Effects of HER Family–targeting Tyrosine Kinase Inhibitors on Antibody-dependent Cell-mediated Cytotoxicity in HER2-expressing Breast Cancer

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ABSTRACT

Purpose: Antibody-dependent cell-mediated cytotoxicity (ADCC) is one mechanism of action of the monoclonal antibody (mAb) therapies trastuzumab and pertuzumab. Tyrosine kinase inhibitors (TKIs), like lapatinib, may have added therapeutic value in combination with mAbs through enhanced ADCC activity. Using clinical data, we examined the impact of lapatinib on HER2/EGFR expression levels and natural killer (NK) cell gene signatures. We investigated the ability of three TKIs (lapatinib, afatinib, and neratinib) to alter HER2/immune-related protein levels in preclinical models of HER2-positive ($HER2^+$) and HER2-low breast cancer, and the subsequent effects on trastuzumab/pertuzumab-mediated ADCC.

Experimental Design: Preclinical studies (proliferation assays, Western blotting, high content analysis, and flow cytometry) employed $HER2^+$ (SKBR3 and HCC1954) and HER2-low (MCF-7, T47D, CAMA-1, and CAL-51) breast cancer cell lines. NCT00524303 provided reverse phase protein array–determined protein levels of HER2/pHER2/EGFR/pEGFR. RNA-based NK cell gene signatures (CIBERSORT/MCP-counter) post-neoadjuvant anti-HER2 therapy were assessed (NCT00769470/NCT01485926). ADCC assays utilized flow cytometry–based protocols.

Results: Lapatinib significantly increased membrane HER2 levels, while afatinib and neratinib significantly decreased levels in all preclinical models. Single-agent lapatinib increased HER2 or EGFR levels in 10 of 11 (91%) tumor samples. NK cell signatures increased posttherapy ($P = 0.03$) and associated with trastuzumab response ($P = 0.01$). TKI treatment altered mAb-induced NK cell–mediated ADCC *in vitro*, but it did not consistently correlate with HER2 expression in $HER2^+$ or HER2-low models. The ADCC response to trastuzumab and pertuzumab combined did not exceed either mAb alone.

Conclusions: TKIs differentially alter tumor cell phenotype which can impact NK cell–mediated response to coadministered antibody therapies. mAb-induced ADCC response is relevant when rationalizing combinations for clinical investigation.

Introduction

The humanized monoclonal antibody (mAb), trastuzumab, has transformed the treatment of breast cancers classified as HER2-positive ($HER2^+$/*ERBB2*$^+$), which account for approximately 20% of breast cancers (1). Trastuzumab containing regimens have significantly improved overall survival and disease-free survival compared with non-trastuzumab regimens in early $HER2^+$ breast cancer, and in $HER2^+$ metastatic beast cancer (MBC) (2, 3). The majority of patients with MBC develop resistance, but long-term follow-up has revealed durable complete responses to trastuzumab in 11% of patients with $HER2^+$ MBC (median follow-up, 64.5 months; ref. 4). Trastuzumab binds subdomain IV of the extracellular domain (ECD) of HER2, exerting its effects through inhibition of HER2 intracellular signaling pathways (MAPK and PI3K/Akt) and antibody-dependent cell-mediated cytotoxicity (ADCC; ref. 5). There is preclinical and emerging clinical data showing that the ADCC response to trastuzumab is an essential factor in its efficacy (6–10). Pertuzumab is a humanized mAb that inhibits dimerization of HER2 by binding subdomain II of the ECD, initiating apoptosis, and tumor growth inhibition in preclinical $HER2^+$ models (11). Pertuzumab is approved in combination with trastuzumab and chemotherapy for the treatment of $HER2^+$ breast cancer in the neoadjuvant, adjuvant, and metastatic settings (12). Like trastuzumab, pertuzumab contains an Fc region capable of engaging FcγR-positive immune cells and initiating ADCC with a comparable potency to trastuzumab in $HER2^+$ models (12–14).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The mAbs, trastuzumab and pertuzumab, are the standard-of-care HER2-targeting therapies for the treatment of early and advanced HER2-positive ($HER2^+$) breast cancer. Trastuzumab and pertuzumab are capable of engaging immune cells, such as natural killer (NK) cells, and inducing tumor cell death through antibody-dependent cell-mediated cytotoxicity (ADCC). Lapatinib (HER2/EGFR-targeting) and neratinib (pan-HER inhibitor) are tyrosine kinase inhibitors (TKIs) approved for the treatment of $HER2^+$ breast cancer in second-line settings. Afatinib is a pan-HER TKI not currently approved for the treatment of $HER2^+$ breast cancer. Greater understanding of the NK cell–mediated immune response to mAb therapies and the immunomodulatory abilities of TKIs could provide rationales for future clinical studies and new treatment strategies for $HER2^+$ and HER2-low breast cancer, especially in the era of antibody–drug conjugates and immune checkpoint inhibition.

Small-molecule tyrosine kinase inhibitors (TKIs) target the intracellular domain (ICD) of HER2, inhibiting kinase activity and downstream signaling (15). The rationale for TKI/mAb combinations arises primarily from the ability of TKIs to overcome trastuzumab resistance by targeting multiple HER family members simultaneously to inhibit alternate signaling strategies and by maintaining activity against the constitutively activated truncated form of HER2, p95 (5). In addition, there is evidence that HER2-targeting TKIs can modulate the mAb-mediated ADCC response. The reversible HER2/EGFR TKI, lapatinib, increases peripheral blood mononuclear cell (PBMC)-mediated trastuzumab-induced ADCC in HER2-overexpressing cell lines and this is associated with increased tumor cell surface levels of HER2 (16–18). A secondary analysis of the NeoALTTO clinical trial revealed the presence of tumor-infiltrating lymphocytes at diagnosis as a positive prognostic marker for response to neoadjuvant trastuzumab, lapatinib, and chemotherapy, highlighting a potential role for immune cells in response to a mAb/TKI combination (19). Lapatinib is approved for second-line treatment of MBC in combination with capecitabine in trastuzumab-refractory patients (20).

Next-generation inhibitors, like the irreversible pan-HER inhibitor, afatinib, displayed preclinical efficacy in $HER2^+$ breast cancer models and activity in heavily pretreated $HER2^+$ MBC (18, 21). The irreversible pan-HER inhibitor, neratinib, is approved for the extended adjuvant treatment of patients with early-stage, $HER2^+$ breast cancer and in combination with capecitabine for the treatment of metastatic $HER2^+$ breast cancer (22).

Approximately 60% of breast cancers are considered HER2-low (23). We have previously shown that trastuzumab is capable of inducing a strong ADCC response against HER2-low breast cancer cell lines *in vitro* (24). TKI-altered HER2 levels may have more impact in HER2-low models that have antigen levels close to ADCC threshold.

Our objective was to investigate immune response–based preclinical rationale for the combination of TKIs and HER2 mAbs in breast cancer using a panel of breast cancer cell lines of varying HER2 expression levels/molecular subtype and relevant clinical data. Membrane ECD-HER2 levels were determined by high-content analysis and flow cytometry following TKI treatment. To assess the clinical relevance of TKI-altered HER family levels observed *in vitro*, we investigated the impact of lapatinib on HER2 protein levels in samples from the neoadjuvant LPT109096 (NCT00524303) trial. We carried out ADCC assays on TKI-treated breast cancer cells with mixed effector cell populations for comparison with previous literature, and with natural killer (NK) cells, based on tumor NK cell gene signatures from neoadjuvant $HER2^+$ breast cancer studies, NCT00769470 and NCT01485926. mAb binding, TKI impact on EGFR, HER3, and HER4, and a preliminary examination of NK cell regulatory proteins (PD-L1, HLA-E, and MHC class I) were performed to further explore the impact of TKI treatment on breast cancer cell lines.

Materials and Methods

Cell lines and reagents

Cell lines were cultured at 37°C in 5% CO_2 without antibiotics and with routine monitoring for *Mycoplasma* contamination. $HER2^+$/estrogen receptor-negative (ER^-) SKBR3, $HER2^+/ER^-$ HCC1954, HER2-low/ER^+ T47D, and HER2-low/ER^+ MCF-7 (ATCC) were maintained in RPMI1640 and 10% heat-inactivated (HI) FCS (25). HER2-low/ER^+ CAMA-1 (ATCC) was maintained in MEM, supplemented with 10% HI FCS, NEAA, sodium pyruvate, and L-glutamine and HER2-low/ER^- CAL-51 (German Tissue Repository DSMZ) was maintained in DMEM, 10% HI FCS, and 2 mmol/L sodium pyruvate (26). Estrogen supplementation was not utilized for ER^+/HER2-low cell lines (MCF-7, CAMA-1, and T47D), and the FCS and phenol red in the culture medium maintained cell growth (27). Cell lines were authenticated by short tandem repeat DNA Profiling (Source BioScience). Lapatinib, afatinib, and neratinib were obtained from Sequoia Chemicals and solubilized in DMSO. Trastuzumab (Herceptin) and rituximab (Rituxan) were obtained from St. Vincent's University Hospital (Dublin, Ireland). Pertuzumab (Perjeta) was obtained from Roche Innovation Center.

Cell proliferation assays

The growth inhibitory effects of TKIs, trastuzumab and pertuzumab, were measured using an acid phosphatase–based proliferation assay, as described previously (28). A description can be found in Supplementary Materials and Methods.

Western blotting

Protein lysates were prepared from 1×10^6 cells seeded in 90-mm petri dishes 24 hours prior to treatment with TKIs or controls. Cells were lysed using RIPA lysis buffer with protease inhibitor, PMSF, and sodium orthovanadate (Sigma). Protein concentrations were determined by the BCA Method (Pierce). Protein samples were prepared at the required concentration in 4X loading buffer (Invitrogen). Samples were denatured (95°C for 5 minutes). Proteins were separated by SDS-PAGE (NuPAGE, Invitrogen) and transferred using the iBlot System (Invitrogen). The following primary antibodies were used: EGFR (Cell Signaling Technology, #4267), HER2 (Lab Vision, #OP-15), pHER2 Y1221/1222 (Cell Signaling Technology, #2249), HER3 (Cell Signaling Technology, #4754), pHER3 Y1289 (Cell Signaling Technology, #4791), pHER2 Y1248 (Upstate, #06229), pEGFR Y992 (Cell Signaling Technology, #2235), pEGFR Y1068 (Millipore, #07-818), MHC Class I (Abcam, #ab52922), HLA-E (Abcam, #ab2216), PD-L1 Clone E1L3N (Cell Signaling Technology, #13684), and *Alpha*-tubulin (Sigma, #T6199). Mouse (Sigma, #A6782) and rabbit (Sigma, #A6154) secondary antibodies were used. Protein bands were visualized using Luminol Detection Reagent (Santa Cruz Biotechnology) or ECL (GE Healthcare). Densitometry was carried out using TotalLab software.

Isolation of effector cells

Ethical approval for collection of blood was obtained from St. Vincent's University Hospital (Dublin, Ireland) Ethics Committee. Whole blood (20 mL) was collected from healthy female volunteers in EDTA Vacutubes (BD Biosciences). The PBMC fraction was isolated using a Ficoll-Paque plus Protocol (GE Healthcare). PBMCs were counted by flow cytometry (Guava ViaCount) and resuspended in ADCC assay medium (RPMI1640/10% HI FCS/0.1 mmol/L penicillin/streptomycin). NK cells were isolated from the PBMC fraction using $CD56^+$ magnetic beads on a midiMACs Column according to the manufacturer's protocol (70%–90% purity; Miltenyi Biotec).

ADCC assays

The method for flow cytometry–based ADCC assays in the absence of TKI pretreatment has been published previously (24, 29). For TKI pretreatment, cells were seeded in T75 flasks to provide 80%–90% confluency after 72 hours. After 24 hours, medium was removed and replaced with fresh medium containing 2 μmol/L lapatinib, afatinib, or neratinib, or 0.2% DMSO for 48 hours. TKI concentration of 2 μmol/L was chosen based on previous studies (30). Treatments were removed, cells were washed in PBS, and painted with carboxyfluorescein diacetate succinimidyl ester (CFSE). Target cells were removed from the flasks with minimum exposure to trypsin and washed twice in PBS. Target cell and NK cell/PBMC concentrations were normalized to a concentration of 2.5×10^5 cells/mL (Guava ViaCount). Target and immune cell aliquots were then prepared in medium alone or medium with 10 μg/mL trastuzumab, 10 μg/mL pertuzumab, or 10 μg/mL trastuzumab and 10 μg/mL pertuzumab. Cells were then plated in duplicate at a final volume of 200 μL in a round-bottomed nontissue culture–treated 96-well plate at three ratios (24). Plates were spun at $50 \times g$ for 3 minutes to start the assay. ADCC assays were started within 6 hours of TKI removal. Following a 12-hour incubation with donor PBMCs, 40-μL 7-AAD was added to each well, mixed, and allowed to incubate for 10 minutes. Percentage cell death was determined on a Guava easyCyte Flow Cytometer using InCyte Software (Millipore). % ADCC = (% target cell cytotoxicity mediated by effector cells with antibody) − (% target cell cytotoxicity mediated by effector cells without antibody). % Direct NK cytotoxicity = (target cell cytotoxicity mediated by effector cells without antibody) – (basal target cell death). Controls included target cells alone +/− antibody, effector cells alone, and dead cell controls. The CD20-specific ADCC capable mAb, rituximab, was utilized as the negative control for ADCC (Supplementary Fig. S1). CFSE-painted K562 cells were utilized as the positive control target cells for direct PBMC or NK cell cytotoxicity.

Reverse phase protein array of tumor samples

Reverse phase protein array (RPPA) data were taken from analyses of LPT109096 (NCT000524303), a phase II randomized clinical trial examining neoadjuvant trastuzumab and/or lapatinib plus chemotherapy [sequential FEC75 (5-fluorouracil, epirubicin. cyclophosphamide) and paclitaxel] in women with HER2-overexpressing invasive breast cancer. RPPA analysis of patient samples is detailed in Holmes and colleagues, 2013 (31). Posttreatment samples were collected after 2 weeks of neoadjuvant trastuzumab, lapatinib, or trastuzumab treatment without chemotherapy.

High-content analysis

SKBR3 (10,000 cells/well), MCF-7/T47D, (4,000 cells/well), and HCC1954 (2,500 cells/well) were seeded in clear-bottomed 96-well plates and dual stained in series using ICD-targeted (Cell Signaling Technology, #2242) and ECD-targeted (Calbiochem, #OP39) HER2 antibodies. Plate preparation is outlined in detail in Supplementary Materials and Methods. Alexa Fluor 488–labeled (A27034) and Alexa Fluor 594–labeled (A11032) secondary antibodies (Invitrogen) were used, respectively. Nuclear staining required Hoechst 33342 (Invitrogen, #H1399) at 4 μmol/L. Hoechst 33342, primary antibodies, and secondary antibodies were diluted in 3% normal goat serum//PBS. Images were acquired on the IN Cell-1000 Analyzer automated imaging platform and analyzed using a Multi-Target Analysis algorithm on InCell analysis software. HER2 expression was determined by measurement of integrated fluorescence intensity of inclusions (area × intensity) in the various cellular compartments analyzed. Membranous ECD HER2 = [(membranous/outer cytoplasmic region excluding nuclei) – (cytoplasmic region excluding nuclei)]. Untreated triplicate wells for medium, DMSO, and primary and secondary antibody controls were included.

Analysis of NK cell gene signatures

DNA microarray data from TRIO-B07 (NCT00769470), a randomized phase II trial examining neoadjuvant trastuzumab (H) or lapatinib (L) or the combination followed by docetaxel (T) and carboplatin (C) with trastuzumab and/or lapatinib in $HER2^+$ breast cancer, are publicly available through Gene Expression Omnibus series accession number GSE130788. Activated NK cell gene signatures (CIBERSORT) in tumor samples ($n = 89$) were examined before and after 2 weeks of neoadjuvant trastuzumab, lapatinib, or trastuzumab/lapatinib therapy without TC. Posttreatment samples were also examined for pathologic complete response (pCR) to subsequent chemotherapy plus targeted therapy. RNA sequencing (RNA-seq) data were obtained from ICORG 10-05 (NCT01485926), a phase II neoadjuvant study assessing TC with trastuzumab and/or lapatinib in $HER2^+$ breast cancer. An NK cell gene signature (MCP-counter) was examined before and after 21 days of therapy for 8 patients. The analysis included TCH ($n = 4$), TCL ($n = 1$), and TCHL ($n = 3$) treated tumor samples. MCP-counter was the appropriate analysis tool for RNA-seq data, while CIBERSORT was appropriate for a DNA microarray–based dataset. Details of DNA microarray and RNA-seq methods are outlined in Supplementary Material and Methods.

Trastuzumab/pertuzumab binding assays

Trastuzumab (Dylight 488) and pertuzumab (Dylight 550) were labeled according to the manufacturer's protocol (Thermo Fisher Scientific). 488- and 550-labeled rituximab acted as background fluorescence controls (Supplementary Fig. S2). Labeled antibody concentrations were determined by NanoDrop (Thermo Fisher Scientific). Cells were pretreated with TKIs and controls for 48 hours in the same manner as the ADCC assays. Cells were washed, trypsinized, and seeded at 2.5×10^5 cells/mL in ADCC assay medium for each of the TKI/MED/DMSO treatments. T0 samples (corresponding to the Western blot analysis and HCA data timepoint) were exposed to trastuzumab-488 (10 μg/mL), pertuzumab-550 (10 μg/mL), trastuzumab-488 (10 μg/mL)/pertuzumab-550 (10 μg/mL), or left untreated. Additional rituximab-488 (10 μg/mL) and rituximab-550 (10 μg/mL) controls were run on DMSO-pretreated samples. Samples were incubated at room temperature for 15 minutes, washed twice with PBS, and resuspended in ADCC assay medium. Cells (200 μL) were plated in single replicates and stained with 5 μmol/L 7-AAD. Dead cell controls were prepared by addition of 5 μL Triton X. Samples were analyzed using Guava easyCyte Flow Cytometer and InCyte Software (Millipore). Rituximab controls were used to set dot plot quadrants. Cell number and mean fluorescence intensity (MFI) values were

determined using rituximab controls. For T6 analysis, corresponding to the start of ADCC assays, samples were incubated at 37°C for 50 minutes followed by incubation at room temperature for 310 minutes, and then treated the same as T0 samples.

Ethical approval

The clinical trials included in this study (NCT00524303/NCT00769470/NCT01485926) were conducted in accordance with the Declaration of Helsinki. All trials were approved by the relevant ethics committees/institutional review boards and health authorities at all participating sites. Informed, written consent was obtained from all participants.

Statistical analysis

Comparative statistical analyses were performed on the quantitative data generated in the study, using Microsoft Excel and MedCalc. Where appropriate, differences between experimental group mean values were evaluated by two-tailed Student *t* tests and considered significant if $P < 0.05$. Patient sample–related statistical methods are outlined in the text or in Supplementary Materials and Methods.

Results

Direct mAb and TKI effects on the cell line panel

HER2 overexpression in the HER2-amplified SKBR3 and HCC1954 cell lines compared with the HER2-low cell lines, T47D, MCF-7, CAMA-1, and CAL-51, is illustrated in **Fig. 1A** and has been quantified previously by ELISA (24). Total EGFR/HER3/HER4 expression levels for the cell lines are also shown. **Figure 1B** highlights the limited direct effects of mAb treatments on proliferation of the cell line panel *in vitro*. The antiproliferative effects of trastuzumab plus pertuzumab did not prove to be statistically different to trastuzumab alone in any cell line. The irreversible pan-HER inhibitors, afatinib and neratinib, were more potent inhibitors of proliferation than lapatinib across all cell lines examined (**Fig. 1C**). HCC1954 was more resistant to all three TKIs compared with SKBR3. The HCC1954 cell line has a PI3K mutation (H1047R) downstream of HER2 that has been associated with innate

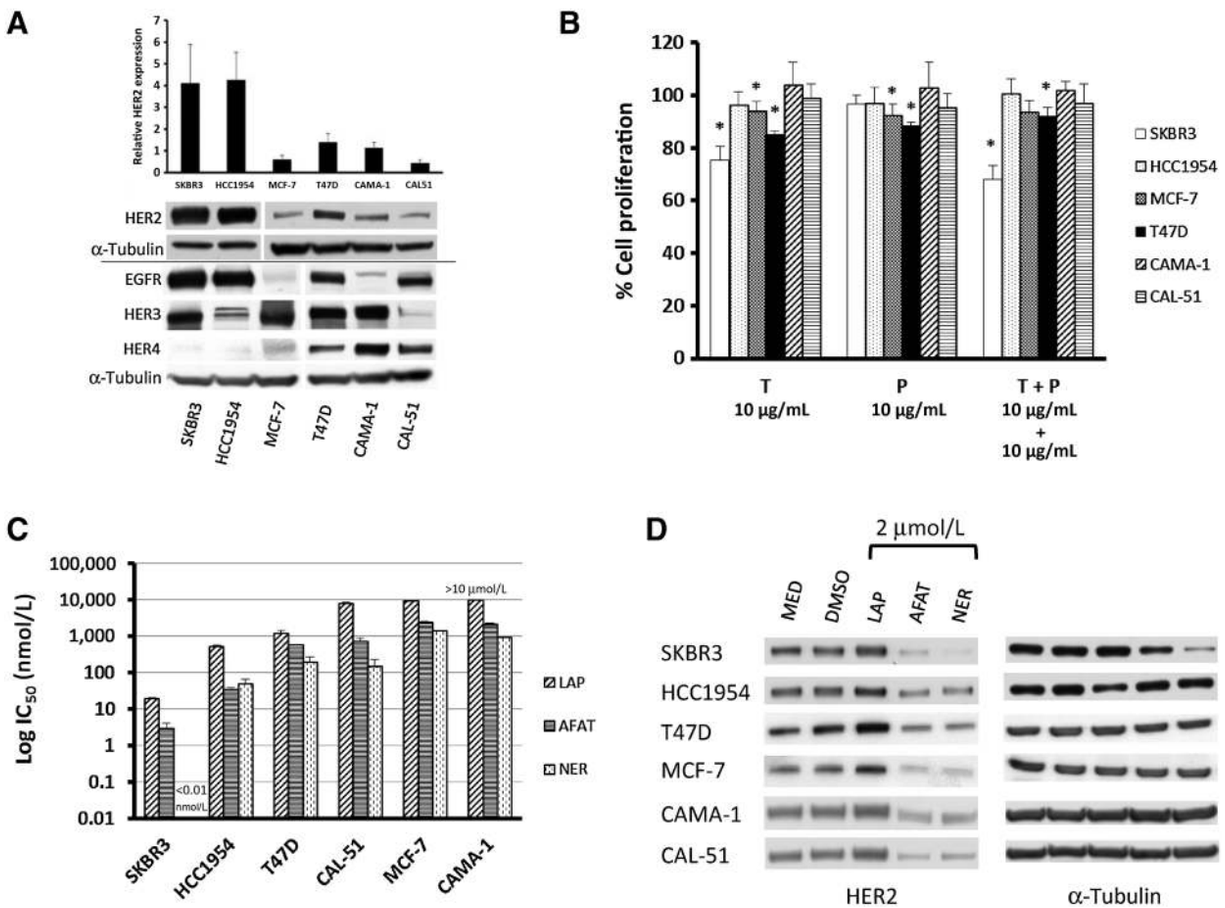

Figure 1.

A, Total HER2 and EGFR, HER3, and HER4 expression by Western blotting in HER2-amplified SKBR3 and HCC1954, and HER2-low MCF-7, T47D, CAMA-1, and CAL-51 breast cancer cell lines. All samples at 30 µg, except SKBR3 and HCC1954 (2.5 µg). Relative HER2 expression determined by densitometry on triplicate blots. **B,** Proliferation for trastuzumab (T), pertuzumab (P), and combination (T + P) in the cell line panel. All data determined in triplicate ± SD. *, $P < 0.05$ versus untreated control (100%). **C,** IC_{50} values for lapatinib (LAP), afatinib (AFAT), and neratinib (NER) ordered by ascending lapatinib resistance. CAMA-1 IC_{50} lapatinib > 10,000 nmol/L and SKBR3 IC_{50} neratinib < 0.01 nmol/L. All data in triplicate ± SD. **D,** Total HER2 expression by Western blotting following 48-hour treatment with 2 µmol/L lapatinib, afatinib, or neratinib, 0.2% DMSO, or medium (MED). Blots shown are representative of three independent experiments.

resistance to trastuzumab (32). In the HER2-low cell lines examined, neratinib proves the most potent antiproliferative agent with significantly lower IC_{50} values than afatinib, followed by lapatinib (**Fig. 1C**). Lapatinib treatment significantly increased total HER2 levels across the cell line panel, while afatinib and neratinib significantly decreased total HER2 levels in all cell lines examined (**Fig. 1D**). Densitometry analysis is shown in Supplementary Fig. S3. The differential effects of lapatinib and neratinib are hypothesized to be driven by the greater induction of HER2 transcription by lapatinib, combined with a higher level of HER2 endocytosis and lysosomal degradation triggered by neratinib (33).

Impact of TKIs on HER2 protein levels in clinical samples and *in vitro*

Preclinical data suggest that lapatinib increases tumor cell surface HER2 and alters ADCC response (16–18). To our knowledge, LPT109096 offers the only dataset available examining patient tumor HER2 protein levels before and after single-agent lapatinib treatment. In support of preclinical findings utilizing short-term exposures, 2 weeks of lapatinib treatment led to more than 50% of matched samples showing an increase in HER2 and/or EGFR expression levels (**Fig. 2A**). A total of 91% (10/11) of samples had increased HER2 or EGFR after lapatinib treatment. Phosphorylation of EGFR and HER2 decreased after treatment with the therapies examined (**Fig. 2A**). In short-term *in vitro* assays, lapatinib and trastuzumab treatment of $HER2^+$ breast cancer cell lines, SKBR3 and HCC1954 (**Fig. 2B**), confirmed the clinical findings (**Fig. 2A**). Lapatinib-induced HER2 increases were maintained on addition of trastuzumab *in vitro* (**Fig. 2B**; additional densitometry analysis in Supplementary Fig. S4). This was not reflected in the clinical samples (**Fig. 2A**), but we did not explore long-term exposure (2 weeks) to both targeted therapies, as a 48-hour timepoint was used for all assays and TKI pretreatment was utilized to induce target cell changes.

TKIs alter membrane-bound ECD-HER2 *in vitro*

To ensure that ADCC relevant membrane HER2 levels, as opposed to cytoplasmic HER2 levels, are altered following TKI treatment, an ECD HER2–targeted antibody was used to assess HER2 levels following TKI treatment by HCA (**Fig. 2C**), and by flow cytometry using fluorescently labelled trastuzumab (Supplementary Table S3). HCA and flow cytometry results confirmed that the global TKI-related changes in HER2 detected by Western blotting (**Fig. 1D**) were representative of cell membrane ECD HER2 level changes. Although 0.2 and 1 μmol/L resulted in significant changes to HER2, 2 μmol/L was retained as the treatment concentration for the remainder of the study because 2 μmol/L neratinib induced the numerically lowest HER2 values in SKBR3 and MCF-7.

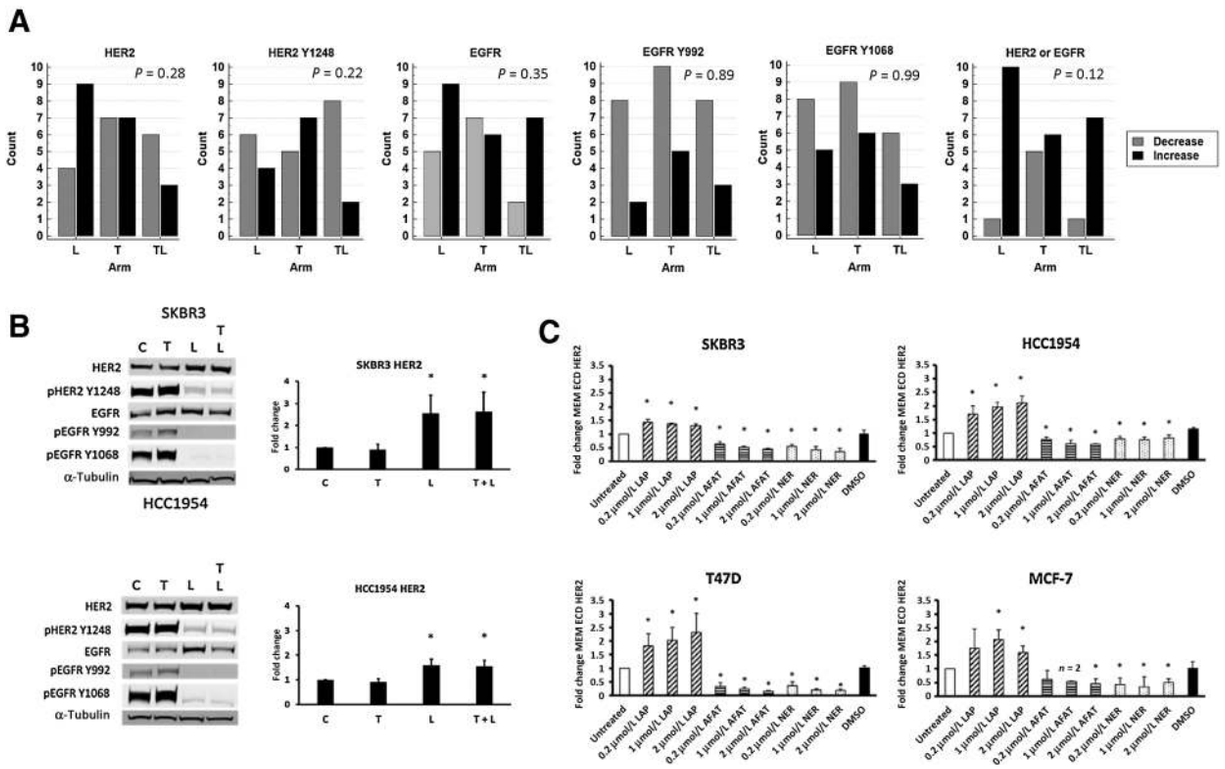

Figure 2.

A, Frequency bar charts showing matched pre- and posttreatment tumor samples from LPT109096 displaying an increase or decrease in protein levels after 2 weeks of treatment with trastuzumab, lapatinib, or both. Readouts for each target were not available for every sample leading to the variation in total numbers. The difference between treatment arms did not reach significance (Fisher exact test). **B,** Impact of 48-hour treatment with 10 μg/mL trastuzumab (T), 2 μmol/L lapatinib (L), or the combination (T + L) on levels of total and phosphorylated (p) HER2/EGFR expression in SKBR3 and HCC1954. Densitometry for HER2 shown. **C,** High-content analysis of membrane HER2 expression in SKBR3, HCC1954, T47D, and MCF-7 following 48-hour TKI treatments using an ECD-targeted antibody. Triplicate data, normalized to untreated control ± SD. *, $P < 0.05$ relative to DMSO control.

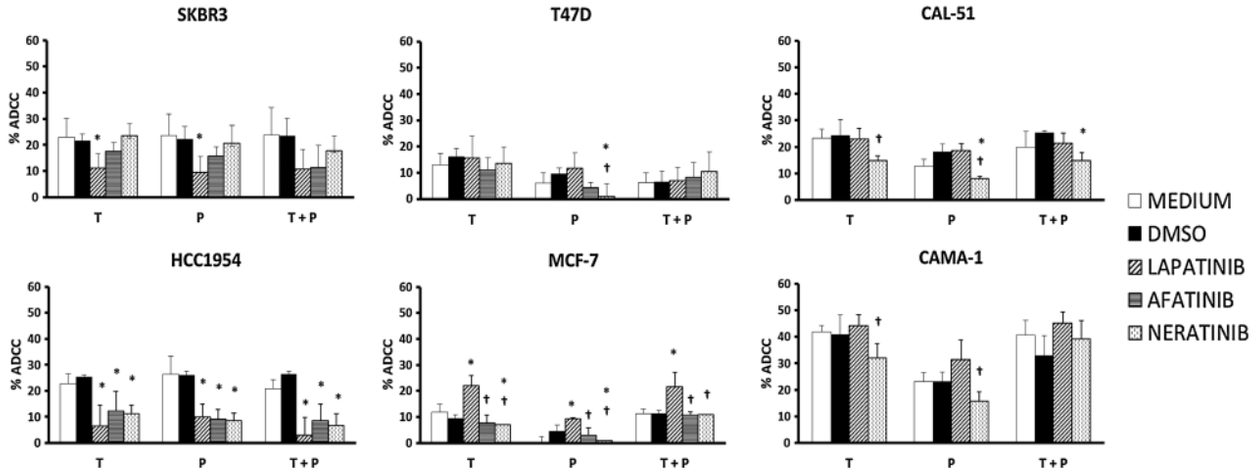

Figure 3.

Trastuzumab (T)-, pertuzumab (P)-, and trastuzumab + pertuzumab (T+P)-mediated ADCC in SKBR3, HCC1954, T47D, MCF-7, CaL-51 and CAMA-1 following 48-hour TKI (2 μmol/L) treatments. Results shown represent PBMC:target ratios of 5:1. Results of three separate experiments ± SD.*, $P < 0.05$ relative to the 0.2% DMSO control; †, $P < 0.05$ relative to lapatinib-treated cells.

TKI treatment alters PBMC-mediated ADCC

PBMCs contain a mix of ADCC-capable cell types, including NK cells and monocytes, and are a commonly used source of effector cells utilized to examine TKIs and ADCC (16–18). We have shown previously that a flow cytometry–based ADCC assay has several advantages over lactate dehydrogenase (LDH)-based assays when assessing the impact of TKIs on ADCC, proving more sensitive, reproducible, and unaffected by TKI-related alterations in LDH levels (16–18, 29). The effects of all three TKIs on ADCC were assessed in $HER2^+$ SKBR3 and HCC1954, and HER2-low MCF-7 and T47D at a 5:1 effector:target cell ratio. Additional PBMC:target cell ratios for each cell line are shown in Supplementary Fig. S5A and S5B. Lapatinib and neratinib were examined in CAMA-1 and CAL-51. Lapatinib-treated SKBR3 displayed reduced trastuzumab- and pertuzumab-mediated ADCC, with afatinib and neratinib treatment having no significant effect on ADCC (**Fig. 3**). In HCC1954, all three TKIs reduced mAb-mediated ADCC and unlike SKBR3, the individual TKIs did not show variable influence on ADCC.

For the HER2-low models, lapatinib treatment resulted in control equivalent mAb-mediated ADCC in three of four HER2-low models examined and increased ADCC (trastuzumab, pertuzumab, and combination) in the remaining HER2-low cell line, MCF-7 (**Fig. 3**). When compared with lapatinib-treated cells, neratinib produced lower trastuzumab-mediated ADCC in three of four HER2-low models and reduced pertuzumab-mediated ADCC in four of four HER2-low models. Afatinib followed a similar trend to neratinib in the two cell lines it was examined (T47D and MCF-7). Interestingly, across $HER2^+$ and HER2-low cell lines, the ADCC response did not correlate directly with the TKI-altered levels of HER2.

Examining control levels of ADCC in the absence of TKI, there was no significant difference between the levels of ADCC induced by trastuzumab, pertuzumab, or the combination of both mAbs in the $HER2^+$ cell lines (Supplementary Fig. S5C). However, the level of pertuzumab-mediated ADCC was lower compared with trastuzumab-mediated ADCC in all HER2-low cell lines, significantly in MCF-7, CAMA-1, and CAL-51, indicating pertuzumab is not as powerful an inducer of ADCC in HER2-low models.

NK cells in early response to neoadjuvant therapy of $HER2^+$ breast cancer

NK cells are the major immune cell subtype within the PBMC fraction contributing to ADCC *in vitro* (34). CIBERSORT analysis of TRIO-B07 samples showed that an activated NK cell signature was significantly increased ($P = 0.03$) following 2 weeks of targeted therapy treatment ($n = 89$; **Fig. 4A**). On breakdown by arm, only the difference in the trastuzumab arm ($n = 26$) proved significant ($P = 0.04$). The posttreatment samples were then analyzed by pCR to the subsequent second part of neoadjuvant therapy (TCH, TCHL, or TCL; **Fig. 4B**). For trastuzumab/lapatinib-treated patients, the activated NK cell signature was significantly lower ($P = 0.04$) for those who went on to achieve a pCR than for nonresponders. This effect was also significant ($P < 0.01$) when the trastuzumab and trastuzumab/lapatinib arms were combined, but not for the lapatinib-only arm ($P = 0.704$). MCP counteranalysis of tumor samples from ICORG 10-05 ($n = 8$) showed that one cycle of chemotherapy (TC) plus an HER2-targeted agent results in a significant increase in NK cell gene signature after 21 days (**Fig. 4C**). This suggests that the targeted therapy–associated increase in NK cell gene signature (**Fig. 4A**) is maintained in the presence of a TC chemotherapy backbone.

TKIs alter NK cell–mediated ADCC

Figure 4 suggests that NK cells may have an early role in response to neoadjuvant treatment in $HER2^+$ breast cancer. An NK cell–enriched effector cell population was used to ascertain the impact of TKIs on direct NK cell cytotoxicity +/− mAb within the same experiments (**Fig. 5**). For SKBR3, lapatinib maintained direct cytotoxicity levels (**Fig. 5A**), but reduced mAb-mediated ADCC (**Fig. 5B**). Afatinib and neratinib did the opposite, suggesting an induced target cell profile more conducive to mAb-mediated ADCC. Afatinib and neratinib produced the same ADCC profile in HCC1954 suggesting that the irreversible TKIs, neratinib in particular, may have advantages over lapatinib when trying to maintain an NK cell–mediated ADCC response to HER2 mAb therapies in $HER2^+$ breast cancer models. Unlike the PBMC-based assays (**Fig. 3**), lapatinib did not produce an increase in mAb-mediated ADCC in MCF-7 (**Fig. 5B**). There was no significant benefit for the addition of trastuzumab to pertuzumab for

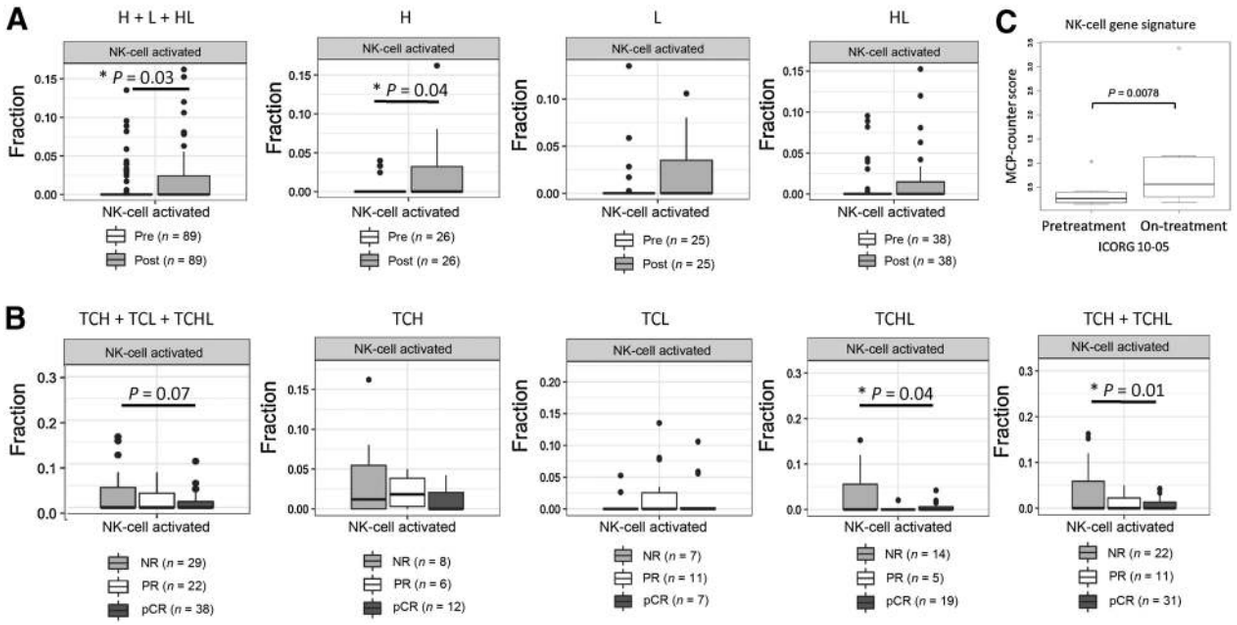

Figure 4.

A, Activated NK cell gene signature fractions (CIBERSORT) from matched pre- and posttreatment [2 weeks of trastuzumab (H), lapatinib (L), or both] TRIO-B07 tumor samples overall, and by treatment arm **B,** Posttreatment samples from **A** broken down by pCR, partial response (PR), or nonresponse (NR) to subsequent docetaxel/carboplatin (TC)H, TCL, or TCHL therapy. **C,** MCP counteranalysis of NK cell gene signature levels in ICORG 10-05 patient samples ($n = 8$) by pretreatment and after one cycle of therapy (TCH/TCL/TCHL). Each individual sample examined showed an increase in NK cell score on-treatment. $P < 0.05$ is significant and corrected.

NK cell–mediated ADCC, and pertuzumab-mediated ADCC was numerically lower than trastuzumab-induced ADCC in MCF-7 and T47D, but not to a significant degree ($P > 0.05$; **Fig. 5B**).

TKI treatment impacts trastuzumab and pertuzumab binding

Trastuzumab and pertuzumab binding was examined in TKI-treated target cells as a key contributing factor to ADCC efficacy. Percentage cell number and MFI values were determined for each treatment. Major TKI-induced changes in mAb binding were found to be stable for at least 6 hours, from T0 (Western blotting and HCA equivalent timepoint) through T6 (commencement of the ADCC assays; Supplementary Tables S1–S4; Supplementary Fig. S6). **Figure 6A** represents the percentage of cells staining positive for mAbs when exposed to trastuzumab and pertuzumab

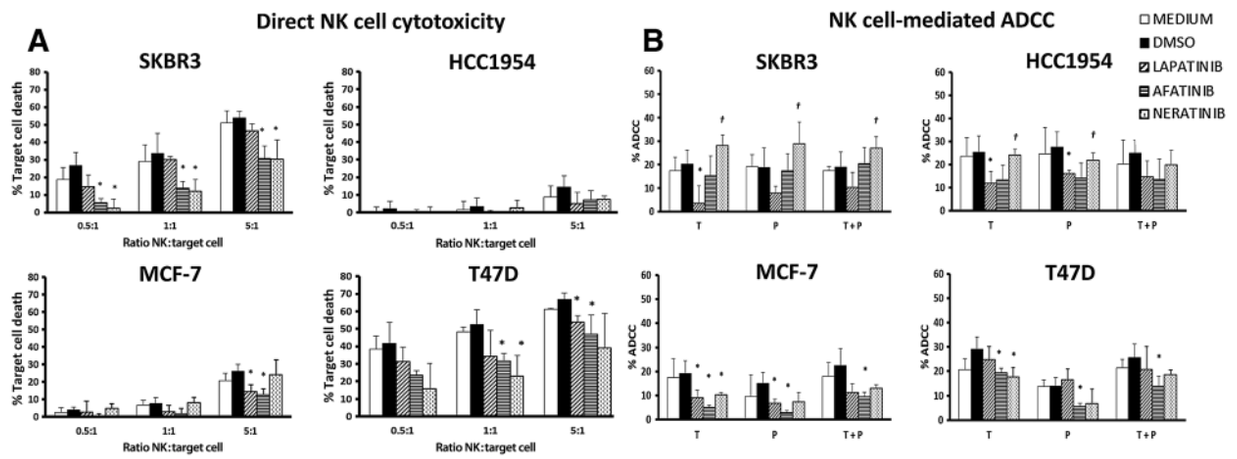

Figure 5.

A, Direct NK cell cytotoxicity against HER2$^+$ (SKBR3 and HCC1954) and HER2-low (MCF-7 and T47D) cell lines following treatment with lapatinib, afatinib, and neratinib (2 μmol/L) for 48 hours. Three ratios of NK cells (NK) to target cells were examined. **B,** NK cell–mediated ADCC induced by trastuzumab (T), pertuzumab (P), or trastuzumab plus pertuzumab (T+P) following treatment with lapatinib, afatinib, or neratinib (2 μmol/L) for 48 hours. Three ratios of NK cells to target cells were examined with the 1:1 ratio shown for SKBR3, HCC1954, and MCF-7. Only the 0.5:1 ratio was available for the T47D cell line due to sensitivity to direct NK cell cytotoxicity. *, $P < 0.05$ is significant versus DMSO-treated control, †, $P < 0.05$ significant relative to lapatinib-treated cells.

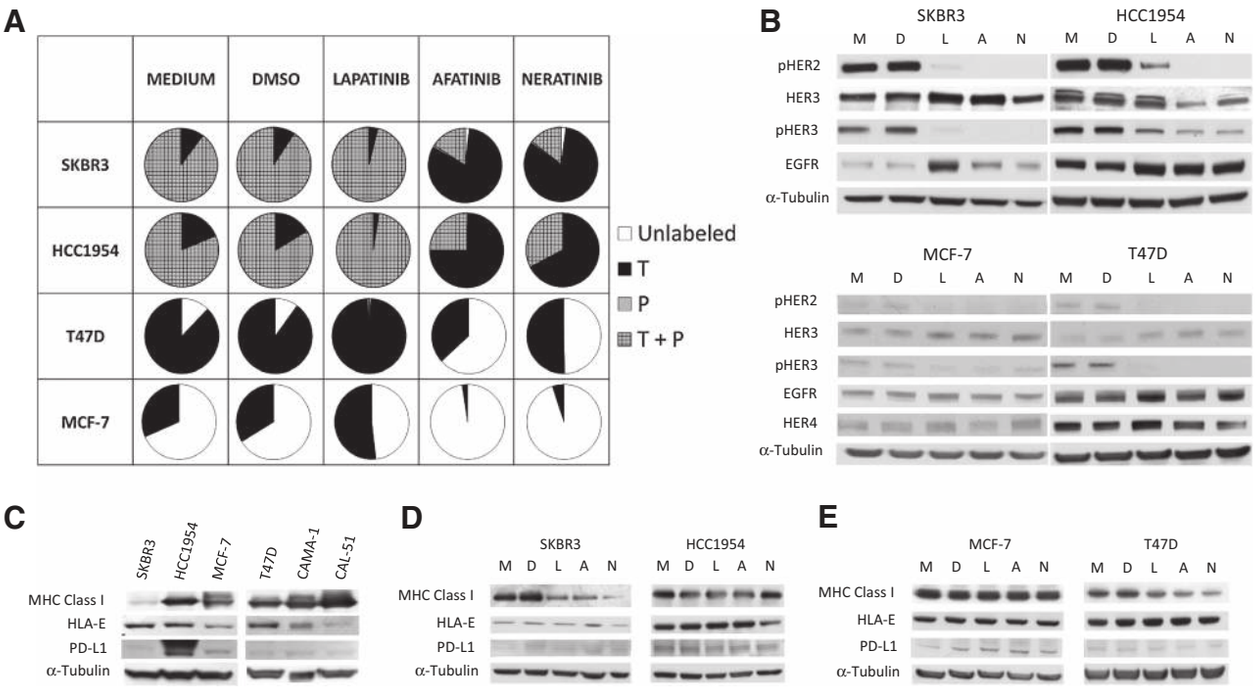

Figure 6.

A, The average percentage cell number staining positive for trastuzumab (T), pertuzumab (P), trastuzumab and pertuzumab (T+P), or those remaining unlabeled immediately after pretreatment (T0) with TKIs (2 μmol/L) for 48 hours. Results show the average of three independent experiments. Original data for coexposure and each individual mAb ± SD and *P* values can be found in Supplementary Table S1. **B,** pHER2, HER3, pHER3, and EGFR expression following treatment with medium (M), DMSO (D), or 2 μmol/L lapatinib (L), afatinib (A), or neratinib (N). All samples at 30 μg. **C**. MHC class I, HLA-E, and PD-L1 expression by Western blotting in all cell lines examined in this study. All samples at 30 μg. MHC class I, HLA-E, and PD-L1 expression levels following treatment with TKIs (2 μmol/L for 48 hours) in SKBR3 and HCC1954 (**D**) and MCF-7 and T47D (**E**). All blots were carried out in triplicate.

simultaneously following TKI treatment. More than 97% of SKBR3 and HCC1954 cells bound trastuzumab and the TKIs did not impact this. However, lapatinib significantly increased the number of cells cobinding pertuzumab, while afatinib and neratinib reduced the number of cells cobinding pertuzumab. These results were consistent when each mAb was examined individually (Supplementary Table S1). Lapatinib can induce a noncanonical ligand binding HER2/HER3 dimer (subdomain II available), while neratinib induces an HER2/HER3 dimer that does not bind ligand (subdomain II not available), further reducing HER2 available for pertuzumab binding (35). Decreased levels of HER2 (**Figs. 1D** and **2C**) and increased levels of EGFR or HER3 (**Fig. 6B**) could also reduce the number of pertuzumab binding sites available through the formation of ligand-induced or ligand-independent heterodimers (36, 37). HER2 subdomain IV was available to bind trastuzumab whether HER2 was homo- or heterodimerized, so trastuzumab binding was not impacted in the same way as pertuzumab (36, 38).

Pertuzumab exposure did not produce a positive cell population for T47D and MCF-7 (Supplementary Table S1), but measurable MFI values confirmed low level binding (Supplementary Tables S3 and S4). This was consistent with the reduced pertuzumab-mediated ADCC in MCF-7 and T47D (**Fig. 3**; Supplementary Fig. S5C). Therefore, T47D and MCF-7 cells only show unlabeled and trastuzumab-stained cell populations (**Fig. 6A**). Lapatinib increased the number of trastuzumab-positive MCF-7 cells by 18.1% and T47D cells by 8.9%. Afatinib and neratinib reduced the percentage trastuzumab-positive cells significantly in both cell lines, concomitant with HER2 levels.

MFI values are a measure of binding intensity within the % positive cell populations. The MFI values associated with (**Fig. 6A**; Supplementary Table S3) change in concordance with the HER2 levels following TKI exposure, as measured by HCA (**Fig. 2**) and Western blotting (**Fig. 1D**), for all four cell lines.

TKIs alter levels of HER2 dimerization partners

Protein levels of pHER2/pHER3, the most potent heterodimer oncogenic driver, and EGFR, HER3, and HER4 were examined to confirm TKI activity and assess levels of HER2 heterodimer partners with potential for impacting pertuzumab binding. All three TKIs significantly reduced pHER3 and pHER2 activity in all cell lines (**Fig. 6B**). The TKIs also altered the total levels of HER family members, particularly EGFR and HER3 (**Fig. 6B**). Densitometry analysis is shown in Supplementary Fig. S7. Lapatinib increased EGFR (in SKBR3, HCC1954, and MCF-7) and HER3 (in SKBR3, MCF-7, and T47D) protein levels. Afatinib treatment increased protein levels of EGFR (in SKBR3 and HCC1954) and HER3 (in SKBR3, MCF-7, and T47D), while neratinib increased EGFR (in HCC1954) and HER3 (in SKBR3, MCF-7, and T47D) expression. All changes were significant. These increases in total HER3 were not associated with increased levels of phosphorylated HER3, but may have implications for pertuzumab binding through increased availability of HER2 dimerization partners

TKIs alter proteins associated with regulation of NK cell cytotoxicity

As a preliminary study of potential mediators of TKI-induced changes in NK cell cytotoxicity, total levels of MHC class I, HLA-E,

and PD-L1 were examined. These proteins are three of many target cell proteins associated with regulation of NK cell cytotoxicity (39). HCC1954 was the only cell line displaying high expression of PD-L1, SKBR3 displayed lower levels of MHC class I than all other cell lines examined, with HLA-E expression present for all study cell lines (**Fig. 6C**). Lapatinib treatment significantly reduced MHC class I expression in SKBR3, and lapatinib and afatinib did the same in HCC1954 (**Fig. 6D**). HLA-E expression was significantly reduced following neratinib treatment in HCC1954 only. For the HER2-low cell lines, afatinib and neratinib reduced MHC class I and PD-L1 expression significantly in T47D (**Fig. 6E**). Reduced trastuzumab-mediated ADCC (**Fig. 5**) was associated with reduced MHC class I expression in SKBR3, HCC1954, and T47D, but not in MCF-7 (**Fig. 6D** and E). Densitometry for **Fig. 6D** and E is presented in Supplementary Fig. S8.

Discussion

Our results suggest that NK cells are key mediators of the early antitumor immune response in neoadjuvant treatment of HER2$^+$ breast cancer, and HER2 antigen levels and antibody load are not the only factors dictating the ADCC response to trastuzumab and pertuzumab following TKI treatment. Compelling evidence for the contribution of an immune response to the efficacy of trastuzumab has been provided by *in vitro* and *in vivo* studies (6, 9, 10). ADCC-capable therapeutic mAbs may be considered a form of passive immunotherapy, tagging antigen-positive tumor cells and priming the immune response directly through engagement of FcR-positive immune cells, and indirectly by sensitizing the tumors to chemotherapy through the inhibition of oncogenic signaling pathways resulting in the release of damage-associated molecular patterns (40). Recent clinical results reporting greater efficacy of margetuximab, an ADCC-enhanced HER2-targeting mAb, over trastuzumab lend support to the relevance of ADCC in clinical mAb response (7). It has also been reported that impaired ADCC capacity leads to reduced trastuzumab efficacy in patients (8).

Strategies for enhancing the immune response to ADCC-capable antibodies are therefore desirable. Altering tumor cell–related factors, such as HER2 antigen levels, is a potential method and is supported by previous preclinical work (16–18, 29). Using the only database available, we have shown that the lapatinib-induced changes in HER2 and EGFR observed *in vitro* are potentially relevant in patient samples (**Fig. 2A**).

Despite increased HER2 levels, we observed reduced trastuzumab-mediated ADCC in lapatinib-pretreated SKBR3 and HCC1954 cells using PBMCs or NK cells. Increased ECD-HER2 shedding (41) is unlikely to be responsible for reduced ADCC on the basis of the HCA (**Fig. 2C**), flow cytometry (Supplementary Table S3), and binding assay (**Fig. 6A**) results. We believe that this work does not contradict previous findings which show lapatinib increases trastuzumab-mediated ADCC (16–18), but provides a perspective on enduring target cell populations, rather than those that have died. The *in vitro* model we used to assess ADCC is robust, employing excess mAb (10 μg/mL), pretreatment with TKIs to avoid direct influence of solvent or TKI on effector cells, and a flow cytometry–based system for single cell–based analysis. The assay measures the proportion of intact cells that are dead or dying due to ADCC or direct cytotoxicity at a particular timepoint. More traditional methods, like LDH release assays, rely on an indirect measurement of a cytosolic factor that is released in to the medium. Such cumulative assays do not report on the status of remaining target cells and in the case of LDH, are impacted by the presence of TKIs (29).

While the magnitude of the ADCC response to pertuzumab alone was similar to trastuzumab alone in HER2$^+$ models (**Figs. 3** and **5**), the addition of pertuzumab to trastuzumab did not enhance ADCC, most likely because of previously reported saturation of the ADCC response in the presence of extreme HER2 overexpression (14). Results also show that pertuzumab-induced ADCC is not equivalent to trastuzumab-mediated ADCC in HER2-low models and pertuzumab binding is more susceptible to TKI-induced changes (**Fig. 6**; Supplementary Fig. S5C). This is potentially due to pertuzumab targeting HER2 subdomain II, which is involved in HER family dimerization (14), and pertuzumab having a lower affinity for HER2 compared with trastuzumab (42).

It has been shown that perioperative lapatinib can result in tumor clearance within 11 days (EPHOS-B; ref. 43). A similar effect with chemotherapy and trastuzumab or lapatinib was observed within 20 days for ICORG 10-05 (44). We report a significantly reduced activated NK cell signature associated with a pCR to trastuzumab and trastuzumab/lapatinib treatment in the TRIO-B07 dataset within 2 weeks. On the basis of data reported for ICORG 10-05 and EPHOS-B, this could indicate the resolution of an active antitumor immune response involving NK cells and trastuzumab that is induced, but is incomplete or ineffective in nonresponders, and does not occur in the presence of lapatinib only (**Fig. 4**). Direct investigation of treatment-related changes in NK cell subpopulations within HER2$^+$ tumors is warranted.

Our data suggest that HER2 antigen level changes in HER2$^+$ cells have minimal impact on an optimal ADCC response that is facilitated by gross HER2 overexpression and excess antibody, in which case regulatory factors other than HER2 antigen levels can play a role. Potential mediators exist within the suite of target cell proteins involved in NK cell activation [e.g., MHC class I, HLA-E (NKG2A receptor), and CD155] and inhibition [e.g., HLA-E (NKG2C receptor) and PD-L1; refs. 39, 45]. Key NK cell–regulating proteins, MHC class I and HLA-E, are altered by TKI treatment (**Fig. 6**). These changes suggest that a more in-depth investigation of NK cell–regulating proteins altered by TKIs is required, given the changes in NK cell–mediated ADCC and direct cytotoxicity observed (**Fig. 5**). It is most likely the balance in expression of a number of these regulators, rather than a lone regulator, that is guiding NK cell response in these assays.

From a purely immunologic view point, afatinib and neratinib pretreatment may prove more beneficial to the mAb ADCC response than lapatinib by maintaining the localized NK cell–mediated ADCC response in HER2$^+$ tumors (**Fig. 5**), with the added benefit of increased pan-HER inhibitory antiproliferative activity (**Fig. 1C**). Neratinib and afatinib improved the antiproliferative effects of trastuzumab combined with pertuzumab in ligand-activated HER2$^+$ cell line models *in vitro* (46). Combinations of neratinib or afatinib with trastuzumab/pertuzumab could be considered as next-generation approaches to neoadjuvant studies like neoALTTO and EPHOS-B (43, 47). Tucatinib is an HER2-specific TKI recently approved in combination with trastuzumab and capecitabine for the treatment of metastatic HER2$^+$ breast cancer (48). As tucatinib is a reversible TKI, it could be expected to behave like lapatinib in our assays, but this remains to be determined.

Of the three TKIs studied, lapatinib in combination with trastuzumab provided the optimal ADCC response in HER2-low breast cancer models. Although HER2 levels were increased in all HER2-low cell lines, lapatinib pretreatment did not increase trastuzumab-mediated ADCC levels compared with control in three of four cell lines (**Fig. 3**) and increased ADCC in one cell line only (MCF-7) when PBMCs, but not NK cells (**Fig. 5**), were used as effector cells. As the

effector cell type used was the only difference between the MCF-7 assays, further investigation in to the immune cell types contributing to ADCC in PBMCs is required.

Clinical evidence to support the use of HER family–targeted TKIs outside $HER2^+$ disease is limited. Afatinib has not shown efficacy in HER2-low/ER^+ or triple-negative breast cancer (49, 50). Neratinib did not show benefit in combination with chemotherapy in triple-negative breast cancer, but may have value in HER2-low/HER2-mutated cancers (15, 51). However, lapatinib may be worth further study in the HER2-low setting in combination with HER2-targeting antibody–drug conjugates, T-DM1 or DS8201, where increased cell surface HER2 levels may be an advantage for antibody–drug conjugate loading.

Immune checkpoint inhibitors are being trialed in all breast cancer subtypes and could potentially enhance the activity of existing antibody therapies through reactivation of checkpoint-inhibited ADCC-capable immune cells. The anti–PD-1 therapy, pembrolizumab, has been shown to improve outcomes for advanced trastuzumab-resistant, PD-$L1^+$ $HER2^+$ breast cancers receiving trastuzumab (52).

Our results show that TKIs differentially alter tumor cell phenotype, which can impact NK cell–mediated response to coadministered antibody therapies. The immune cell milieu (**Figs. 3** and **5**), the ADCC capacity of effector cells (29), and the expression of immune regulatory proteins on the surface of tumor cells (**Fig. 6**; ref. 53) may all have a bearing on this response. Other factors not addressed in this study, such as HER family ligand expression, extended TIL profile of the tumor microenvironment, or varying immune modulating properties of concomitant chemotherapy regimens, will also influence TKI impact on NK cell–mediated mAb-induced ADCC.

Authors' Disclosures

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Authors' Contributions

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