

# Inhibition of IGF1R activity enhances response to trastuzumab in HER-2-positive breast cancer cells

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**Background:** Although trastuzumab has improved the prognosis for HER-2-positive breast cancer patients, not all HER-2-positive breast tumours respond to trastuzumab treatment and those that initially respond frequently develop resistance. Insulin-like growth factor-1 receptor (IGF1R) signalling has been previously implicated in trastuzumab resistance. We tested IGF1R inhibition to determine if dual targeting of HER-2 and IGF1R improves response in cell line models of acquired trastuzumab resistance.

**Materials and methods:** HER-2, IGF1R, phospho-HER-2, and phospho-IGF1R levels were measured by enzyme-linked immunosorbent assays in parental and trastuzumab-resistant SKBR3 and BT474 cells. IGF1R signalling was targeted in these cells using both small interfering RNA (siRNA) and the tyrosine kinase inhibitor, NVP-AEW541.

**Results:** IGF1R levels were significantly increased in the trastuzumab-resistant model, SKBR3/Tr, compared with the parental SKBR3 cell line. In both the SKBR3/Tr and BT474/Tr cell lines, inhibition of IGF1R expression with siRNA or inhibition of tyrosine kinase activity by NVP-AEW541 significantly increased response to trastuzumab. The dual targeting approach also improved response in the parental SKBR3 cells but not in the BT474 parental cells.

**Conclusions:** Our results confirm that IGF1R inhibition improves response to trastuzumab in HER-2-positive breast cancer cells and suggest that dual targeting of IGF1R and HER-2 may improve response in HER-2-positive tumours.

**Key words:** breast cancer, HER-2, Herceptin, IGF1R, NVP-AEW541, trastuzumab

## introduction

HER-2 gene amplification or overexpression occurs in ~20 to 25% of human breast cancers and is associated with poor prognosis [1, 2]. Trastuzumab (Herceptin™, Genentech), a humanised monoclonal antibody directed against the extracellular domain of HER-2 [3], has shown activity both as a single agent and in combination with chemotherapy in HER-2-overexpressing breast cancer [4, 5]. However, not all HER-2 positive patients benefit from trastuzumab and those that initially respond can develop resistance [6].

Several mechanisms of resistance to trastuzumab have been proposed (for review, see [6, 7]), including reduced receptor antibody binding, increased cellular signalling through alternative receptor tyrosine kinases, and altered intracellular signalling involving loss of PTEN, reduced p27<sup>kip1</sup>, or increased Akt activity.

Increased signalling via the insulin-like growth factor-1 receptor (IGF1R) has been implicated in reduced response to trastuzumab in breast cancer cells *in vitro*. Lu et al. [8] reported that transfection of IGF1R into trastuzumab-sensitive SKBR3 cells conferred almost complete resistance to trastuzumab. Sensitivity to trastuzumab was restored by reducing IGF1R signalling with recombinant IGF binding protein 3 (rhIGFBP3) [9]. Jerome et al. [10] also reported that combined treatment with rhIGFBP3 and trastuzumab had significant growth inhibitory effects in trastuzumab-resistant BT474/HerR cells, which had acquired resistance following long-term exposure to trastuzumab. This combined treatment also had significant anti-tumour effects on MCF7/HER-2-18 xenografts. Furthermore, inhibition of IGF1R signalling with an anti-IGF1R antibody  $\alpha$ IR3 or a specific IGF1R tyrosine kinase inhibitor (TKI) also sensitised trastuzumab-resistant SKBR3/HerR cells to trastuzumab [11].

To further elucidate the role of IGF1R in the development of trastuzumab resistance, we examined two *in vitro* models of acquired resistance to trastuzumab and tested the effects of

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IGF1R inhibition on response to trastuzumab using both small interfering RNA (siRNA) molecules targeting IGF1R and an IGF1R TKI (NVP-AEW541).

## materials and methods

### cells and reagents

BT474 and SKBR3 cell lines were obtained from the American Tissue Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 medium with 10% fetal calf serum. Trastuzumab-conditioned cell lines BT474/Tr and SKBR3/Tr were established by continuous exposure to 1.4  $\mu$ M trastuzumab for 9 months [12]. Trastuzumab (Herceptin™, Roche) was obtained from the Department of Pharmacy, St Vincent's University Hospital. The IGF1R TKI NVP-AEW541 was obtained from Novartis Pharma (Basel, Switzerland).

### proliferation assays

Cells ( $2.5\text{--}3.75 \times 10^3$ ) were plated in wells of 96-well plates. After 24 h, cells were treated with or without trastuzumab (10 or 100 nM) and NVP-AEW541 (1  $\mu$ M). Proliferation was measured after 5 days by cell counting or acid phosphatase assays. Prior to cell counting, cells were resuspended in RPMI-1640 containing Guava Viacount reagent (Guava Technologies, Hayward, CA) and counted using a Guava EasyCyte (Guava Technologies). For acid phosphatase assays, media was removed and cells were rinsed with phosphate-buffered saline (PBS), 100  $\mu$ l of acid phosphatase substrate [7.25 mM *p*-nitrophenol phosphate (Sigma-Aldrich, Dublin, Ireland) in sodium acetate buffer] was then added to each well followed by incubation at 37°C for 45 min. Fifty microliters of 1 M NaOH was added to each well and the absorbance was read at 405 nm with 620 nm as a reference. Proliferation or inhibition of proliferation was calculated relative to untreated controls. Each assay was carried out in triplicate.

### protein extraction

Cells were washed twice with ice-cold PBS and 100–500  $\mu$ l RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 1% Triton X-100] containing 1 $\times$  protease inhibitor cocktail (Merck, Darmstadt, Germany), 2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), and 1 mM sodium orthovanadate (Sigma-Aldrich) was added and cells were incubated on ice for 10 min. Lysates were centrifuged at 16 000 $\times$ g for 10 min at 4°C. The supernatants were stored at –80°C. Protein quantification was carried out using the BCA quantitation kit (Pierce Biotechnology, Rockford, IL).

### immunoprecipitation

Five hundred micrograms of protein lysate was diluted in 900  $\mu$ l RIPA buffer and incubated with 3  $\mu$ g antibody [monoclonal anti- $\alpha$ -HER-2 (Merck) or polyclonal anti-IGF1R $\beta$  (Santa Cruz Biotechnology, Santa Cruz, CA)] at 4°C, shaking, for 30 min. Fifty microliters of packed protein-A/G agarose beads (Santa Cruz Biotechnology) were added and the samples were incubated overnight, shaking, at 4°C. The samples were centrifuged at 16 000 $\times$ g for 25 s, the supernatants were removed, and the pellets were washed with immunoprecipitation (IP) wash buffer [mild lysis buffer (Cell Signaling Technology, Danvers, MA) with protease inhibitors]. The wash was repeated twice and the samples were centrifuged for 35 s. The supernatants were removed and the pellets were resuspended in 50  $\mu$ l denaturing buffer and denatured at 95°C for 5 min. Denatured samples were then centrifuged at 16 000 $\times$ g for 3 min and the supernatants were collected and stored at –20°C.

### western blotting

Proteins, 5–50  $\mu$ g, were electrophoretically resolved on denaturing polyacrylamide gels (Lonza Workingham Ltd, Berkshire, UK), transferred

to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK), which were blocked with either bovine serum albumin (Sigma-Aldrich) or skimmed-milk powder (Bio-Rad Laboratories, Cambridge, MA) in PBS-Tween (0.1%), and incubated overnight at 4°C with the following primary antibodies: monoclonal anti- $\alpha$ -HER-2 (Merck), polyclonal anti-phosphorylated HER-2/epidermal growth factor receptor (EGFR) (Cell Signaling Technology), polyclonal anti-IGF-IR $\beta$  (Santa Cruz Biotechnology), and monoclonal anti- $\alpha$ -tubulin (Sigma-Aldrich). Proteins were visualised using horseradish peroxidase-conjugated anti-mouse (Sigma-Aldrich) or anti-rabbit antibodies (Sigma-Aldrich) and luminol reagent (Santa Cruz Biotechnology).

### enzyme-linked immunosorbent assays

Total HER-2 protein levels were measured in protein lysates using quantitative enzyme-linked immunosorbent assays (ELISA) (Merck) according to the manufacturer's instructions. For each protein lysate 0.5  $\mu$ g was tested and HER-2 protein levels were calculated as nanogram HER-2 per milligram of total protein. Total IGF1R and EGFR and phosphorylated HER-2, IGF1R, and EGFR were measured by ELISAs (R&D Systems, Minneapolis, MN). Fifty to one hundred micrograms of protein was used for IGF1R and 30  $\mu$ g was used for EGFR measurement. Protein levels were calculated as nanogram/milligram of total protein. Seven micrograms, 140  $\mu$ g, and 30  $\mu$ g were used for phosphorylated HER-2, phosphorylated IGF1R, and phosphorylated EGFR measurements, respectively. Arbitrary values were calculated relative to levels in positive control cell lines.

### siRNA transfection

A validated siRNA molecule targeting exon 2 and two pre-designed siRNA molecules targeting exons 2 and 4 of the *IGF1R* gene were obtained from Applied Biosystems (Foster City, CA). A kinesin siRNA and a scrambled sequence siRNA molecule (Applied Biosystems) were used as controls. Each siRNA molecule was transfected at a final concentration of 30 nM. Cells ( $6.5\text{--}8 \times 10^3$  and  $3\text{--}3.5 \times 10^5$ ) were resuspended in 10% RPMI for 96-well and 6-well plates, respectively. Each siRNA and NeoFX™ transfection agent (Applied Biosystems) was diluted in Gibco™ Opti-MEM reduced serum medium (Invitrogen, Carlsbad, CA) and incubated at room temperature (RT) for 10 min. Diluted NeoFX™ was then added to each siRNA and incubated for a further 10 min at RT. The transfection mix was added to the wells followed by the cell suspension. After 24 h, the transfection media was replaced with 10% RPMI with or without 10 nM trastuzumab. Six-well plates were used to prepare lysates after 72 h. Cells in 96-well plates were harvested after 4 days and counted using the Guava EasyCyte as described above.

## results

### characterisation of trastuzumab-resistant cell lines

Trastuzumab-conditioned SKBR3/Tr and BT474/Tr cells displayed significantly reduced response to trastuzumab compared with the parental cells in proliferation assays (Table 1). The reduced response to trastuzumab was stable for at least 12 weeks, in the absence of trastuzumab (supplemental Figure 1, available at *Annals of Oncology* online). HER-2 and phospho-HER-2 levels, determined by ELISA, were not significantly altered in the SKBR3/Tr cells but were significantly increased in the BT474/Tr cells compared with the parental BT474 cells (HER-2,  $P = 0.016$ ; phospho-HER-2,  $P = 0.043$ ) (Table 1). IGF1R levels were significantly higher in the SKBR3/Tr cells than in the parental SKBR3 cells ( $P = 0.012$ ), while they were unchanged in BT474/Tr cells. No significant difference in

**Table 1.** Expression and phosphorylation levels of HER-2 and IGF1R and sensitivity to trastuzumab in SKBR3 and BT474 parental and trastuzumab-resistant cell lines

	% Growth in trastuzumab (100 nM)	HER-2, ng/mg	pHER-2 <sup>a</sup>	IGF1R, ng/mg	pIGF1R <sup>b</sup>
SKBR3	57.4 ± 0.9	228.1 ± 37.6	0.34 ± 0.02	0.7 ± 0.1	0.34 ± 0.03
SKBR3/Tr	75.9 ± 4.0*	184.7 ± 16.8	0.34 ± 0.06	1.8 ± 0.3*	0.34 ± 0.03
BT474	38.1 ± 7.6	147.9 ± 13.3	0.44 ± 0.05	3.7 ± 0.5	0.50 ± 0.03
BT474/Tr	73.6 ± 4.4*	411.4 ± 78.2*	0.62 ± 0.05*	3.1 ± 0.4	0.57 ± 0.09

<sup>a</sup>Phospho-HER-2 and phospho-IGF1R levels are expressed relative to a positive control cell line (HCC1419).

\*indicates *P* < 0.05 for comparisons of resistant cell lines with respective parental cell lines, as determined by the Student's *t*-test.

IGF1R, insulin-like growth factor-1 receptor.

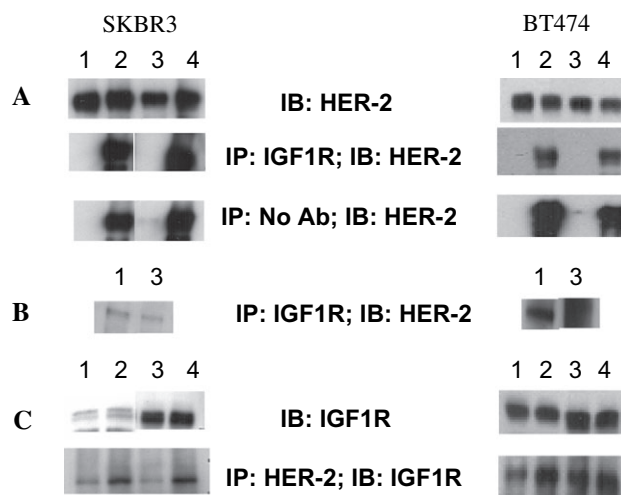
phospho-IGF1R levels was observed in the SKBR3/Tr or BT474/Tr cells compared with the parental cells.

### IGF1R/HER-2 heterodimerization

We examined the formation of IGF1R/HER-2 heterodimers by performing IP for HER-2 and immunoblotting for IGF1R and vice versa (Figure 1). Following IGF1R IP, HER-2 was detected in both parental and resistant SKBR3 and BT474 cells that were treated with trastuzumab. To determine if trastuzumab treatment induced heterodimer formation or if trastuzumab in the medium (and subsequent lysate) was responsible for immunoprecipitating HER-2, the same IP experiment was carried out in the absence of the IGF1R antibody. HER-2 was detected in all cell lysates prepared from trastuzumab-treated cells (Figure 1), suggesting that the HER-2 observed in the IGF1R IP experiment was due to direct IP by trastuzumab. SKBR3 and BT474 parental cells and SKBR3/Tr and BT474/Tr cells grown in the absence of trastuzumab were immunoblotted separately to facilitate detection of HER-2, in the absence of the high levels of HER-2 observed in samples from cells treated with trastuzumab (Figure 1). Low levels of HER-2 were detected, suggesting that low levels of HER-2/IGF1R heterodimers are present in both parental and resistant cells. The presence of the heterodimers was confirmed by IP for HER-2, followed by immunoblotting for IGF1R (Figure 1). Interestingly, HER-2 immunoprecipitated samples showed higher levels of IGF1R in both the parental and resistant cells after treatment with trastuzumab. This may be due to more efficient IP of HER-2/IGF1R heterodimers due to the presence of trastuzumab or may suggest that trastuzumab treatment increases HER-2/IGF1R heterodimer formation.

### IGF1R siRNA

IGF-IR siRNA treatment reduced IGF1R levels by 34 (± 6) % in SKBR3 cells, 11 (± 7) % in SKBR3/Tr cells, 24 (± 4) % in BT474 cells, and by 21 (± 9) % in BT474/Tr cells, as determined by ELISA. Decreased IGF1R levels caused up to 20% growth inhibition (Figure 2). Two additional siRNAs, targeting different exons of IGF1R, were also tested and produced similar results (data not shown). Combined treatment with IGF1R siRNA and trastuzumab showed

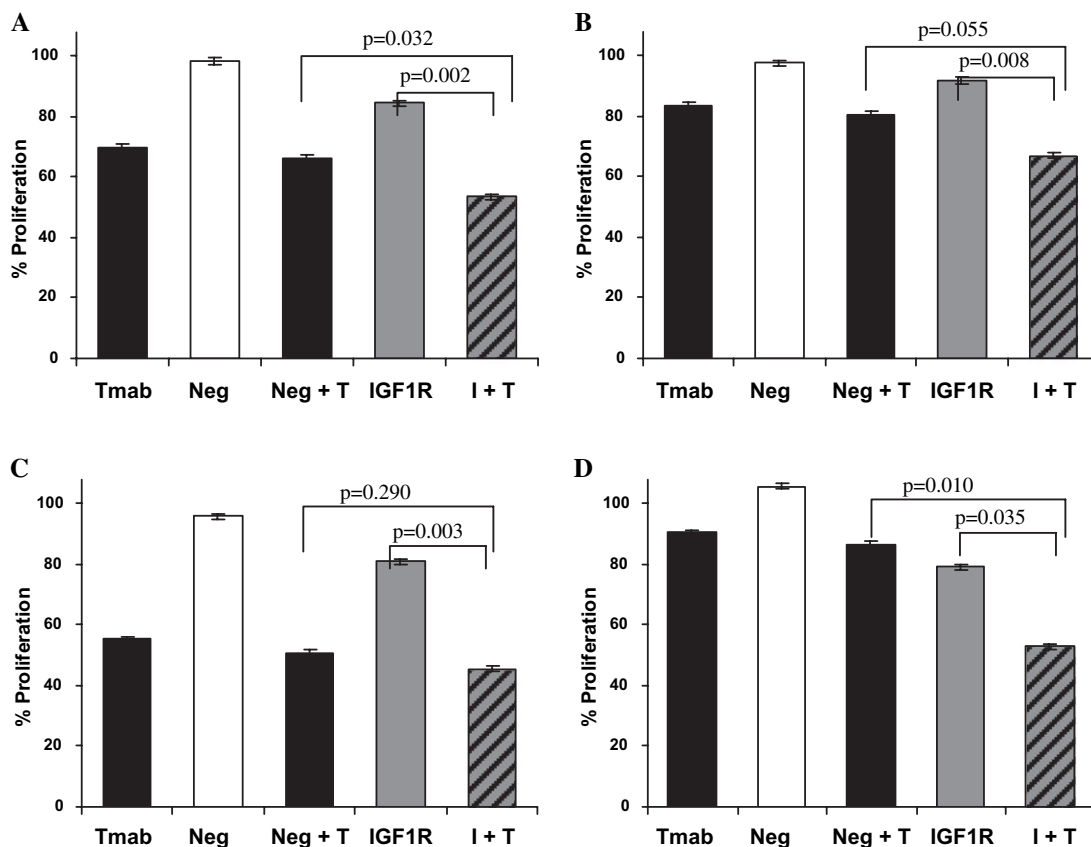


**Figure 1.** Immunoblotting for HER-2 and insulin-like growth factor-1 receptor (IGF1R) in SKBR3 and BT474 parental and trastuzumab-resistant cells: parental cells untreated (1), parental cells treated with trastuzumab (100 nM) for 24 h (2), trastuzumab-resistant (SKBR3/Tr and BT474/Tr) cells grown in the absence of trastuzumab for 7 days (3), and trastuzumab-resistant cells grown in the absence of trastuzumab for 6 days followed by trastuzumab (100 nM) treatment for 24 h (4). (A) Immunoprecipitation (IP) for IGF1R followed by immunoblotting for HER-2. Samples were also immunoprecipitated in the absence of primary antibody (No Ab) and immunoblotted for HER-2. (B) Repeat IGF1R IP was carried out in parental (1) and resistant cells grown in the absence of trastuzumab for 7 days (3), followed by immunoblotting for HER-2. (C) IP for HER-2 followed by immunoblotting for IGF1R.

improved response compared with either trastuzumab or IGF1R siRNA alone, in both SKBR3 and SKBR3/Tr cells (Figure 2). In BT474 cells, combined treatment with IGF1R siRNA and trastuzumab did not improve response compared with treatment with trastuzumab alone. However, in the BT474/Tr cells, the combination of IGF1R siRNA with trastuzumab resulted in significantly improved response compared with either agent alone (Figure 2).

### IGF1R tyrosine kinase inhibition

SKBR3 and SKBR3/Tr cells showed similar growth inhibition when treated with the IGF1R TKI NVP-AEW541 (1 μM)



**Figure 2.** Proliferation of (A) SKBR3, (B) SKBR3/Tr (C) BT474 and (D) BT474/Tr cells treated with trastuzumab (Tmab, T) (10 nM) alone, negative control scrambled siRNA (Neg) with and without trastuzumab, anti-insulin-like growth factor-1 receptor (IGF1R) siRNA (IGF1R, I) alone, and combined with trastuzumab. Proliferation is expressed relative to untreated control. Error bars represent standard error of triplicate experiments. Statistical significance was evaluated using the Student's *t*-test.

(Figure 3). NVP-AEW541 treatment decreased pIGF1R levels by  $12 (\pm 5) \%$  and  $11 (\pm 5) \%$  in SKBR3 and SKBR3/Tr cells, respectively, and by  $29 (\pm 5) \%$  and  $10 (\pm 6) \%$  in BT474 and BT474/Tr cells, respectively, as measured by ELISA. Addition of NVP-AEW541 to trastuzumab had a greater inhibitory effect on cell growth than either treatment alone in both SKBR3 and SKBR3/Tr cells (Figure 3). NVP-AEW541 was not as effective in BT474 and BT474/Tr cells, achieving only  $\sim 10\%$  growth inhibition at  $1 \mu\text{M}$  (Figure 3). However, the combination of NVP-AEW541 and trastuzumab showed a significantly greater effect on growth than trastuzumab alone in the BT474/Tr cells ( $P = 0.021$ ).

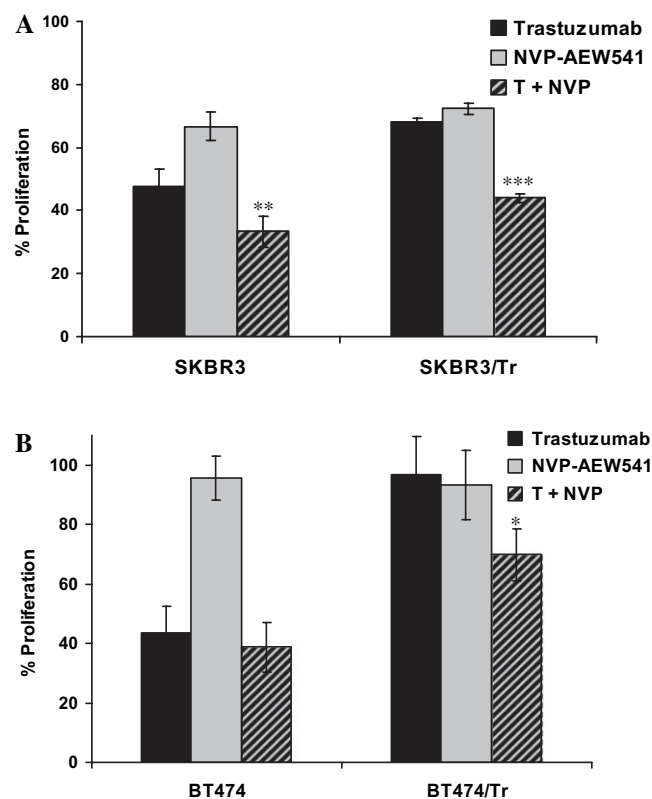
## discussion

Not all patients with HER-2-overexpressing breast cancer benefit from trastuzumab therapy, and those that initially respond frequently develop resistance [6]. Previous studies suggest that alternative receptor tyrosine kinase (RTK) signalling, such as IGF1R or EGFR signalling, may play a role in trastuzumab resistance [8, 10, 13, 14]. Our studies of two models of acquired trastuzumab resistance suggest that different mechanisms of resistance can develop in HER-2-positive breast cancer cells. In the SKBR3/Tr model of acquired trastuzumab resistance, we observed an increase in IGF1R

levels, which suggests that the development of trastuzumab resistance in these cells is associated with increased expression of IGF1R, with no significant change in HER-2 levels. In the BT474/Tr model, no change in IGF1R levels was detected but both the levels and phosphorylation of HER-2 were increased and may play a role in acquired trastuzumab resistance in this model. EGFR and phosphorylated EGFR levels are also significantly increased in BT474/Tr cells compared with in BT474 cells [15]. Heterodimerization between the EGFR/HER-2 and the subsequent signalling from this complex may be responsible for the trastuzumab resistance in this model. This hypothesis is consistent with previous reports of increased EGFR/HER-2 signalling in BT474 cells selected for resistance to trastuzumab *in vivo* [14].

IGF1R has been shown to heterodimerize with HER-2 in MCF-7-HER2 cells [16] and in trastuzumab-resistant cells [11, 17]. In contrast to previous findings [11, 17], we detected HER-2/IGF1R heterodimers in both the parental and trastuzumab-resistant cells, suggesting that heterodimerization is not unique to trastuzumab-resistant cells and does not appear to play a causative role in the development of acquired trastuzumab resistance in the SKBR3 and BT474 models. However, blocking this interaction may improve response to trastuzumab treatment both in sensitive and resistant cells.





**Figure 3.** Proliferation of (A) SKBR3 and SKBR3/Tr and (B) BT474 and BT474/Tr treated with trastuzumab (T) (10 nM) and insulin-like growth factor-1 receptor tyrosine kinase inhibitor NVP-AEW541 (NVP) (1  $\mu$ M). Cells ( $2.5\text{--}3.75 \times 10^3$ ) were plated in 96-well plates in RPMI-1640 with 2% serum. Proliferation is expressed relative to untreated control. Error bars represent standard deviation of triplicate experiments. Student's *t*-tests were carried out to evaluate the statistical significance of the effects of trastuzumab treatment alone compared with trastuzumab combined with NVP-AEW541: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

While the SKBR3/Tr cells show reduced response to trastuzumab compared with parental cells, combined treatment with trastuzumab and IGF1R siRNA enhanced growth inhibition in both SKBR3 parental and SKBR3/Tr cells. The combination of trastuzumab and the IGF1R inhibitor, NVP-AEW541, also achieved similar growth inhibition in both cell lines. The improved response seen in both the SKBR3 parental cell line and the trastuzumab conditioned SKBR3/Tr cell line suggest that IGF1R inhibition may be beneficial in HER-2-positive tumours that express IGF1R, regardless of sensitivity to trastuzumab.

Although the levels of IGF1R are higher in BT474 than in SKBR3 cells, neither the IGF1R siRNA nor the TKI enhanced response to trastuzumab in these cells. Esparis-Ogando et al. [18] tested NVP-AEW541 with trastuzumab in BT474 cells and showed that the combination was synergistic in these cells, using concentrations of 2.5 and 5  $\mu$ M NVP-AEW541. Therefore, the concentration used in our study (1  $\mu$ M) may have been too low to observe an enhanced response to trastuzumab in the BT474 cells. However, while NVP-AEW541 is selective for IGF1R, with a concentration that causes 50% inhibition of growth ( $IC_{50}$ ) of 0.086  $\mu$ M in cellular activity

assays, the  $IC_{50}$  for its effect on the insulin receptor is 2.3  $\mu$ M [19], suggesting that use of the inhibitor at higher concentrations may result in non-specific effects.

While targeting IGF1R was ineffective in BT474 cells, the combination of either IGF1R siRNA or NVP-AEW541 with trastuzumab achieved significantly enhanced growth inhibition in the BT474/Tr cells compared with trastuzumab alone. Therefore, although IGF1R expression and phosphorylation was not significantly altered in this model of acquired trastuzumab resistance, co-targeting HER-2 and IGF1R enhances response to trastuzumab. Similar results were obtained with the IGF1R siRNA and the TKI in combination with trastuzumab, suggesting that the enhanced response was due to targeting IGF1R and not due to non-specific effects.

Two immunohistochemical studies have reported that IGF1R is expressed in 48.5% (33/68) and 54.0% (39/72) of HER-2-positive tumours [20, 21]. Phosphorylated IGF1R has been detected in ~64% of HER-2-positive breast tumours [22]. Targeting IGF1R may be a rational approach to improve response to trastuzumab in the sub-group of HER-2-positive breast tumours that express IGF1R. A number of IGF1R monoclonal antibodies are currently being tested in breast cancer, and more recently, a number of IGF1R TKIs have entered phase I trials [23]. A phase I/II trial of trastuzumab in combination with the IGF1R TKI, BMS-754807, is currently recruiting patients with advanced or metastatic HER-2-positive breast cancer.

In conclusion, our results provide further evidence that IGF1R inhibition may enhance response to trastuzumab in HER-2-positive tumour cells that also express IGF1R and in tumour cells that have developed resistance following trastuzumab treatment. Clinical trials of HER-2 and IGF1R inhibitors should also include analysis of potential predictive biomarkers to identify HER-2-positive tumours that may respond to the dual targeting approach.

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