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# HER-targeted tyrosine kinase inhibitors enhance response to trastuzumab and pertuzumab in HER2-positive breast cancer

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## Summary

Despite trastuzumab and pertuzumab improving outcome for patients with HER2-positive metastatic breast cancer, the disease remains fatal for the majority of patients. This study evaluated the anti-proliferative effects of adding anti-HER2 tyrosine kinase inhibitors (TKIs) to trastuzumab and pertuzumab in HER2-positive breast cancer cells. Afatinib was tested alone and in combination with trastuzumab in HER2-positive breast cancer cell lines. TKIs (lapatinib, neratinib, afatinib) combined with trastuzumab and/or pertuzumab were tested in 3 cell lines, with/without amphiregulin and heregulin-1β. Seven of 11 HER2-positive cell lines tested were sensitive to afatinib (IC$_{50}$ < 80 nM). Afatinib plus trastuzumab produced synergistic growth inhibition in eight cell lines. In trastuzumab-sensitive SKBR3 cells, the TKIs enhanced response to trastuzumab. Pertuzumab alone did not inhibit growth and did not enhance trastuzumab-induced growth inhibition or antibody-dependent cellular cytotoxicity. Pertuzumab enhanced response to trastuzumab when combined with lapatinib but not neratinib or afatinib. In two trastuzumab-resistant cell lines, the TKIs inhibited growth but adding trastuzumab and/or pertuzumab did not improve response compared to TKIs alone. Amphiregulin plus heregulin-1β stimulated proliferation of SKBR3 and MDA-MB-453 cells. In the presence of the growth factors, neither antibody inhibited growth and the TKIs showed significantly reduced activity. The triple combination of trastuzumab, pertuzumab and a TKI showed the strongest anti-proliferative activity in all three cell lines, in the presence of exogenous growth factors. In summary, addition of anti-HER2 TKIs to combined anti-HER2 monoclonal antibody therapy results in enhanced anticancer activity. These data contribute to the rationale for studying maximum HER2 blockade in the clinic.

**Keywords** HER2-positive · Breast cancer · erbB2 · Afatinib · Neratinib · Lapatinib

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Alexandra Canonici and Laura Ivers contributed equally to this work.

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s10637-018-0649-y) contains supplementary material, which is available to authorized users.

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## Introduction

The therapy for patients with breast carcinoma that contains an alteration in human epidermal growth factor receptor 2 (HER2) has evolved dramatically over the last two decades. The addition of trastuzumab to conventional chemotherapy resulted in improved response rates and survival for patients with overtly metastatic disease [1]. A proportion of these patients achieved durable remissions and some may be cured [2]. The incorporation of pertuzumab, a second anti-HER2 monoclonal antibody, into a regimen of conventional chemotherapy plus trastuzumab resulted in a further improvement in disease control, with median survival greater than four years [3]. However, for the overwhelming majority of patients with HER2-positive metastatic breast cancer the disease remains fatal.

The impact of trastuzumab in the adjuvant therapy of patients with HER2-altered early stage disease has been even greater [4]. Pertuzumab is also being studied in this setting and when added to pre-operative chemotherapy plus trastuzumab for patients with HER2-altered locally advanced disease, pertuzumab has resulted in improved rates of pathological complete response [5]. In the recently presented APHINITY trial, the addition of pertuzumab to adjuvant chemotherapy and trastuzumab prolonged the duration of distant relapse free survival, without affecting overall survival [6].

Another group of HER2-targeted agents, small molecule inhibitors that interact with the tyrosine kinase domain of HER family members, have also been studied. In random assignment trials, lapatinib, a reversible inhibitor of HER2 and epidermal growth factor receptor (EGFR) [7], has been shown to augment the effect of chemotherapy in both the first-line setting and in patients with trastuzumab pre-treated disease [8, 9]. In the NeoALTTO random assignment trial, addition of lapatinib to trastuzumab and chemotherapy produced more frequent pathological complete remissions than trastuzumab plus chemotherapy [10]. However, survival was not improved in this trial [9], nor in the ALTTO trial where lapatinib was studied as a post-operative adjuvant therapy [11]. Lapatinib has also been shown to be inferior to trastuzumab as a companion to chemotherapy in first line treatment for metastatic disease [12].

Afatinib and neratinib are both irreversible HER tyrosine kinase inhibitors (TKIs) [13, 14]. In the I-SPY-2 adaptive randomisation design, the addition of neratinib to chemotherapy and trastuzumab resulted in increased frequency of pathological complete response in HER2-positive breast cancer [15]. Neratinib also conferred additional progression free survival advantage for patients with early stage disease following conventional chemotherapy and trastuzumab therapy in the ExteNET trial [16].

The impact of pertuzumab in metastatic disease, together with the somewhat greater toxicity profile of the TKIs compared to monoclonal antibodies, has curtailed the development of these potentially useful drugs. However, preclinical data suggest that resistance mechanisms for lapatinib and trastuzumab differ, and that lapatinib given in combination with trastuzumab could result in improved rates of cytotoxicity [17–19]. Similarly, combined treatment with trastuzumab and neratinib enhances growth inhibition both in vitro and in vivo [20].

In this study, we investigated the effect of afatinib, alone and in combination with trastuzumab, in a panel of 11 HER2-positive breast cancer cell lines. Furthermore, we hypothesized that addition of the TKIs to trastuzumab and pertuzumab might result in a further anticancer effect for patients with HER2-positive disease. Thus, we tested combinations of trastuzumab and/or pertuzumab with TKIs in HER2-positive breast cancer cell lines.

## Materials and methods

### Cell lines and reagents

HER2-positive breast cancer cell lines BT474, SKBR3, HCC1419, HCC1569, HCC1954, MDA-MB-361, MDA-MB-453, JIMT-1, UACC-732, UACC-812 and EFM-192-A were obtained from the American Tissue Culture Collection (LGC Standards). The BT474, SKBR3, HCC1419, HCC1569, HCC1954, MDA-MB-361, MDA-MB-453 and EFM-192-A cells were cultured in RPMI-1640 medium (Sigma-Aldrich) containing 10% foetal calf serum (FCS) (PAA Laboratories GmbH); the JIMT-1 cells were cultured in Dulbecco's minimal essential medium (DMEM) (Sigma-Aldrich) containing 10% FCS; the UACC-812 cells were cultured in Leibovitz L15 (Sigma-Aldrich) containing 15% FCS; and the UACC-732 cells were cultured in minimum essential medium (MEM) (Sigma-Aldrich) containing 10% FCS, 4 nM glutamine (Life Technologies) and 1 mM sodium pyruvate (Life Technologies). SKBR3-TRes trastuzumab-resistant cells (SKBR3-TRes) were developed at UCLA following continuous exposure to trastuzumab (100 μg/ml) for 9 months as previously described [19]. SKBR3-TRes cells were cultured in RPMI containing 10% FCS and 100 μg/ml trastuzumab.

Afatinib (provided by Boehringer Ingelheim GmbH), lapatinib and neratinib (Carbosynth Limited) were prepared as 10 mM stocks in dimethyl sulfoxide (DMSO). Trastuzumab (Roche) was purchased from the Pharmacy Department, St Vincent's University Hospital, Dublin. Pertuzumab was obtained from Roche Diagnostics (Germany). Cell line identity was verified by short tandem repeat (STR) typing (Source Bioscience).

### 2D proliferation assays

Cell proliferation was determined using the acid phosphatase assay. Briefly, $5 \times 10^4$ cells/well for BT474 and MDA-MB-361 cells, and $3 \times 10^4$ cells/well for the other cell lines were seeded in 96-well plates. Following overnight incubation at 37 °C, drugs were added and incubated for 5 days at 37 °C. For assays with exogenous HER ligands added, cells were cultured in medium with 5% FCS plus amphiregulin (5 ng/mL) and heregulin-1β (20 ng/mL) (R&D Systems). Media was removed and cells were washed once with PBS. Acid phosphatase substrate (10 mM p-nitrophenyl-phosphate (Sigma-Aldrich) in sodium acetate buffer) was added to each well and incubated at 37 °C for 1 h. Following addition of 1 M NaOH, absorbance was read at 405 nm with 620 nm as the reference wavelength. Inhibition of proliferation was calculated relative to untreated controls. $IC_{50}$, the dose that inhibits 50% of growth, and combination index (CI) values at $ED_{50}$ were determined using CalcuSyn software. CI < 1 implies synergism, CI > 1 antagonism. Resistance to the TKIs was

classified as an $IC_{50}$ value >1 µM for lapatinib, >130 nM for neratinib and > 80 nM for afatinib, based on achievable plasma concentrations in patients.

## 3D growth assays

$3 \times 10^4$ cells/well for JIMT-1 and $7 \times 10^4$ cells/well for BT474 were seeded in media containing 2% growth factor reduced matrigel (VWR International) in 96-well plates previously coated with polyHEMA (Sigma-Aldrich). Following overnight incubation at 37 °C, drugs were added and incubated for 7 days at 37 °C. Presto Blue (Life Technologies) was added to each well and incubated at 37 °C (5 h for JIMT-1 and 7 h for BT474). Fluorescence was read at 544 nm (excitation wavelength) and 590 nm (emission wavelength). Inhibition of proliferation was calculated relative to untreated controls.

## Immunoblotting

Protein (30 µg) was separated on 4–12% gels (ThermoScientific), transferred to a nitrocellulose membrane using the iBlot 2 (ThermoScientific), and blocked in 1X NET (1.5 M NaCl, 0.05 M EDTA, 0.5 M Tris pH 7.8, 0.5% Triton X-100, 0.25% gelatin) for 1 h at room temperature. The membrane was incubated in primary antibody overnight at 4 °C (anti-EGFR (ThermoScientific); anti-HER2 (Millipore); anti-pEGFR; anti-pHER2; anti-HER3; anti-pHER3 (Cell Signalling Technology (CST)). Following three NET washes, membranes were incubated in secondary antibody (rabbit/ mouse 1:1000 in NET) (Sigma-Aldrich) for 90 min. Following three 10 min NET washes protein bands were visualized using ECL clarity (Bio-Rad). Alpha-tubulin (Sigma-Aldrich) was used as a loading control. Densitometry was performed using Total LabQuant on triplicate blots.

## Antibody-dependent cell-mediated cytotoxicity (ADCC) assays

ADCC assays were performed in triplicate as previously described [21]. Approval was granted by St Vincent's University Hospital Ethics Committee. Natural killer (NK) cells were isolated from healthy volunteers' blood using Ficoll-Paque plus (GE Healthcare) followed by selection of CD56 positive NK cells utilising magnetic beads (Miltenyi Biotec). ADCC was determined using the Guava Cell Toxicity Kit on a Guava Easycyte using Cytosoft™ software (Millipore). K562 cells were used as a positive control for direct NK cell cytotoxicity.

## Statistical analysis

Relationships between response to afatinib alone, or in combination with trastuzumab, and HER2, p95, EGFR, HER3, insulin-like growth factor receptor I (IGF-IR), protein kinase B (PKB/Akt), p27, phosphatase and tensin homolog (PTEN), phosphoinositide 3-kinase (PI3K) and oestrogen receptor (ER) (from [17]) were examined using the Spearman-Rank correlation, Mann-Whitney $U$ test and Chi-squared test on StatView for Windows (version 5.0.1; SAS Institute Inc). $P < 0.05$ was considered statistically significant.

**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

# Results

## Sensitivity to afatinib in HER2-positive breast cancer cell lines

In a panel of 11 HER2-positive breast cancer cell lines, afatinib $IC_{50}$ values ranged from 7.4 nM to greater than 5 µM (Fig. 1, Table 1). Using a cut-off of 80 nM, based on the achievable peak plasma concentration of afatinib [22], 7 of the 11 cell lines are sensitive to afatinib while 3 are resistant (UACC-732, JIMT-1, MDA-MB-453), with $IC_{50}$ values greater than 1 µM. UACC-812 cells show borderline afatinib sensitivity with an $IC_{50}$ value of 86 nM (Table 1). The 3 cell lines that are resistant to lapatinib (UACC-732, JIMT-1 and MDA-MB-453) are also resistant to afatinib. In terms of potency, afatinib and neratinib $IC_{50}$ values are significantly lower than lapatinib, with neratinib being slightly more potent than afatinib. In contrast, 6 of the 11 cell lines are innately resistant to trastuzumab (Table 1), but three of the trastuzumab-resistant cell lines are sensitive to the TKIs.

## Dual targeting with afatinib and trastuzumab

To examine the anti-proliferative benefit of combining afatinib with trastuzumab, we tested the combination in 7 HER2-positive cell lines, 4 sensitive to both trastuzumab and afatinib (BT474, SKBR3, EFM-192-A, MDA-MB-361), 2 resistant to both trastuzumab and afatinib (JIMT-1 and MDA-MB-453) and one cell line sensitive to afatinib but resistant to trastuzumab (HCC1419) (Fig. 2, Online Resource 1:Supplementary Table 1). In the four trastuzumab/afatinib sensitive cell lines, combining trastuzumab and afatinib significantly improved response compared to either single agent. In the trastuzumab-resistant HCC1419 cells, the combination enhanced response but to a lesser degree than in the trastuzumab-sensitive cell lines. In the trastuzumab/afatinib resistant cell lines, the combination did not enhance response compared to afatinib alone. We also tested afatinib in combination with trastuzumab in a cell line model of acquired trastuzumab resistance, SKBR3-TRes (Fig. 2, Online
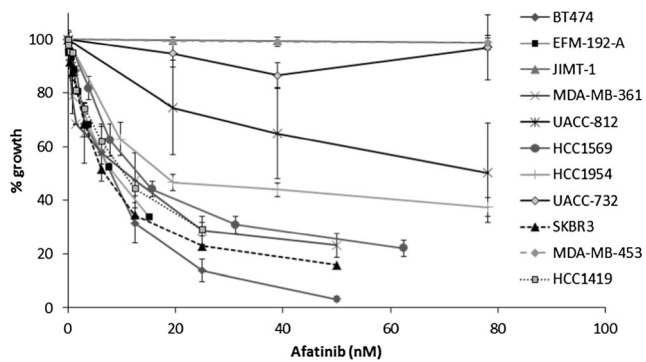
**Fig. 1** Growth inhibitory effect of afatinib in HER2-positive breast cancer cell lines. HER2-positive breast cancer cell lines were incubated with increasing doses of afatinib for 5 days and cell viability was determined using the acid phosphatase method. Data represents the mean $\pm$ SD of triplicate independent experiments. [◆= BT474, ■ = EFM-192-A, ▲ = JIMT-1, ✕ = MDA-MB-361, ✳ = UACC-812, ● = HCC1569, | = HCC1954, ◇ = UACC732, ▲ = SKBR3, ◆ = MDA-MB-453, ☐ = HCC1419]

Resource 1: Supplementary Table 1). Although the SKBR3-TRes cells show cross resistance to afatinib ($IC_{50} = 48.0 \pm 5.6$ nM), the combination of trastuzumab and afatinib showed a significant increase in growth inhibition compared to either drug alone.

As previous studies have reported differences in response to trastuzumab in two-dimensional (2D) and three-dimensional (3D) growth assays, we also examined the effect of the combination treatment in 3D assays for the BT474 and JIMT-1 cell lines. For the BT474 cells, the results obtained in the 3D assays were comparable to the 2D assays (Online Resource 2: Supplementary Figure 1). Interestingly the JIMT-1 cells were more sensitive to both afatinib and trastuzumab in 3D.

However, similar to the results observed in the 2D assays, the combination did not enhance response compared to afatinib alone (Online Resource 2: Supplementary Figure 1).

## Biomarkers of afatinib sensitivity

To identify predictive biomarkers of afatinib sensitivity, we examined the relationship between sensitivity to afatinib (defined as an $IC_{50}$ value less than 80 nM) and expression/activation of key signalling pathways in the panel of HER2-positive breast cancer cell lines, using previously published protein expression/activation data [17]. Sensitivity to afatinib significantly correlates with higher levels of HER2 ($p = 0.02$), phospho-HER2 ($p = 0.04$) and phospho-EGFR ($p = 0.05$) (Online Resource 1: Supplementary Table 2). No association was observed between response to afatinib and activation of the PI3K/Akt signalling pathway. The only factor examined that was predictive of enhanced response to combined afatinib and trastuzumab treatment was innate trastuzumab sensitivity ($p = 0.03$) (Online Resource 1: Supplementary Table 2).

## Sensitivity to HER2-targeted TKIs combined with Trastuzumab and/or Pertuzumab

Three HER2-positive cell lines, which also express EGFR and HER3 (Online Resource 2: Supplementary Figure 2), and represent models of trastuzumab sensitivity (SKBR3) and resistance (MDA-MB-453, HCC1569), and TKI sensitivity (SKBR3, HCC1569) and resistance (MDA-MB-453) (Table 1) were selected to test combinations of the TKIs with trastuzumab and/or pertuzumab. Combining the two antibodies, trastuzumab and

**Table 1** Sensitivity to afatinib, neratinib, lapatinib and trastuzumab in HER2-positive breast cancer cell lines

| | Afatinib | | Neratinib | | Lapatinib | | Trastuzumab[b] |
|---|---|---|---|---|---|---|---|
| | $IC_{50} \pm SD$ (nM) | Response[a] | $IC_{50} \pm SD$ (nM) | Response | $IC_{50} \pm SD$ (nM) | Response | Response |
| BT474 | $7.4 \pm 1.1$ | S | $0.84 \pm 0.11$ | S | $23.9 \pm 19.6$ | S | S |
| SKBR3 | $7.4 \pm 0.8$ | S | $1.26 \pm 0.24$ | S | $25.9 \pm 2.9$ | S | S |
| EFM-192-A | $8.4 \pm 0.6$ | S | $2.38 \pm 0.23$ | S | $20.2 \pm 18.6$ | S | S |
| MDA-MB-361 | $10.0 \pm 3.6$ | S | $7.36 \pm 2.65$ | S | $323.5 \pm 50.8$ | S | S |
| HCC1419 | $10.5 \pm 0.3$ | S | $3.94 \pm 0.5$ | S | $63.1 \pm 29.7$ | S | R |
| HCC1569 | $15.5 \pm 3.9$ | S | $36.24 \pm 4.85$ | S | $242.0 \pm 8.7$ | S | R |
| HCC1954 | $19.6 \pm 7.5$ | S | $24.73 \pm 5.42$ | S | $582 \pm 31$ | S | R |
| UACC-812 | $86.5 \pm 53.5$ | S/R | $4.03 \pm 0.14$ | S | $80.4 \pm 32.7$ | S | S |
| JIMT-1 | $1691.2 \pm 592.6$ | R | $141.5 \pm 9.61$ | R | $1416.0 \pm 371.9$ | R | R |
| MDA-MB-453 | $2613.6 \pm 214.7$ | R | $267.23 \pm 22.52$ | R | $5186.3 \pm 1542.8$ | R | R |
| UACC-732 | $> 5000$ | R | $132.23 \pm 19.66$ | S/R | $3180 \pm 939$ | R | R |

[a] S: sensitive, R: resistant

[b] [17]

**Fig. 2** Effect of afatinib in combination with trastuzumab on proliferation of HER2-positive breast cancer cell lines. Seven HER2-positive and one acquired trastuzumab-resistant (SKBR3-TRes) breast cancer cell lines were treated with increasing doses of afatinib, trastuzumab or the combination, at the indicated concentrations for 5 days. Cell viability was determined using the acid phosphatase method. Data represents the mean ± SD of triplicate independent experiments.[♦ = afatinib, ■ = trastuzumab, ▲ = trastuzumab + afatinib]
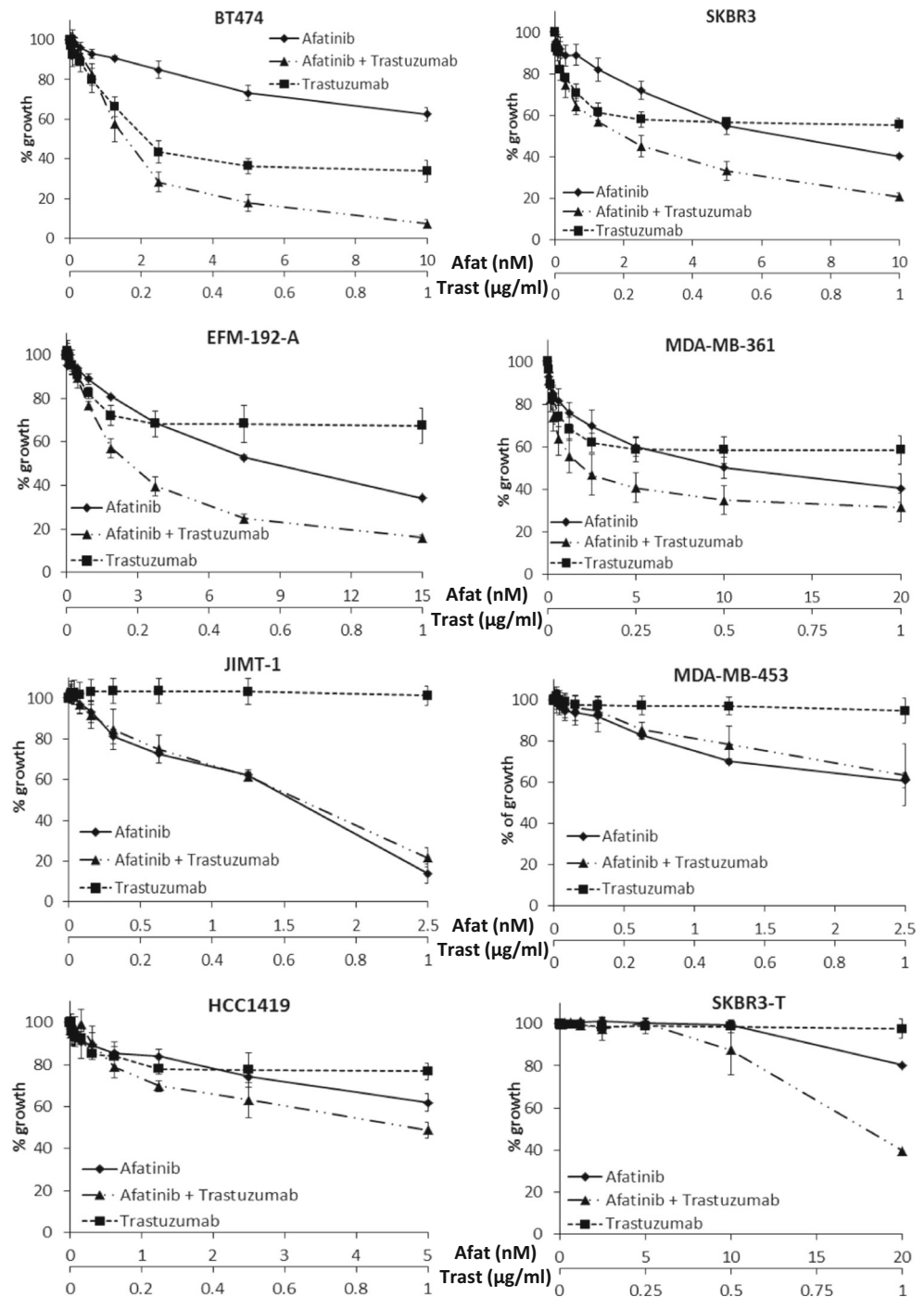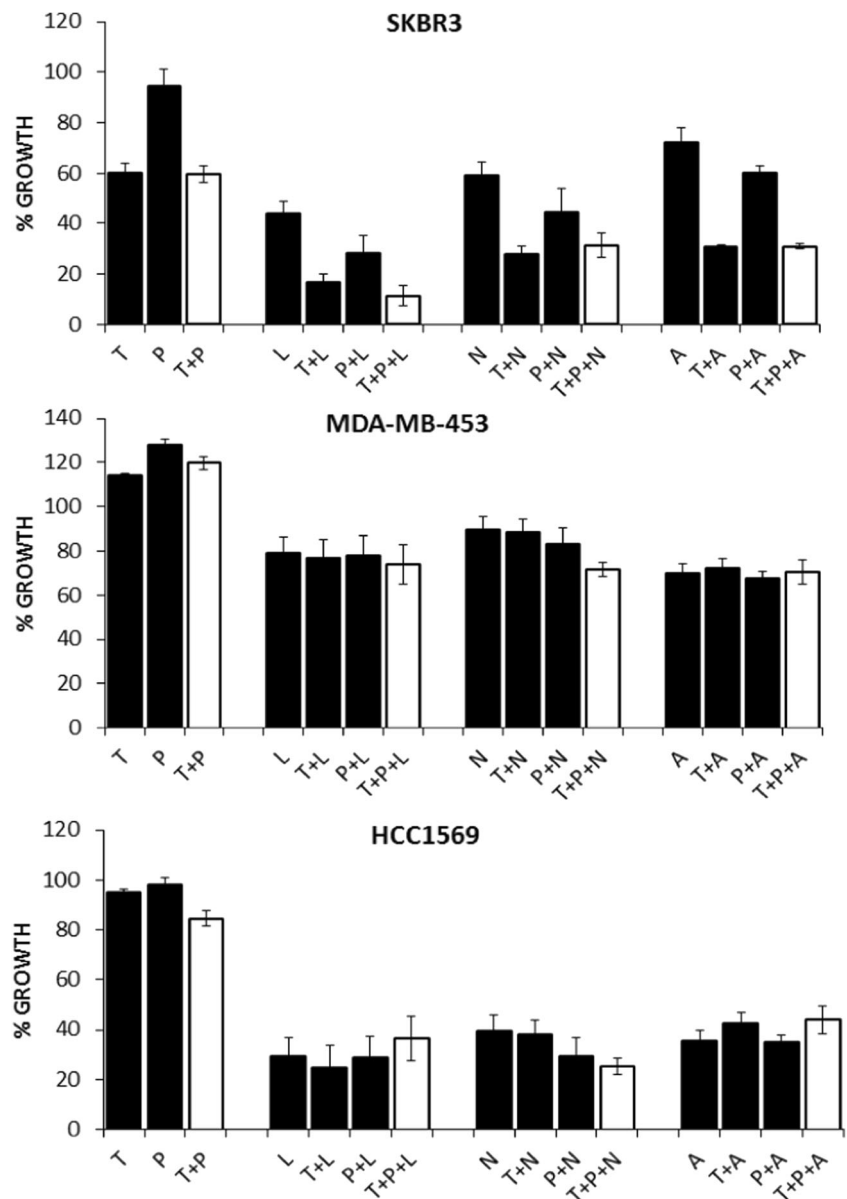


pertuzumab, did not enhance response in any of the three cell lines (Fig. 3).

Lapatinib combined with trastuzumab enhanced response compared to trastuzumab ($p = 0.004$) and lapatinib ($p = 0.002$) alone in SKBR3 cells (Fig. 3). In MDA-MB-453 and HCC1569 cells, lapatinib plus trastuzumab did not significantly enhance response compared to lapatinib alone ($p = 0.119$, $p = 0.609$, respectively) (Fig. 3). Pertuzumab combined with lapatinib also produced an enhanced response in SKBR3

cells, compared to lapatinib ($p = 0.035$), and addition of pertuzumab to trastuzumab and lapatinib slightly enhanced growth inhibition compared to trastuzumab plus lapatinib (88.5% versus 83.1%, $p = 0.057$) (Fig. 3).

Similar to lapatinib, neratinib or afatinib combined with trastuzumab significantly enhanced growth inhibition in SKBR3 cells, compared to the TKI alone ($p = 0.003$, $p = 0.006$ respectively), but not in MDA-MB-453 and HCC1569 cells (Fig. 3). In SKBR3 cells, the triple combination of

**Fig. 3** Proliferation assays for SKBR3, MDA-MB-453 and HCC1569 cells treated with trastuzumab (T) (5 μg/mL), pertuzumab (P) (5 μg/mL) and/or lapatinib (L)/neratinib (N) /afatinib (A), in RPMI plus 10% FBS. White bars highlight the combinations of trastuzumab plus pertuzumab with/without a TKI. Error bars represent the standard deviations of triplicate experiments



trastuzumab and pertuzumab plus either neratinib or afatinib did not enhance response compared to trastuzmab plus the TKI (Fig. 3). In contrast, in both MDA-MB-453 and HCC1569 cells, the triple combination of trastuzumab and pertuzumab plus neratinib showed the strongest anti-proliferative effect (MDA-MB-453: p = 0.05; HCC1569: $p$ = 0.04 versus pertuzumab plus neratinib) (Fig. 3). Addition of trastuzumab and/or pertuzumab did not enhance response to afatinib in MDA-MB-453 or HCC1569 cells (Fig. 3).

To determine if the TKIs have an impact on trastuzumab/pertuzumab-mediated ADCC we performed in vitro NK cell ADCC assays on SKBR3 cells pre-treated with the TKIs. Both trastuzumab and pertuzumab alone induced NK cell-mediated ADCC, however combined treatment did not increase the ADCC response. Furthermore, pre-treatment with the TKIs did

not alter the trastuzumab and/or pertuzumab induced ADCC response (Online Resource 2: Supplementary Figure 3).

## HER ligands significantly alter response to HER2-targeted therapies

The combination experiments were repeated in medium supplemented with ligands amphiregulin (5 ng/mL) and heregulin-1β (20 ng/mL). The ligands stimulated proliferation of SKBR3 and MDA-MB-453, but not HCC1569 cells (Fig. 4). In the presence of the ligands, trastuzumab and/or pertuzumab did not inhibit growth of the three cell lines (Fig. 4). The TKIs also showed significantly reduced activity in the presence of the growth factors. Interestingly, in SKBR3

**Fig. 4** Proliferation response of HER2-positive breast cancer cell lines cultured in medium (with 5% FCS) and supplemented with growth factors (amphiregulin (AREG) and heregulin (HRG)) (C), and treated with trastuzumab (T), pertuzumab (P) and/or lapatinib (L), neratinib (N) or afatinib (A), relative to normal growth medium containing 10% FCS. White bars highlight the combinations of trastuzumab plus pertuzumab with/without a TKI. Error bars represent the standard deviations of triplicate experiments
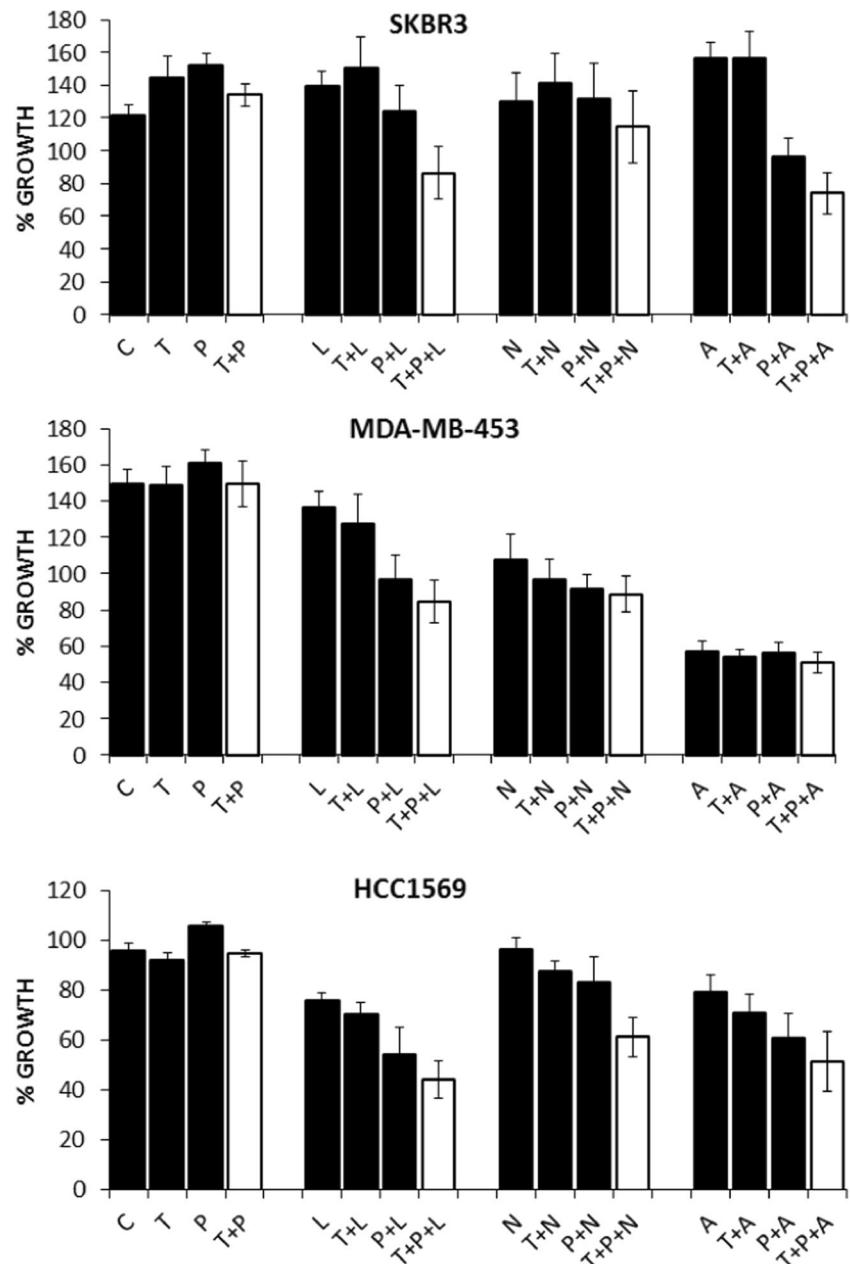
cells the two antibodies and the TKIs stimulated growth in the presence of the growth factors (Fig. 4).

In SKBR3 cells, trastuzumab plus pertuzumab and lapatinib or afatinib produced the strongest anti-proliferative response (Fig. 4). To determine if the effects of the growth factors would be overcome by higher concentrations of therapies, we tested trastuzumab (50 μg/mL) and pertuzumab (50 μg/mL) and neratinib (25 nM) in the SKBR3 cells (Fig. 5). The higher concentrations of trastuzumab, pertuzumab or neratinib had no impact on cell growth individually. Combined treatment with trastuzumab and pertuzumab or trastuzumab and neratinib also showed no effect on growth. However, at the higher

concentrations pertuzumab combined with neratinib ($p = 0.001$), and the triple combination (trastuzumab, pertuzumab and neratinib) significantly inhibited growth ($p = 0.0004$).

In the presence of the growth factors, MDA-MB-453 cells retained sensitivity to afatinib (1 μM) but were not sensitive to lapatinib (1 μM) or neratinib (270 nM) (Fig. 4). For each of the TKIs, the triple combination with trastuzumab, pertuzumab and the TKI showed the strongest anti-proliferative effect. In HCC1569 cells, the triple combinations also showed the strongest anti-proliferative effect, significantly stronger than trastuzumab plus pertuzumab or trastuzumab plus each of the TKIs ($p < 0.05$) (Fig. 4).

**Fig. 5** SKBR3 cells treated with trastuzumab (50 μg/mL), pertuzumab (50 μg/mL) and 25 nM neratinib with exogenous amphiregulin (5 ng/mL) and heregulin (20 ng/mL) in RPMI plus 5% FBS. White bars highlight the combinations of trastuzumab plus pertuzumab with/without a TKI. Error bars represent the standard deviations of triplicate experiments
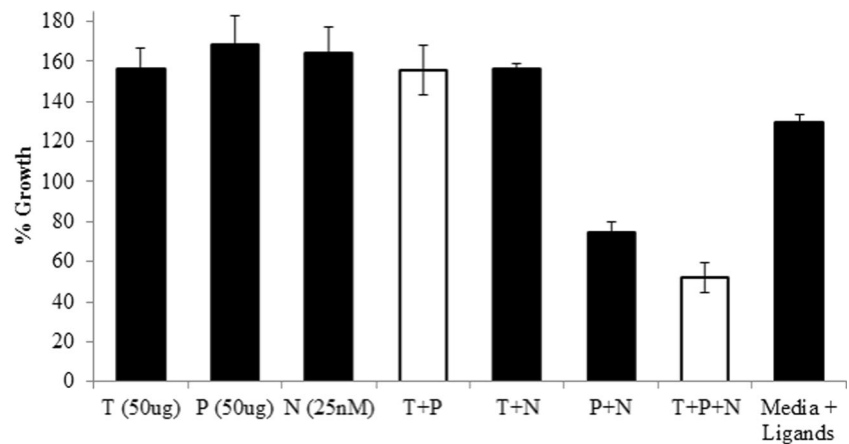


## Discussion

In this study, we first tested the irreversible pan-HER inhibitor afatinib in a panel of HER2-positive breast cancer cell lines, including cell lines which are innately resistant to trastuzumab and a cell line model of acquired trastuzumab resistance. A direct comparison of the $IC_{50}$ values for lapatinib, neratinib and afatinib shows that neratinib and afatinib are significantly more potent than lapatinib in the panel of cell lines, which is most likely attributable to the fact that they are irreversible inhibitors, whereas lapatinib is a reversible inhibitor of HER2 and EGFR. Neratinib is generally more potent than afatinib across the panel of cell lines, in vitro. When the cell lines are classified as sensitive or resistant to each of the TKIs, using the achievable peak plasma concentrations reported in humans as cut-off values (afatinib 80 nM [22], neratinib 130 nM [23], lapatinib 1 μM [19]), the profile of sensitivity/ resistance is similar with three cell lines displaying innate resistance to all three TKIs.

Addition of the TKIs to trastuzumab may overcome resistance to trastuzumab by blocking compensatory signalling pathways. Similar to our previously reported data for the addition of lapatinib or neratinib to trastuzumab [18, 20], combined treatment with trastuzumab and afatinib improves response compared to trastuzumab alone in all of the cell lines tested, including a cell line model of acquired trastuzumab resistance (SKBR3-TRes).

As pertuzumab is now approved in combination with trastuzumab for first line treatment of metastatic HER2-positive breast cancer, we performed a preclinical evaluation of trastuzumab and/or pertuzumab combined with the HER-targeted TKIs, in three HER2-positive breast cancer cell lines. The cell lines selected represent models of trastuzumab/ lapatinib sensitivity (SKBR3), trastuzumab/lapatinib resistance (MDA-MB-453) and lapatinib sensitive but trastuzumab resistant (HCC1569). Both SKBR3 and HCC1569 cells are sensitive to neratinib and afatinib. The lapatinib resistant MDA-MB-453 cells are resistant to afatinib and neratinib.

Pertuzumab did not inhibit growth in vitro and did not enhance response to trastuzumab. Consistent with our results, pertuzumab showed no significant anti-proliferative activity in SKBR3 cells in 2D [24] or 3D [25] assays. Based on the fact that pertuzumab inhibits receptor heterodimerisation and has been shown to inhibit proliferation in a cell line model of autocrine heregulin-stimulated HER2-HER3 signalling (MDA-MB-175) [26], we tested the effects of the HER ligands, amphiregulin, which binds to EGFR, and heregulin, which binds to HER3 and HER4 [27]. However, in the presence of the ligands, pertuzumab alone did not inhibit growth of any of the cell lines, in fact it stimulated growth of all three cell lines to varying degrees. In the presence of ligands, trastuzumab did not inhibit growth of SKBR3 cells. Weigelt et al. [25] reported that SKBR3 cells are resistant to trastuzumab when grown in 3D. Growth factors present in matrigel may contribute to the resistance observed in 3D assays. Several previous studies have implicated high levels of heregulin and/or amphiregulin in resistance to HER2-targeted therapies [28–30].

Nahta et al. [31] reported that combined treatment with trastuzumab and pertuzumab inhibited proliferation and induced apoptosis in BT474 cells in vitro. In a KPL-4 xenograft model of HER2-positive inflammatory breast cancer, trastuzumab plus pertuzumab exhibited significantly enhanced anti-tumour activity and induced tumour regression [32]. Scheuer et al. [32] also reported that the combination enhanced ADCC activity in vitro. However, in our study pertuzumab did not enhance response to trastuzumab in SKBR3 or MDA-MB-453 cells, with/without exogenous amphiregulin and heregulin. Furthermore, pertuzumab did not enhance trastuzumab-induced ADCC against SKBR3 cells. In HCC1569 cells, addition of pertuzumab led to a small, but statistically significant, enhanced response compared to trastuzumab alone, in the absence of amphiregulin/heregulin. Interestingly, addition of the ligands did not significantly enhance the growth of HCC1569 cells, in contrast to the SKBR3 and MDA-MB-453 cells, suggesting that they may not require

exogenous ligands and thus, similar to the MDA-MB-175 and KPL-4 models, may possess autocrine EGFR/HER2/HER3 signalling. Thus, one could speculate that the benefit of adding pertuzumab to trastuzumab may be greatest in tumours driven by autocrine EGFR/HER2/HER3 signalling.

In culture medium containing 10% serum, addition of a TKI enhanced response to trastuzumab and/or pertuzumab in the SKBR3 cells. Trastuzumab combined with a TKI showed similar anti-proliferative activity compared to trastuzumab plus pertuzumab and a TKI, in this model. As both trastuzumab and pertuzumab induce ADCC it may be argued that the dual antibody combination may have greater benefit based on improved ADCC response. However, we did not observe any increase in ADCC response with the addition of pertuzumab in ADCC assays performed in vitro with SKBR3 cells. In the two trastuzumab-resistant cell lines tested, the TKIs showed anti-proliferative activity but addition of the TKIs to the monoclonal antibodies did not improve response compared to the TKIs alone.

In the presence of the exogenous ligands, the TKIs showed significantly reduced anti-proliferative activity. Under these conditions, the only combination that showed significant anti-proliferative activity across all three cell lines was trastuzumab plus pertuzumab plus a TKI. Interestingly, in HCC1569 cells, pertuzumab combined with a TKI also showed anti-proliferative activity, which was not observed with trastuzumab. This may be related to autocrine EGFR/HER2/HER3 signalling in HCC1569 cells as discussed above.

Our results suggest that the addition of small molecule TKIs to antibody/chemotherapy based therapies may result in improved anti-tumour activity in HER2-altered metastatic breast cancer. We believe that there is a sound scientific rationale for performing a prospective clinical trial to test this hypothesis. However, organisational hurdles may make such a trial difficult to initiate.

## Compliance with ethical standards

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