Acquired resistance to tamoxifen is associated with loss of the type I insulin-like growth factor receptor (IGF1R): implications for breast cancer treatment

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Abstract

The role of the insulin-like growth factor (IGF) system in breast cancer is well defined, and inhibitors of this pathway are currently in clinical trials. The majority of anti-IGF1R clinical trials are in estrogen receptor-positive patients who have progressed on prior endocrine therapy; early reports show no benefit for addition of IGF1R inhibitors to endocrine therapy in this setting. In this study, we examined the effectiveness of IGF1R inhibitors in vitro by generating tamoxifen resistant (TamR) cells. We found that TamR cells had diminished levels of IGF1R with unchanged levels of insulin receptor (IR), and failed to respond to IGF-I-induced Akt activation, proliferation, and anchorage-independent growth while retaining responsiveness to both insulin and IGF-II. The IGF1R antibody dalotuzumab inhibited IGF-I-mediated Akt phosphorylation, proliferation, and anchorage-independent growth in parental cells, but had no effect on TamR cells. An IGF1R tyrosine kinase inhibitor, (AEW541) with equal potency for the IGF1R and IR, inhibited IGF-I-, IGF-II-, and insulin-stimulated Akt phosphorylation, proliferation, and anchorage-independent growth in parental cells. Interestingly, AEW541 also inhibited insulin- and IGF-II-stimulated effects in TamR cells. Tamoxifen-treated xenografts also had reduced levels of IGF1R, and dalotuzumab did not enhance the effect of tamoxifen. We conclude that cells selected for tamoxifen resistance in vitro have downregulated IGF1R making antibodies directed against this receptor ineffective. Inhibition of IR may be necessary to manage tamoxifen-resistant breast cancer.
Introduction

The first and arguably most effective targeted therapy for breast cancer involves inhibition of estrogen receptor (ER) function. Tamoxifen, a selective estrogen receptor modulator (SERM), has proven effective in both early and advanced stages of breast cancer (1). Additionally, depriving receptors of ligand using aromatase inhibitors and degrading receptors through pure non-steroidal anti-estrogens have also proven effective. Unfortunately, after initial success, a large portion of these tumors will develop resistance. This has led to the exploration and identification of additional targeted therapies, namely against growth factor receptors, such as EGFR, HER2, and IGF1R.

The IGF1R is a receptor tyrosine kinase that exerts its biological effects through binding of the ligands IGF-I and IGF-II. Following, ligand binding and receptor activation, adaptor molecules are recruited, leading to activation of downstream pathways, including the MAPK and PI3K pathways, ultimately leading to proliferation, angiogenesis, resistance to apoptosis, and metastasis (2, 3). The closely related insulin receptor behaves in a similar manner, through its ligands insulin and IGF-II.

Crosstalk between the IGF1R and estrogen receptor has been well-documented and has led to clinical trials investigating the combined use of IGF1R and ER-inhibitors. Multiple studies have shown that ERα can enhance IGF1R signaling through transcriptional upregulation of IGF1R, IRS-1, and IGF-II (4-8). Reciprocally, IGF1R has been shown phosphorylate and activate ER on serine-167 through a S6-kinase mechanism (9). In addition to current IGF1R inhibitor clinical trials examining combined anti-IGF1R, anti-ER therapies, trials are also being performed in endocrine resistant populations.
The role of the IGF1R in cancer has been established and clinical trials evaluating inhibitors to this pathway are currently underway (10). As noted, preclinical studies have documented crosstalk between IGF1R and ER pathways (11), yet clinical trials conducted primarily in endocrine resistant patients have been disappointing (12). *In vitro* and *in vivo* evaluation has been performed using endocrine sensitive cells, with relatively little evidence demonstrating the effectiveness of anti-IGF1R therapy in endocrine resistant cells.

Two strategies of targeting the IGF1R are currently being evaluated in clinical trials. Monoclonal antibodies bind to the IGF1R, leading to receptor internalization and downregulation. Tyrosine kinase inhibitors bind to the ATP catalytic domain of the internal tyrosine kinase domain of the IGF1R and the closely related insulin receptor. While some view targeting of the IR dangerous due to metabolic consequences, recent data suggest a benefit to targeting the IR (13, 14). Multiple reports have demonstrated a role for the insulin receptor in cancer biology (15-17). Further, phase I clinical trials have shown limited metabolic consequences that can be treated using metformin (18). Thus, the clinical benefit of using IGF1R/IR TKI’s may outweigh their potential metabolic side effects.

The overall aim of our study was to investigate the effectiveness of anti-IGF therapies using an endocrine resistant model. Herein, we reveal tamoxifen-resistant cells lack expression of IGF1R, and hence, are unaffected by IGF1R monoclonal antibodies. Tamoxifen treated xenografts also have reduced levels of IGF1R and mice do not benefit from combined treatment with tamoxifen and dalotuzumab. Furthermore, complete and successful suppression of IGF1R signaling may require dual-inhibition of IGF1R and PI3K targets, as is currently under study in the clinic. Alternatively, endocrine resistant patients may require the use of tyrosine kinase inhibitors, which are effective through inhibition of IR signaling.
Materials and Methods

Reagents. All chemical reagents were purchased from Sigma unless otherwise indicated. IGF-I, IGF-II, and insulin were purchased from Novozymes GroPep Limited (Denmark) and Eli Lilly (USA) respectively.

Cell lines and culture. All cells were grown at 37° C in a humidified atmosphere containing 5% CO₂ and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin. MCF-7 cells were provided by C. Kent Osborne (Baylor College of Medicine, Houston, TX) and maintained in improved MEM Richter's modification medium (zinc option) supplemented with 5% FBS and 11.25 nM insulin. MCF-7 TamR cells were generated by culturing MCF-7 in phenol-red free IMEM (zinc option) supplemented with 11.25 nM insulin, 5% charcoal/dextran-treated FBS, and 100 nM 4-OH-tamoxifen. T47D cells were obtained from ATCC and maintained in MEM supplemented with 5% FBS and 6 ng/ml insulin. T47D TamR cells were generated by culturing T47D cells in phenol-red free IMEM supplemented with 5% charcoal/dextran-treated FBS, and 100 nM 4-OH-tamoxifen. TamR cells were grown in the presence of 4-OH-tamoxifen for 6 months to allow resistance to develop prior to characterizing cells. As a control, parental cells were cultured for the same amount of time in regular media. Following the establishment of resistance, cells were passed for no more than 3 months.

Antibodies. Horseradish peroxidase-conjugated antiphosphotyrosine (PY-20) was purchased from BD Biosciences (Sparks, MD). The ERα antibody used for Western blot analysis was purchased from Neomarkers Lab Vision (Fremont, CA). The IRβ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phosphorylated Akt, IGF1Rβ, and total and phospho-p44/42 (MAPK/ERK) were purchased from Cell Signaling Technology.
(Beverly, MA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce (Rockford, IL).

**Growth Curve Analysis.** Cells were plated at a density of $1 \times 10^4$ in 6-well plates and allowed to equilibrate overnight. Full medium was replaced with phenol-red free IMEM supplemented with 1% dextran-coated-charcoal (DCC)-FBS. 4-OH-tamoxifen was added to cells at concentration and time as indicated in the figures. Cells were stained with trypan blue and counted using a hemacytometer.

**Immunoblot.** Cells were plated at a density of $3 \times 10^5$ in 60-mm-diameter dishes and allowed to equilibrate overnight. Full medium was replaced with dextran-coated-charcoal (DCC)-treated fetal calf serum for the next 3–5 d, after which cells were switched to serum-free medium (SFM) for 24 h. Upon reaching 70% confluency, cells were treated, placed on ice, washed twice with ice-cold PBS, and lysed with lysis buffer of 50 mM Tris-Cl (pH 7.4), 1% Nonidet P-40, 2 mM EDTA (pH 8.0), 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin). Lysates were clarified by centrifugation at $12,000 \times$ rpm for 15 min at 4 C. Protein concentrations were determined using the bicinchoninic acid protein assay reagent kit (Pierce). Cellular protein (50 μg) was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted according to manufacturer guidelines.

**Monolayer growth assay.** Cells were plated in 24-well plates at a density of 30,000 cells per well, allowed to equilibrate overnight and starved in SFM media for 24 hours. After 5 days of treatment, growth was assessed via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (19). 60 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution in SFM was added to each well. After incubation for 3 h at
37°C, wells were aspirated and formazan crystals were lysed with 500 µL of solubilization solution (95% DMSO + 5% IMEM). Absorbance was measured with a plate reader at 570 nm using a 650 nm differential filter to assess growth.

**Anchorage-independent growth.** A 1-ml layer of 0.8% SeaPlaque-agarose (BioWhittaker, Rockland, ME) in 1% FBS-containing growth media was solidified into each well of a six-well plate. The bottom layer was overlaid with 0.8 ml of a 0.45% top agar mixture for 10,000 cells per well with appropriate treatment. All plates were incubated at 37°C. After 12 days colony number was assessed on a light microscope with an ocular grid. Five random fields were counted per well and only colonies exceeding two thirds of a grid square were scored.

**RNA Isolation and qRT-PCR.** Cells were plated at a density of $1 \times 10^6$ in 100-mm-diameter dishes, allowed to equilibrate overnight, DCC starved for 3 d, and incubated overnight in SFM. Cells were treated with SFM or 1 nM estradiol for 4 hours. Cellular RNA was isolated using TriPure Reagent according to the manufacturer (Roche). For quality control and to determine concentration, a 260:280 assay was performed on a spectrophotometer. Forward and reverse primers were designed to target the following transcripts: RPLP0 and IGF1R. A total of 2 µg of RNA was reverse transcribed using the Transcriptor reverse transcriptase kit, and quantitative PCR was performed using the Universal SYBR Green kit according to the manufacturer's recommended protocol (Roche) on an Eppendorf Mastercycler Realplex 4 machine (Hamburg, Germany). The relative concentration of mRNA was calculated using cycle threshold values that were derived from a standard curve and normalized to ribosomal protein, large, P0 as an internal control.

**Xenograft growth.** All animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. MCF-7L cells ($5 \times 10^6$) were injected into the
mammary fat pad of 5-week-old female ovariectomized athymic mice. One day prior to injection, mice were administered estrogen via drinking water at a concentration of 1 µM as described previously (20). Tumors were allowed to achieve an average volume of 200 mm³ prior to beginning treatment. Tamoxifen citrate (Sigma) was subcutaneously administered at a dose of 500 µg in a peanut oil emulsion daily for 5 of 7 days per week. Dalotuzumab was administered twice weekly via intraperitoneal injection at a dose of 500 µg. Control animals were injected with histidine-based buffer and peanut oil alone. Tumor growth was measured bidirectionally and tumor volumes were calculated using the formula length x breadth²/2.

Results

Tamoxifen resistant cells are refractory to tamoxifen treatment, but respond to estrogen treatment. In order to examine the effect of anti-IGF therapy in endocrine resistance, tamoxifen-resistant MCF-7L and T47D cells were generated. After selection, TamR cells survived in the presence of increasing concentrations of tamoxifen; however, parental cells were inhibited with as little as 1 nM tamoxifen (figure 1A). Thus, TamR cells continued to survive and grow in the presence of tamoxifen, even up to concentrations of 1 µM, demonstrating resistance to the drug. Similar to some tamoxifen resistant cancers, TamR cells maintained expression of estrogen receptor (figure 1B). Further, TamR cells were able to proliferate in response to estrogen (figure 1C). When we examined gene expression regulated by ER in TamR cells, we found basal levels of estrogen regulated genes such as KIAA0575 (GREB1), PGR (figure 1D), TFF1, AREG, CTSD, and IGF1R (data not shown) were downregulated; however, estrogen was still able to stimulate transcription of these genes. Similar to the clinical situation
of tamoxifen resistance where some tumors remain dependent on estradiol, our cells maintained estrogen receptor expression and responded to estrogen treatment.

**Tamoxifen resistant cells expressed low levels of IGF1R.** Prior to examining the effectiveness of anti-IGF therapy in TamR cells, we examined the IGF signaling pathway and its components. Interestingly, IGF1R protein levels were diminished as measured by Western blot (figure 2A). Further, TamR cells failed to phosphorylated Akt and MAPK after IGF-I treatment. The cells retained expression of IR and insulin and IGF-II ligand treatment resulted in phosphorylation of Akt and MAPK. To examine whether this change in IGF1R expression was due to decreased transcription, we performed qRT-PCR to examine the message level of IGF1R. Indeed, IGF1R mRNA was decreased in TamR cells compared to parental cells (figure 2B). Treating TamR cells with estrogen resulted in a small increase in IGF1R mRNA, but did not restore the receptor to parental levels (figure 2B). Insulin receptor mRNA levels were not significantly different between parental and resistant cells (figure 2C). Further, estrogen treatment did not affect IR levels in either cell line. These data demonstrate that tamoxifen resistant cells lack IGF1R expression, but maintain expression of IR and are able to signal through IR.

**Dalotuzumab inhibited signaling, proliferation, and anchorage-independent growth in parental, but not TamR cells.** Dalotuzumab (MK-0646) is a humanized monoclonal antibody that binds the IGF1R. It has been shown to downregulate IGF1R in vitro and in vivo (21, 22). In order to examine the ability of the antibody to inhibit IGF-induced signaling, we pretreated MCF-7L parental and TamR cells with 20 ug/ml dalotuzumab for 24 hours prior to stimulating cells with ligand. Dalotuzumab inhibited IGF-I signaling, as measured via Akt and MAPK
phosphorylation, in MCF-7L (figure 3A) and T47D (data not shown) parental cells and had a minimal effect on both insulin and IGF-II signaling. TamR cells did not respond to IGF-I, but Akt was activated by IGF-II and insulin. Dalotuzumab did not affect response to any of the ligands in TamR cells, presumably due to lack of IGF1R expression. In order to examine if this difference was also biologically relevant, we examined the effect of dalotuzumab on proliferation and anchorage-independent growth using the MTT and soft agar assays, respectively. All IGF system ligands tested induced proliferation in MCF-7L and T47D (data not shown) parental cells; however, only proliferation in response to IGF-I was inhibited in the presence of dalotuzumab (figure 3B). In contrast, insulin and to a lesser extent IGF-II stimulated the proliferation of TamR cells and this proliferation was not inhibited by dalotuzumab. Similarly, all ligands induced the anchorage-independent growth of MCF-7L parental cells (figure 3C) and dalotuzumab inhibited growth in response to IGF-I and IGF-II. In agreement with the signaling data, both insulin and IGF-II induced the anchorage-independent growth of TamR cells. This growth was not inhibited by dalotuzumab. Thus, dalotuzumab inhibited IGF-I induced signaling, proliferation, and anchorage-independent growth in MCF-7L parental cells, but had no effect in TamR cells, presumably due to their lack of IGF1R expression.

**AEW541 inhibited signaling, proliferation, and anchorage-independent growth in parental and TamR cells.** AEW541 is a dual tyrosine kinase inhibitor (TKI) that targets both IGF1R and insulin receptor. In order to examine the effect of IGF1R TKI’s in endocrine resistance, we pretreated MCF-7L parental and TamR cells for three hours with 0.3 uM AEW541 prior to stimulating cells with ligands. AEW541 inhibited insulin, IGF-I, and IGF-II signaling in MCF-7L cells (figure 4A) and T47D cells (data not shown). Further, AEW541 was also able to inhibit
insulin and IGF-II stimulated phosphorylation of Akt and MAPK in TamR cells. To investigate whether this inhibition was also biologically important, we again examined proliferation and anchorage-independent growth. AEW541 was able to inhibit insulin, IGF-I, and IGF-II stimulated proliferation in MCF-7L and T47D (data not shown) cells and insulin and IGF-II stimulated proliferation in TamR cells (figure 4B). Additionally, AEW541 was also able to inhibit insulin, IGF-I, and IGF-II stimulated anchorage-independent growth in MCF-7L parental cells and insulin and IGF-II stimulated anchorage-independent growth in TamR cells (figure 4C). Thus, AEW541 was able to inhibit signaling, proliferation, and anchorage-independent growth by suppressing both IGF1R and IR function in MCF-7L parental cells. Interestingly, AEW541 was also able to inhibit the growth of TamR cells presumably via suppression of IR signaling. These data show that TKI’s, which target both IGF1R and IR, are effective in parental and resistant cells, due to inhibition of IR signaling.

**Dalotuzumab inhibited estrogen stimulated growth but did not add to tamoxifen-mediated growth inhibition in vivo.**

We next examined the effect of dalotuzumab on the *in vivo* growth of MCF-7L cells. Ovariectomized athymic mice were injected in the second mammary fat pad with MCF-7L cells as previously described (23). Mice were administered estrogen to stimulate tumor growth and tumors were allowed to establish (tumor volume of ~ 200 mm³) prior to beginning treatment. Dalotuzumab (administered beginning at day 32) inhibited the growth of estrogen stimulated tumors (figure 5A). To study the combination of tamoxifen and daltozumab, estradiol was withdrawn on day 32 and tamoxifen was started. Dalotuzumab treatment began simultaneously with tamoxifen (Tam+Dalotuzumab) or when tumors began to grown on tamoxifen alone (Tam →Dalotuzumab) at approximately day 74. Tamoxifen by itself inhibited the growth of tumors;
however, dalotuzumab co-administered with tamoxifen did not further suppress tumor growth.

Further, dalotuzumab did not significantly inhibit the growth of tamoxifen-resistant tumors when administered after the tumors began to grow on tamoxifen.

We next sought to determine whether this lack of efficacy of dalotuzumab in tamoxifen treatment was similar to the lack of IGF1R expression as observed in vitro. When tumors reached 1000 mm$^3$, mice were sacrificed and tumors were harvested for RNA isolation. Expression of IGF1R mRNA was significantly reduced in tamoxifen treated xenografts when compared to estrogen treated xenografts regardless of dalotuzumab treatment (figure 5B). Thus, tamoxifen treated xenografts do not benefit from dalotuzumab treatment, due to decreased IGF1R expression. However, estrogen treated xenografts express significantly more IGF1R and benefit from dalotuzumab treatment. These data suggest that the level of receptor expression is important in determining response to dalotuzumab treatment and that estrogen receptor plays an important role in regulating IGF1R expression.

**Discussion**

The recently published results of IGF1R antibodies in clinical trials demonstrating limited success in endocrine-resistant populations prompted us to investigate their efficacy using an endocrine resistant model. Previous investigations into the efficacy of anti-IGF therapies have been performed using endocrine-sensitive cell lines and xenograft models. Since IGF1R is an ER transcriptional target, understanding if IGF1R expression was affected by resistance to tamoxifen has clinical relevance. We found our tamoxifen-resistant cell lines lacked both protein and mRNA expression of IGF1R, but maintained expression of IR. This is in contrast to a report by Westley, et. al. showing that tamoxifen resistance in MCF-7 cells was associated with a
dependence on IGF-I (24). This contrary finding may be a result of a difference in the way the tamoxifen-resistant cells were generated; these investigators used low serum conditions during generation of tamoxifen resistance while we used compete media. When cells were selected in this manner, tamoxifen became an ER agonist. While seemingly contradictory, these findings are consistent with our own. IGF1R expression requires agonism of ER. In Westley et al, their “tamoxifen resistant” cells tamoxifen stimulated ER function. In our tamoxifen resistant cells, we saw no evidence of agonistic activity stimulated by tamoxifen (Figure 1D).

Based on prior reports of ER transcriptional regulation of IGF1R, it is not surprising that IGF1R expression would be decreased following acute treatment with a selective estrogen receptor modulator such as tamoxifen (25-27). Interestingly, studies performed by Massarweh, et. al. using tamoxifen-resistant xenografts demonstrate decreased total levels of IGF1R, but basal phosphorylation of the receptor (28). This discordance may be explained by a difference in dosage of tamoxifen in model systems. In our model, tamoxifen is continuously administered to cells, whereas, in the Massarweh study, animals are given tamoxifen five times weekly, leading to the possibility that ER function is not completely suppressed in this model. Furthermore, this study did not clearly distinguish between IGF1R or IR phosphorylation since the “phospho-specific” antibody detects both receptors.

The finding that tamoxifen resistant cells were refractory to IGF1R antibody treatment underscores the importance of using model systems similar to the patient populations the drug will be used in. Although several studies have demonstrated the efficacy of IGF1R monoclonal antibodies in breast cancer cells, these cells have been endocrine sensitive (21, 23). The effect of combined anti-estrogen/anti-IGF1R treatment should also take into consideration whether the dose of anti-estrogen is sufficient, in and of itself, to suppress IGF1R function via receptor
downregulation. Our in vivo results demonstrate that tamoxifen treatment results in decreased IGF1R mRNA levels. Initial results examining the effectiveness of IGF1R antibodies in endocrine resistant breast cancer populations have not demonstrated a definitive positive result (29, 30). This may be due to the lack of IGF1R expression in these patient populations. A recent study examining mRNA expression in a cohort of tamoxifen-resistant breast cancer patients has demonstrated a decrease in IGF1R levels in the recurrent tumors, suggesting our findings in vitro may correlate with the clinical scenario (31).

The efficacy of TKI’s in our tamoxifen resistant cells underscores the importance of co-targeting the IR, along with the IGF1R. Initially, development of IGF1R inhibitors aimed to avoid targeting the IR, due to potential metabolic consequences. However, numerous studies by us and others have demonstrated the IR does indeed play a role in cancer biology (14, 18, 29, 32). Specifically, work by Hanahan and colleagues showed that IGF1R inhibition using antibodies is only successful in tumors/cells where the IGF1R/IR ratio is high. Additionally, they demonstrate the IR can actually serve as an escape mechanism, providing resistance to IGF1R antibodies (17). Further, work by Haluska and colleagues has shown that when figitumumab (an IGF1R monoclonal antibody) is administered to patients, there is an associated increase in plasma insulin (33). This increase in insulin levels could potentially lead to increased IR signaling in tumor cells, providing yet another escape mechanism for the cancer cells to survive. These data are supported by a case report demonstrating increased copy number of IR in a woman with metastatic hormone refractory breast cancer (34). The role of the IR in cancer biology has been clearly defined, and the metabolic consequences of its inhibition are actively being investigated. A recent study performed in mice demonstrated that an IGF1R/IR TKI alone or in combination with tamoxifen did not lead to a significant change in glucose homeostasis,
suggesting the drugs are tolerable. This study also demonstrated the efficacy of the TKI in letrozole resistant xenografts, supporting our data that suggest TKI’s are more effective than antibodies in tamoxifen resistant cells (35).

Although our data indicate IGF1R antibodies may not be effective in an endocrine resistant system, this does not mean they have little utility. Multiple trials are underway, examining the use of IGF1R antibodies in endocrine sensitive populations. One promising area of investigation is inhibiting both the upstream (IGF1R) and downstream (mTOR) components of the IGF1R pathway, leading to maximal inhibition of signaling. Inhibition of IGF1R has been shown to sensitize cells to mTOR inhibition (36). Additionally, clinical trials are underway examining the efficacy of combining IGF1R antibodies with mTOR inhibitors (37, 38).

Overall, our data highlight the importance of using model systems that will match the patient population the drug will ultimately be used in. Additionally, when evaluating IGF1R therapies, it will be important to carefully select the proper patient population, as well as to verify the target is present. Finally, our data suggest dual IGF1R/IR TKI’s may be more effective than IGF1R antibodies, due to inhibition of IR. Combination therapy using IGF1R antibodies may require use of an mTOR inhibitor for complete suppression of the target, as is currently being evaluated in the clinic.

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References

Figure Legends

**Figure 1. Generation of TamR Cells.** A. MCF-7L and TamR (left panel) or T47D and TamR (right panel) cells were plated in monolayer at a density of 10,000 cells/plate in the presence of 1% charcoal stripped serum and increasing concentrations of tamoxifen as indicated. Cells were collected and stained with trypan blue prior to counting using a hemacytometer.

B. Cell lysates were collected from MCF-7L and TamR cells and were separated by SDS-PAGE. Total protein levels of estrogen receptor (ER) and MAPK were assessed using specific antibodies by immunoblotting.

C. MCF-7L and TamR (left panel) or T47D and TamR (right panel) cells were grown in charcoal stripped serum prior to serum starving cells overnight. Cells were treated with 1 nM E2 or 5% FBS and growth was assessed after 5 days using the MTT assay.

D. Cells were plated and exposed to charcoal stripped serum prior to serum starving and treating with estradiol for 4 hours. Total RNA was isolated from MCF-7L and TamR (left panel) or T47D and TamR (right panel) cells and was reverse transcribed. Expression of PGR and KIAA0575 was analyzed using qRT-PCR and was normalized to the RPLP0 housekeeper gene. One way ANOVA with a Tukey’s post-test was used to analyze the data. * p<0.01

**Figure 2. TamR cells have decreased IGF1R levels and fail to respond to IGF-I treatment.**

A. MCF-7L and TamR (upper panel) or T47D and TamR (lower panel) cells were serum starved overnight, then treated with 10 nM insulin, 5 nM IGF-I or 10 nM IGF-II for 10 minutes. Cellular lysates were separated by SDS-PAGE and levels of IGF1R, IR, phosphorylated Akt and MAPK, and total MAPK protein levels were assessed using specific antibodies by immunoblotting.

B. Cells were plated and exposed to charcoal stripped serum prior to an overnight starvation and a 4 hour estradiol treatment. Total RNA was isolated from MCF-7L and TamR (left panel) or
T47D and TamR (right panel) cells and was reverse transcribed and IGF1R levels were analyzed using qRT-PCR. Data was normalized to the RPLP0 housekeeper gene. One way ANOVA with Tukey’s post-test was done to compare the statistical significance between the cell lines. *p<0.05, **p<0.01

C. Cells were plated and exposed to charcoal stripped serum, serum starved overnight, and treated for 4 hours with estradiol. Total RNA was isolated from MCF-7L and TamR cells and was reverse transcribed and IR levels were analyzed using qRT-PCR. Data was normalized to the RPLP0 housekeeper gene.

**Figure 3. Dalotuzumab can inhibit the growth of MCF-7L parental, but not TamR cells.**

A. MCF-7L and TamR cells were serum starved overnight and pre-treated with 20 ug/ml antibody for 24 hours prior to treating the cells with 10 nM insulin, 5 nM IGF-I, or 10 nM IGF-II for 10 minutes. Cellular lysates were separated by SDS-PAGE and levels of IGF1R, IR, phosphorylated Akt and MAPK, and total MAPK protein levels were assessed using specific antibodies by immunoblotting.

B. MCF-7L and TamR cells were serum starved and treated with anti-IGF antibody along with ligand. Proliferation was evaluated using MTT assay, with results displayed as absorbance at 570 nm. Two way ANOVA with Bonferroni comparison was used to compare the difference between antibody pre-treatment and un-treated samples. *p<0.01

C. MCF-7L and TamR cells were serum starved and treated with anti-IGF antibody and ligand in 1% FBS in 0.45% agar and overlaid on 0.8% bottom agar. Colony growth in agarose was assessed after 14 days. Colonies formed were counted and averaged from 5 individual microscopic fields. Results displayed are the average number of colonies in 5 fields of 3 wells.
Two way ANOVA with Bonferroni comparison was performed to compare the difference between antibody pre-treated and un-treated samples. *p<0.01

**Figure 4. AEW541 can inhibit the growth of MCF-7L and TamR cells.** A. MCF-7L and TamR cells were serum starved overnight and pre-treated with 0.3 uM TKI for 3 hours prior to treating the cells with 10 nM insulin, 5 nM IGF-I, or 10 nM IGF-II for 10 minutes. Cellular lysates were separated by SDS-PAGE and levels of IGF1R, IR, phosphorylated Akt and MAPK, and total MAPK protein levels were assessed using specific antibodies by immunoblotting.

B. MCF-7L and TamR cells were serum starved and treated with anti-IGF1R/IR TKI along with ligand. Proliferation was evaluated using MTT assay, with results displayed as absorbance at 570 nm. Two way ANOVA with Bonferroni comparison was used to compare the difference between TKI treatment and un-treated samples. *p<0.05, **p<0.005

C. MCF-7L and TamR cells were serum starved and treated with anti-IGF1R/IR TKI and ligand in 1% FBS in 0.45% agar and overlaid on 0.8% bottom agar. Colony growth in agarose was assessed after 14 days. Colonies formed were counted and averaged from 5 individual microscopic fields. Results displayed are the average number of colonies in 5 fields of 3 wells. Two way ANOVA with Bonferroni comparison was performed to compare the difference between TKI treated and un-treated samples. *p<0.01

**Figure 5. Tamoxifen treated MCF-7L xenografts have reduced IGF1R levels and do not respond to dalotuzumab treatment.** A. Ovariectomized athymic mice were given estrogen to stimulate MCF-7L xenograft tumor growth. At day 32, estrogen was withdrawn (unless indicated) and treatments began. For the Tam→dalotuzumab group, tamoxifen was started at day 32 and dalotuzumab was started when tumors began to grow despite tamoxifen treatment at approximately day 74. Tumor volumes were measured weekly and average volume was plotted.
B. Xenografts were harvested from mice and total RNA was isolated using TriPure Reagent. RNA was reverse transcribed and analyzed using qRT-PCR. Results were normalized to the RPLP0 housekeeping gene. An unpaired t test was used to compare the difference between treatment groups. *p<0.005
Figure 1

A. 

![](image)

B. 

MCF-7L TamR 

T47D TamR 

C. 

![](image)

D.
Figure 2

A.

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![IGF1Rβ](image1)

![IRβ](image2)

![pAkt](image3)

![pMAPK](image4)

![tMAPK](image5)

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![IGF1Rβ](image6)

![IRβ](image7)

![pAkt](image8)

![pMAPK](image9)

![tMAPK](image10)

B.

**IGF1R**

![Expression of IGF1R](image11)

C.

**INSR**

![Expression of INSR](image12)
Figure 3

A. 

B.

C.
Figure 4

A.

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<th>NVP AEW541</th>
<th>NVP AEW541</th>
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<tr>
<td></td>
<td>0.3 uM</td>
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<tr>
<td>Insulin</td>
<td>+++-</td>
<td>+++-</td>
</tr>
<tr>
<td>IGF-I</td>
<td>+---</td>
<td>+---</td>
</tr>
<tr>
<td>IGF-II</td>
<td>-+++</td>
<td>-+++</td>
</tr>
<tr>
<td>IGF1Rβ</td>
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<tr>
<td>pAkt</td>
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<tr>
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<tr>
<td>tMAPK</td>
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</tbody>
</table>

B.

Absorbance @ 570 nm

Day 5

SFM Insulin IGF-I IGF-II FBS

MCF-7L MCF-7L + AEW TamR TamR + AEW

C.

Colonies per well

SFM Insulin IGF-I IGF-II 5% FBS

MCF-7L MCF-7L + AEW TamR TamR + AEW
Figure 5

A.

![Graph showing tumor volume over time for different treatments.]

- E2
- E2+Dalotuzumab
- Tam+Dalotuzumab
- Tam
- Tam→Dalotuzumab

B.

![Bar graph showing amount of SYBR (normalized to RPLP0) for different treatments.]

- E2
- E2 + Dal
- Tam + Dal
- Tam
- Tam→Dal

*IGF1R*
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