu\text{g}/\text{mL}$ trastuzumab, vehicle control DMSO, or 20 $\mu\text{g}/\text{mL}$ trastuzumab plus 1 μM PI3K inhibitor LY294002. Each group was run in triplicate; error bars represent standard deviation between triplicates. P-values were determined by student's t-test. The experiment was performed twice with reproducible results. Pharmacologic inhibition of PI3K improved sensitivity to trastuzumab in the presence of 3T3 CM. **B)** BT474 cells transiently transfected with 1 μg pcDNA3 empty vector control (C), pcDNA3-Akt-T308D/S473D plasmid (CA-Akt), which expresses constitutively active Akt, or pcDNA3-Akt-K179A plasmid (KD-Akt), which expresses kinase dead Akt. After 24 h, media was changed to DMEM or 3T3 CM $\pm$ 20 $\mu\text{g}/\text{mL}$ trastuzumab. After 48 h, cells were counted by trypan blue exclusion assay. Each group was run in triplicate. Error bars represent standard deviation between triplicates; p-values were determined by student's t-test. The experiment was performed twice with reproducible

results. Expression of kinase-dead Akt restored sensitivity to trastuzumab in the presence of 3T3 CM. C, control untreated; Tr, trastuzumab-treated; CM, untreated cells cultured in 3T3 CM; CM + Tr, trastuzumab-treated cells cultured in 3T3 CM

Fig. 6.

Conditioned media from differentiated human omental adipocytes was analyzed using human leptin-specific or human GDF15-specific ELISA. Concentrations of each cytokine are shown as an average of triplicate cultures per group. Error bars represent standard deviation between triplicates. ELISA was performed once per cytokine. Leptin and GDF15 were detected in omental CM. Leptin and GDF15 were undetectable in DMEM alone; leptin was undetectable in serum starvation media alone.

Fig. (7A)

Fig. (7B)

Fig. 7.

A) SKBR3 HER2-over-expressing breast cancer cells were pre-treated with vehicle control or recombinant human leptin (1000 ng/mL) for 24 hours, followed by treatment with control, leptin, lapatinib (0.1 μ M), and/or trastuzumab (10 μ g/mL) for an additional 72 hours. Cells were then counted by trypan blue exclusion. Error bars represent standard deviation between triplicates; p-values were determined by student's t-test. Experiments were performed twice with reproducible results. Leptin reduced sensitivity to lapatinib but not trastuzumab. **B)** BT474 cells were treated with vehicle control, recombinant human GDF15 (100 ng/mL), lapatinib (3 μ M), and/or trastuzumab (20 μ g/mL). After 72 h, cells were counted by trypan blue exclusion assay. Error bars represent standard deviation between triplicates; p-values were determined by student's t-test. Experiments were performed at least twice with reproducible results. GDF15 reduced sensitivity to trastuzumab but not lapatinib.

Fig. 8.

A schematic representation of mechanisms of CM-mediated trastuzumab resistance is shown. Increased adiposity may confer resistance to trastuzumab due to increased secretion of adipocytokines such as GDF15, which activate PI3K signaling and cell survival, abrogating trastuzumab-mediated growth inhibition. High BMI and increased levels of GDF15 should be studied to determine if they serve as novel therapeutic targets or predictors of trastuzumab resistance in HER2-positive breast cancer. Questions requiring further study are highlighted.