Tyrosine kinase inhibitors as modulators of trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity in breast cancer cell lines.

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Abstract

Background: Trastuzumab is an anti-HER2 monoclonal antibody (mAb) therapy capable of antibody-dependent cell-mediated cytotoxicity (ADCC) and used in the treatment of HER2+ breast cancer. Through interactions with FcγR+ immune cell subsets, trastuzumab functions as a passive immunotherapy. The EGFR/HER2-targeting tyrosine kinase inhibitor (TKI) lapatinib and the next generation TKIs afatinib and neratinib, can alter HER2 levels, potentially modulating the ADCC response to trastuzumab. Using LDH-release assays, we investigated the impact of antigen modulation, assay duration and peripheral blood mononuclear cell (PBMC) activity on trastuzumab-mediated ADCC in breast cancer models of maximal (SKBR3) and minimal (MCF-7) target antigen expression to determine if modulating the ADCC response to trastuzumab using TKIs may be a viable approach for enhancing tumor immune reactivity.

Methods: HER2 levels were determined in lapatinib, afatinib and neratinib-treated SKBR3 and MCF-7 using high content analysis (HCA). Trastuzumab-mediated ADCC was assessed following treatment with TKIs utilising a colorimetric LDH release-based protocol at 4 and 12 hour timepoints. PBMC activity was assessed against non-MHC-restricted K562 cells. A flow cytometry-based method (CFSE/7-AAD) was also used to measure trastuzumab-mediated ADCC in medium-treated SKBR3 and MCF-7.

Results: HER2 antigen levels were significantly altered by the three TKIs in both cell line models. The TKIs significantly reduced LDH levels directly in SKBR3 cells but not MCF-7. Lapatinib and neratinib augment trastuzumab-related ADCC in SKBR3 but the effect was not consistent with antigen expression levels and was dependent on volunteer PBMC activity (vs. K562). A 12 hour assay timepoint produced more consistent results.

Trastuzumab-mediated ADCC (PBMC:target cell ratio of 10:1) was measured at 7.6 ± 4.7% (T12) by LDH assay and 19 ± 3.2 % (T12) using the flow cytometry-based method in the antigen-low model MCF-7.**Conclusions:**In the presence of effector cells with high cytotoxic capacity, TKIs have the ability to augment the passive immunotherapeutic potential of trastuzumab in SKBR3, a model of HER2+ breast cancer. ADCC levels detected by LDH-release assays are extremely low in MCF-7; the flow cytometry-based CFSE/7-AAD method is more sensitive and consistent for the determination of ADCC in HER2-low models.

1.Introduction

Trastuzumab is an ADCC-capable mAb therapy used in combination with chemotherapy to treat the 20-30 % of breast cancers that are clinically defined as HER2+(HER2-positive/HER2-overexpressing/HER2-amplified) [1]. Trastuzumab has an Fc portion capable of engaging FcγR+ (FcγRIII/CD16, FcγRII/CD32, FcγRI/CD64) immune cells and initiating antibody-dependent cell-mediated cytotoxicity (ADCC) resulting in tumor cell death [2, 3]. The presence of tumor infiltrating lymphocytes (TILs) has been associated with improved response to trastuzumab containing chemotherapy [4] but the composition and ADCC capability of all tumor-associated immune subsets has not been fully assessed. Recent clinical developments have led to interest in the combination of HER family targeted TKIs and trastuzumab as a potential mechanism of overcoming resistance to trastuzumab through abrogation of compensatory HER family signaling [5]. The HER2/EGFR-targeting reversible TKI lapatinib is approved for treatment of trastuzumab-refractory HER2+ breast cancer [6]. The next generation irreversible TKIs afatinib and neratinib are being investigated in breast cancer but are not approved for HER2+ breast cancer [7, 8]. All three TKIs have been shown to alter HER2 levels on HER2-overexpressing and HER2-low (HER2-negative) breast cancer cells and these changes are associated with altered mAb-mediated ADCC [9-12]. Lapatinib has also been shown to increase mAb-mediated ADCC in gastric cancer and mesothelioma cell lines [13, 14].

The LDH release assay has been used to determine necrotic cell death related to immune cell cytotoxicity since the 1980s [15, 16]. As an alternative to the ^{51}Cr and ^{3}H-thymidine release assays, it has become one of the benchmark non-radioactive methods for the measurement of immune cell cytotoxicity and mAb-mediated ADCC *in vitro* due to its

high-throughput and reproducibility [17]. LDH is a key component of cellular metabolism remaining relatively stable in cell line models. The basis of the LDH assay is the indirect measure of cell death through quantification of LDH released from dead and late-stage necrotic target cells in which the plasma membrane has become porous [18]. The instability of the plasma membrane of these cells is also the basis for dye exclusion based measures of cell death such as 7-AAD and propidium iodide [19]. We have previously used a flow cytometry-based method to measure trastuzumab-mediated ADCC in HER2+ and HER2-low breast cancer cell lines [20]. The method involves the staining of target cells with CFSE to distinguish them from effector cells and the use of the dead cell stain 7-AAD to discriminate between live and dead cells. This method can be used for adherent or non-adherent cells and takes measurements directly on a single cell basis [19].

The advent and success of immune checkpoint inhibitors has placed a renewed emphasis on exploiting the immune response in cancer [21]. TKIs may have the ability to enhance the passive immunotherapeutic potential of trastuzumab. In this manuscript, we use lapatinib, afatinib and neratinib to modulate HER2 antigen levels in the HER2+ cell line SKBR3 (antigen-high) and the HER2-low cell line MCF-7 (antigen-low). We have shown previously that these cell lines represent breast cancer models at the opposite extremes of HER2 expression [20]. Using LDH-based ADCC assays, we determine the impact of TKI-treatment on HER2 levels and on trastuzumab-mediated ADCC in both cell lines at two time points. The direct cytotoxicity of PBMCs against the non-adherent leukemic cell line K562 is used as a measure of the innate activity of PBMCs from individual healthy volunteers. These results inform the suitability of the LDH-release assay for determining

trastuzumab-mediated ADCC in TKI-treated breast cancer cell line models with high and low antigen levels and explore if modulating the ADCC response to trastuzumab using small molecule inhibitors may be a viable approach for enhancement of immune reactivity.

2. Materials and Methods

2.1 Cell Lines and Reagents

SKBR3 (HER2+ subtype, HER2-amplified/ER-), MCF-7 (luminal A subtype/HER2-low/ER+) and K562 (leukemic cell line HER2 negative/ER-) were cultured (37ºC in 5% CO_2) without antibiotics and with routine monitoring for *Mycoplasma* contamination. Cell lines were maintained in RPMI 1640 and 10% heat-inactivated FCS. Cell line identities were authenticated by short tandem repeat DNA profiling (Source Bio-science). Lapatinib, afatinib and neratinib (Sequoia Chemicals) were solubilized in DMSO. Trastuzumab (Herceptin ®) and rituximab (Rituxan ®) were obtained from St. Vincent's University Hospital.

2.2 TKI treatment of cell lines

A 2µM concentration and a 48 hour timepoint for TKI exposure were chosen following studies examining 0.2, 1 and 2 uM TKI at 12, 24 and 48 hour timepoints by Western blot [[10]/unpublished results]. High content analysis of 0.2, 1 and 2 uM TKI was carried out at the 48 hour timepoint only (Figure 1A and 1B/ 0.1 and 1 uM high content analysis results are unpublished). The largest impact on HER2 antigen levels in SKBR3 and MCF-7, whether an increase or decrease, occurred at 48 hours using a concentration of 2µM TKI.

2.3 High Content Analysis

Total HER2 protein levels in SKBR3 and MCF-7 were determined by high content analysis (HCA). Cells were plated (SKBR3 - 10,000 cells/well, MCF-7 - 4,000 cells/well) in glass-bottomed 96 well-plates (Matrical). 24 hours later, triplicate wells were treated with medium, 0.2% DMSO, lapatinib, neratinib or afatinib (2 μM) for 48 hours. Treatments were removed and cells were rinsed with wash buffer (30 mM glycine/PBS) and fixed with 4% paraformaldehyde. Following permeabilisation (ice-cold 100% methanol), cells were blocked with 3% normal goat serum (NGS) at room temperature (NGS-Vector in PBS). Wells were stained using HER2 extracellular domain -targeted OP39 (Calbiochem) and Alexa Fluor 594-labeled secondary Ab. Hoechst 33342 (Invitrogen Molecular Probes #H1399 – 4 μM 3% NGS/PBS) was used for nuclear staining. Primary and secondary antibodies were diluted in 3% NGS/PBS. Images were acquired on the IN Cell-1000 Analyser automated imaging platform. Images were analysed using a Multi Target Analysis algorithm on the InCell Analysis software. Total HER2 expression was determined as Inclusion Area x Intensity in the whole cell. Final results are presented as fold change relative to medium-treated cells.

2.4 Laser Scanning Confocal Microscopy

SKBR3 cells were TKI-treated and prepared as for the HCA. HER2 extracellular domain and nuclear staining were carried out as described for the HCA. HER2 intracellular domain

was targeted with Ab #2242 (Cell Signalling) and an Alexa Fluor 488-labeled secondary Ab. Images were obtained using a plan-apochromat 63X oil (NA=1.4) lens on a Zeiss 510 confocal microscope. The same settings were maintained for each image taken.

2.5 Cell Viability Assays

5 x 10^6 cells were seeded in vented T75 flasks. 24 hours later, medium was removed and replaced with medium alone or medium containing 2 μM lapatinib, afatinib or neratinib or 0.2% DMSO. After 48 hours, the drug-containing medium was removed from the flasks and the cells washed twice with PBS. Cells were trypsinised and re-suspended in medium. Cell counts for each treatment were determined on a Guava Easycyte flow cytometer using Guava Viacount (Millipore) and expressed as a percentage of the medium control flask cell number. The remaining cells were utilised for ADCC assays after the cell concentration was normalised to the required assay concentration.

2.6 Isolation of PBMCs

Whole blood was collected from healthy volunteers using EDTA vaccutubes (Becton Dickinson). Ethical approval for collection of blood was obtained from St. Vincent's University Hospital Ethics Committee. Informed consent was obtained from all individual participants included in the study. Healthy volunteer samples were taken if they had no acute illness in the month preceding the sample donation. A Ficoll-Paque plus-based protocol (GE Healthcare) was utilised to isolate PBMCs. PBMCs were counted by flow cytometry (Guava Viacount) and re-suspended in assay medium (RPMI-1640/10% HI FCS/0.1 mM pen/strep).

2.7 LDH-release ADCC Assays

The LDH assays were carried out according to the manufacturer's instructions (Pierce LDH Cytotoxicity kit). A colorimetric kit was chosen due to greater sensitivity reported over a fluorescence-based alternative [22]. Assay cell number optimisation studies were performed in SKBR3, MCF-7 and K562 (Supplementary Figure 1). 3.5 x 10^4 cells/ml (17500 cells /well) was chosen as the fixed target cell concentration. TKI- and medium only / 0.2% DMSO-treated target cells were prepared as previously described and normalised to 3.5 x 10^4 cell/ml. PBMC numbers were standardised to 3.5, 17.5 and 35 x 10^4 cells/ml. Target cells (50 μl) were plated in round-bottomed 96-well plates in triplicate together with PBMCs (50 μl) at 1:1, 5:1 and 10:1 ratios, with and without trastuzumab (10 μg/ml). Controls included: target cells ± trastuzumab or rituximab (spontaneous target controls (**A**)); PBMCs alone at each ratio (spontaneous effector controls (**B**)); culture medium alone (background control (**BC)**); maximum LDH release control (**MX**); and associated medium control (**MC**) to adjust for addition of lysing agent. The addition of trastuzumab did not affect LDH release from PBMCs alone. Rituximab (10 μg/ml) was used as a negative control for HER2-targeted ADCC. Plates were centrifuged (250 x g for 3 minutes) and incubated for 4 or 12 hours. 45 minutes before the supernatant was removed from the plates for quantification of LDH activity, 10 μl of lysis buffer was added to the maximum LDH release control (MX) and the associated medium control (MC). At the 4 and 12 hour timepoints, the plates were centrifuged and 50 μl supernatant was removed from each well and transferred to a flat-bottomed 96 well plate. 50 μl INT reaction mixture was added and after 30 minutes, 50 μl of stop solution.

Plate absorbance was read at 490 and 680nm (correction value). Following subtraction of the background control **(BC)** from all values obtained (excluding MX and MC)), % cytotoxicity was determined using:

$$\frac{(\text{Experimental value} - A - B)}{(MX - MC)} \times 100$$

Trastuzumab-mediated ADCC was determined by subtracting (% cytotoxicity in the absence of trastuzumab) from (% cytotoxicity in the presence of trastuzumab).

2.8 Flow cytometry-based ADCC assays

The flow-cytometry-based method for determining ADCC has been described in detail previously [20]. Briefly, target cells were painted with carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the Guava Celltoxicity kit protocol. Following trypsinisation and re-suspension in assay medium, cell viability counts were carried out using Guava Viacount to allow target and effector cells to be adjusted to fixed concentrations. Three ratios of effector/target cell combinations were plated in duplicate in round-bottomed 96 well plates. The assay was initiated by centrifuging plates at 50G for 3 minutes. The assay was terminated 12 hours later by addition of 40 µl 7-AAD. Live/dead effector or target cells were determined on a Guava Easycyte flow cytometer using InCyte ™ software (Millipore). % ADCC = (% target cell cytotoxicity mediated by effector cells + antibody) - (% target cell cytotoxicity mediated by effector cells alone). Controls consisted of target cells ± antibody, effector cells only and dead cell controls. Rituximab (CD20-specific ADCC-capable mAb) was used as the ADCC negative

control. CFSE-painted K562 cells were included as the positive control target cells for direct PBMC cytotoxicity.

2.9 Statistical Analysis of Data

Statistical analyses were performed on the quantitative data generated in the study using Microsoft Excel. Differences between experimental group mean values were evaluated by two tailed Student's t-test and considered significant if $p < 0.05$.

3. Results

3.1 TKI exposure impacts pre-assay SKBR3 and MCF-7 viability

48 hour exposures to 2 μM TKI significantly reduced the number of viable SKBR3 cells available for use in the ADCC assays by >60 % (Figure 1). Attached viable cells were harvested and counted. Viability of SKBR3 was 31 ± 7%, 33 ± 2% and 21 ± 2% when treated with lapatinib, afatinib or neratinib respectively. TKI exposure had an effect on MCF-7 cells (% viability following TKI exposure - lapatinib 82 ± 2.5%, afatinib 81 ± 13% and neratinib 72 ± 13%) but these changes were not significant when compared to DMSO-treated controls. Target cell numbers for each treatment condition were normalized to the assay optimized cell number (17,500/well) (Supplementary Figure 1) for use in ADCC assays.

3.2 TKIs alter total HER2 levels in SKBR3 and MCF-7

High content analysis using an extracellular domain-targeted HER2 antibody confirmed that lapatinib increased HER2 levels in SKBR3 (2.1 ± 0.6 fold increase) and MCF-7 (1.3

± 0.1 fold increase) (Figure 2A/2B). Both of the irreversible pan-HER family TKIs significantly decreased HER2 levels in SKBR3 (afatinib0.8 ± 0.1 fold and neratinib 0.6 ± 0.1 fold) and in MCF-7 (afatinib 0.7 ± 0.2 fold and neratinib 0.3 ± 0.1 fold). Confocal microscopy images of SKBR3 provide a visual representation of the TKI-induced changes in HER2 levels using an intracellular domain-targeted HER2 antibody and an extracellular domain-targeted HER2 antibody (Figure 2C). Visualization of HER2 levels in MCF-7 did not produce useful images due to significantly lower levels of HER2. Membrane and cytosolic increases in HER2 can be seen in response to lapatinib with the opposite occurring for afatinib and neratinib. To ensure HER2 changes were relevant to the ADCC assays, TKI-related effects on HER2 in SKBR3 cells were shown to last up to 18 hours in the absence of TKI (Supplementary Figure 2). Additional studies using fluorescently-labelled trastuzumab have confirmed TKI-effects on HER2 levels in MCF-7 last a minimum of 6 hours (unpublished data).

3.3 The effect of TKIs on LDH levels in SKBR3 and MCF-7

Determining ADCC levels by LDH release requires maximal (100%) values for LDH levels in the cell population under investigation. Figure 3 shows the average absorbance values for the maximal LDH controls (minus background control) in TKI-treated SKBR3 and MCF-7 following 4 hour and 12 hour assays. TKI treatment for 48 hours prior to the assay significantly reduces LDH levels in the TKI-sensitive SKBR3 (36% (lapatinib), 34% (afatinib), 51% (neratinib) reductions following 12 hour assay) (Figure 3), highlighting the importance of treatment specific controls in these assays. TKI treatment does not affect MCF-7 LDH levels as dramatically. Without appropriate treatment

controls, the TKI-related changes in LDH values would significantly impact on the resulting ADCC levels reported.

Publicly available cDNA microarray data from E-MEXP-440, previously utilised by our group, suggests that other cell lines may also be susceptible to TKI-related alterations in LDH levels (Supplementary Figure 6) [23, 24]. LDHA and LDHB gene expression are significantly reduced in lapatinib-treated SKBR3, BT474 (ER+, HER2 over-expressing) and MDA-MB-468 (triple negative, EGFR-over-expressing) vs. DMSO control but not to the same extent in T47D (ER+, HER2-low) after 12 hours. These results suggest that lapatinib is capable of altering gene expression of LDHA and LDHB in SKBR3 within a short timeframe, and is likely to have similar effects in other HER-family over-expressing cell lines (BT474, MDA-MB-468) but more limited impact in HER2-low cell lines similar to MCF-7, such as T47D.

3.4 ADCC does not directly correlate with TKI-related changes in HER2 antigen levels

Lapatinib increases HER2 levels in SKBR3 but this did not lead to a consistent increase in ADCC levels (Figure 4 A/B/C/Supplementary Figure 3/4). Also in SKBR3, afatinib produced no change in ADCC individually or when averaged (Figure 4C) but neratinib treatment resulted in increased trastuzumab-mediated ADCC in two individual samples (Supplementary Figure 4). Afatinib and neratinib effects on ADCC are contrary to the decreased levels of HER2 antigen detected following exposure to these drugs.

Although responsible for elevated levels of HER2 in MCF-7, lapatinib did not significantly affect trastuzumab-mediated ADCC relative to DMSO control. However, afatinib and

neratinib did reduce trastuzumab-mediated ADCC, corresponding to the decreased HER2 levels (Figure 5). This effect was most clearly evident at the 12 hour timepoint, 10:1 ratio but did not achieve statistical significance, (afatinib p=0.167, neratinib p=0.146 (Figure 5)).

3.5 The influence of assay duration on trastuzumab-mediated ADCC

Trastuzumab-mediated ADCC was examined in SKBR3 at 4 hour (Figure 4A) and 12 hour (Figure 4B) timepoints. Untreated healthy volunteer PBMCs were used in all ADCC assays. Rituximab levels of ADCC were negligible at 4 and 12-hours for both cell lines (Supplementary Figure 5). The 12 hour timepoint resulted in higher levels of ADCC in medium-treated SKBR3 (15.7 ± 6.7%) compared to the 4 hour timepoint (8.7 ± 6.3 %) (Figure 4). The LDH-release assays in MCF-7 produced very low basal (medium-treated) ADCC levels (4 hour- 3.8 ± 1.1%, 12 hour – 7.6 ± 4.7%) at the highest ratio examined (10:1), but the 12 hour timepoint was higher (Figure 5). Overall, TKI-related changes in ADCC were more pronounced at the 12 hour timepoint (Figure 4 A/B/C, Figure 5).

3.6 PBMC activity and ADCC

Lapatinib treatment of SKBR3 does not significantly affect trastuzumab-mediated ADCC when examining the average of three independent experiments (Figures 4A/4B/4C). The SKBR3 LDH experiments are shown individually in Supplementary Figures 3 and 4. Interestingly, for PBMC samples displaying higher direct cytotoxicity against K562, lapatinib treatment results in an increase in trastuzumab-mediated ADCC, most consistently at the 12 hour timepoint (Supplementary Figure 3). This suggests that

increases in trastuzumab-mediated ADCC in lapatinib-treated SKBR3 are associated with the activity of the PBMC sample as measured against K562 and an assay incubation time greater than 4 hours. Three further assays carried out using a new heat-inactivated serum batch in lapatinib-treated SKBR3 resulted in only one increase in trastuzumab-mediated ADCC, in one sample at the highest ratio with the highest K562 cytotoxicity (Supplementary Figure 4).

3.7 Levels of ADCC detected by a flow cytometry method

We have previously examined trastuzumab and cetuximab-mediated ADCC in HER2+ and HER2-low breast cancer models using a flow cytometry-based method [20]. Figure 6 shows the average of three independent experiments examining trastuzumab- and rituximab-mediated ADCC in SKBR3 and MCF-7 after 12 hours using the flow cytometry method. In SKBR3, ADCC levels range from 16.5 ± 3 % at 1:1, 28.7 ± 2.8 % at 5:1 through to 38.7 ± 7.7 % at 10:1, with all ratios significantly greater than the rituximab control. K562 cytotoxicity at 10:1 for the three volunteer samples used ranged from 6.5 – 22.7 %. The same assay in MCF-7 resulted in greater than negative control levels of trastuzumab-mediated ADCC at 5:1 (14.7 ± 0.4 %) and 10:1 (19 ± 3.2 %) ratios with K562 cytotoxicity (10:1) ranging from 5.3 – 22.6 %. The standard deviation of three independent flow cytometry-based assays was less than 10% for SKBR3 and MCF-7. The flow cytometry values reported are approximately double the levels of trastuzumab-mediated ADCC measured by the LDH method in MCF-7 suggesting the flow cytometry method may be more capable of measuring TKI-related changes in mAb-mediated ADCC in antigen low cell lines.

4. Discussion

Using LDH-release assays to successfully measure trastuzumab-mediated ADCC in breast cancer cell lines with high and low antigen load we show that 1) TKIs can influence LDH, the parameter being assayed to determine ADCC 2) TKI-altered antigen levels do not directly correlate with ADCC response 3) Effector cell activity is associated with TKI-induced changes in ADCC which are most detectable at a 12 hour assay timepoint and 4) the level of ADCC reported for the low antigen model (MCF-7) is too low to determine changes related to TKI exposure. Collectively, these results suggest that TKIs have potential as modulators of the passive immunotherapy trastuzumab and a more sensitive flow cytometry-based method may be required to assess this potential in greater detail.

The TKIs examined consistently alter HER2 levels (Section 3.2). Zhang *et al.* have proposed a hypothesis to explain that the opposite impact of lapatinib and neratinib on HER2 levels under normal culture conditions is due to neratinib-augmenting endocytic degradation of HER2 with only moderate increases in HER2 transcription, while lapatinib treatment is associated with strong upregulation of HER2 transcription and limited levels of HER2 endocytosis [25]. Our results suggest that, as an irreversible inhibitor like neratinib that decreases HER2 levels, afatinib is likely to follow the neratinib model proposed by Zhang *et al.* This would require further investigation to confirm.

The significant reduction in levels of LDH in SKBR3 due to TKI exposure has an impact on assay procedures (Section 3.3), requiring inclusion of specific controls for each TKI

treatment. The lowered LDH values in SKBR3 are most likely due to reduced metabolic activity following TKI inhibition of HER family members in a cell line highly dependent on HER2 signaling for growth and proliferation [26-28]. The impact of lapatinib on LDH gene expression suggests that this occurs at the transcriptional level and will be of consequence in cell lines dependent on HER family signaling for proliferation (Supplementary Figure 6). Lapatinib, afatinib and neratinib treatment have been reported to induce senescence in SKBR3, a process by which cell metabolism is slowed and the cell enters a reduced metabolic state [29, 30]. LDH levels in the MCF-7 cell line were not altered as dramatically but TKI-treatment controls still had to be included for this cell line.

In general, the inclusion of TKIs in LDH-based assays may not be advantageous, particularly in TKI-sensitive cell lines, unless controls are included to compensate for the direct cytotoxicity of the TKI, the effects on LDH levels and the potential impact of the TKI on HER-family expressing effector cell function. The alterations to HER2 resulting from TKI exposure last at least 18 hours in SKBR3 and at least 6 hours in MCF-7 (Section 3.2), which allows the TKI impact on trastuzumab-mediated ADCC to be ascertained in the absence of the TKIs in the assays reported in this study. Removing the TKIs from the cell lines prior to the assays reduces any potential impact the compounds or DMSO could have on HER-expressing PBMCs [19, 20]. We acknowledge that the cells remaining after TKI treatment are a selected population, as a tumor cell population would be following exposure to a TKI *in vivo,* but of primary interest in this study is the increased (lapatinib) or decreased (afatinib and neratinib) levels of HER2 antigen.

Antigen levels did not directly correlate with ADCC response in SKBR3 and MCF-7 (Section 3.4). This study concentrates solely on the impact of increased or decreased levels of total antigen on ADCC. As we have shown that TKI modulation of mAb-mediated ADCC is not directly related to antigen levels, other factors require investigation. Sanchez-Martin *et al.* report that the disruption of HER family dimers by lapatinib and neratinib in the presence of NRGβ1 could enhance trastuzumab-mediated ADCC by increasing the amount of HER2-antigen available to bind [31]. We have not tested the impact of the TKIs on receptor dimerization in this study and can only assume an innate level of ligand-induced HER family dimerization related to the FCS used. Levels of proteins associated with NK cell activity, such as the cognate ligands of killer immunoglobulin-like receptors (KIRs) also require investigation [32].

Timepoints greater than 4 hours, up to 8 and 10 hours, have been utilized in mAb-mediated ADCC assays reporting lapatinib-related increases in ADCC [16, 28]. The experimental set-up used in this study suggest a 12 hour incubation produced higher levels of ADCC in both models examined (Section 3.5). However, the MCF-7 cell line has been used as a low level, and sometimes negative, control for trastuzumab-mediated ADCC assays using LDH and ^{51}Cr techniques, suggesting the level of ADCC detected using these techniques in an antigen-low model is inadequate to detect significant changes at any timepoint [32, 33].

Effector cell activity rather than increased HER2 levels may be more relevant to the level of lapatinib-related augmentation of trastuzumab-mediated ADCC in HER2+ cell line models like SKBR3 (Section 3.6). The variation in PBMC cytotoxicity, as measured

against the non-MHC-restricted K562 cell line, resulted in large standard deviations between replicates. NK cells are reported as the main mediators of ADCC in the PBMC population but monocytes, although present in lower numbers, are also capable of trastuzumab-mediated ADCC [33]. Studies differentiating the composition and activation of tumor-infiltrating FcγR+ immune cells, of lymphoid- or myeloid-lineage, in trastuzumab-treated HER2+ tumor samples could provide novel insights in to the contribution of the immune response to trastuzumab efficacy.

The variation in response to lapatinib treatment in individual SKBR3 assays highlights potential factors that need to be considered when using an unstimulated mixed effector cell population like PBMCs. Stimulating the PBMCs with IL-2 may provide more cytotoxic effector cells but it does not allow for the innate levels of effector cell cytotoxicity and ADCC to be determined [12, 34]. Additionally, the reduced levels of HER2 following afatinib and neratinib treatment did not lower trastuzumab-mediated ADCC in SKBR3, most likely due to saturation of effector cell:antibody engagement in an antigen-high model in the presence of excess antibody.

The low ADCC values detected in MCF-7 cells by the LDH method limits the detection of TKI-related changes in ADCC. The flow cytometry-based CFSE/7-AAD ADCC assay reported measures of ADCC in MCF-7 approximately twice that of the LDH assay (Section 3.7). This increased sensitivity resulted in lower deviations when combining three individual experiments using PBMC samples from different individuals.

The LDH and flow cytometry assays are not the only methods available to measure mAb-mediated ADCC. The chromium (^{51}Cr) release assay, mentioned previously, is commonly used to determine mAb-mediated ADCC in breast cancer cell lines [12, 35-37]. This method has shown lapatinib-related increases in trastuzumab-mediated ADCC using IL-2 stimulated PBMCs [12, 34]. Safety and non-specific release of ^{51}Cr are two drawbacks of this method. The xCelligence system is an impedance-based system, measuring changes in electrical conductance of cells detaching due to cell death [33]. This method is sensitive and offers real time measurements of proliferation or mAb-mediated cell death in adherent cell lines. The culture surface requires coating with substrates to facilitate measurement of non-adherent cell death [38]. Immune effector cell toxicity against non-adherent cell lines (e.g. K562) using this procedure has not been reported to date.

Conclusions: This study shows, in the absence of antibody concentration as a limiting factor, trastuzumab-mediated ADCC is more dependent on effector cell activity than absolute antigen levels in SKBR3, a HER2+ model of breast cancer and TKIs can augment the ADCC response. Alterations to antigen levels may have more impact on ADCC in the HER2-low MCF-7 model but a more sensitive method for determining ADCC is required to explore this. The impact of TKIs on LDH levels and the % ADCC levels reported in our antigen-low model suggest that using a flow cytometry-based method would provide data that is independent of TKI-influence, shows better consistency between volunteer PBMC samples and shows greater sensitivity to enable detection of ADCC response in HER2-low cell lines.

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (St. Vincent's University Hospital) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figure 1

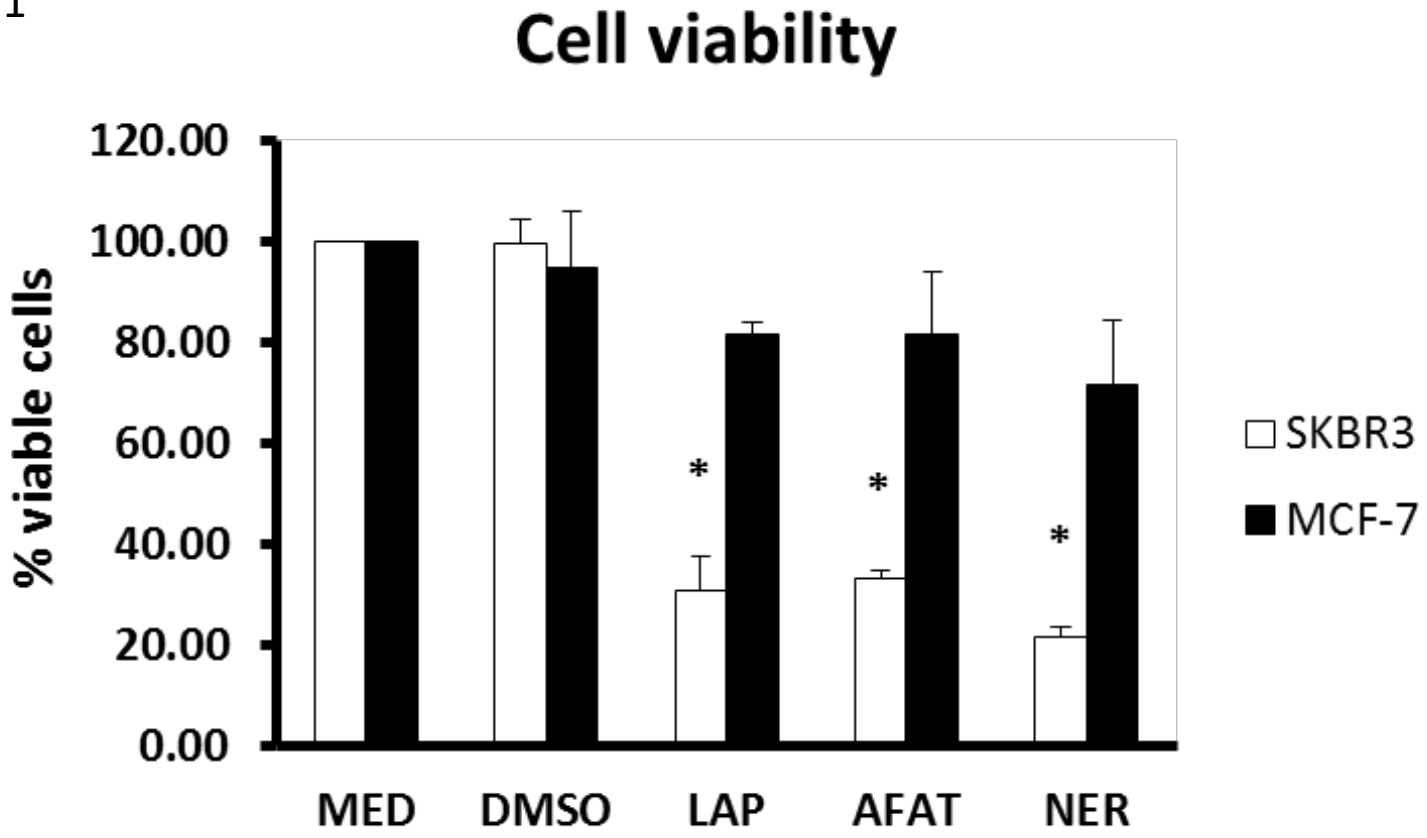
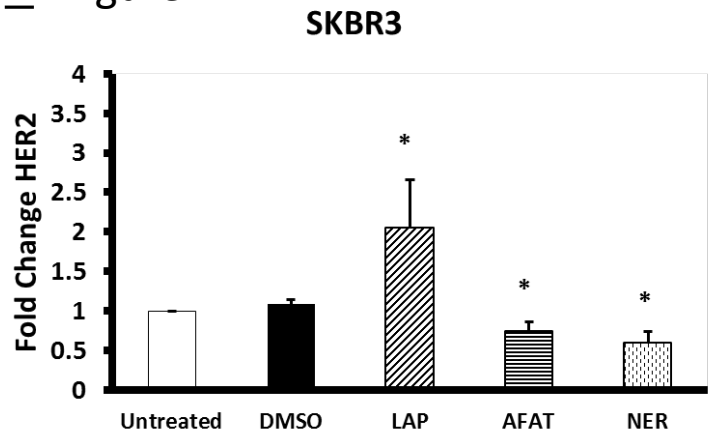


Fig. 1 Effects of lapatinib (LAP), afatinib (AFAT) and neratinib (NER) treatment (2 µM for 48 hours) on pre-assay cell viability. Viability is expressed as a percentage of Medium control and 0.2% DMSO control values are also given. The results show three independent experiments ± std. deviation. * $p<0.05$ relative to 0.2% DMSO control

A Figure 2



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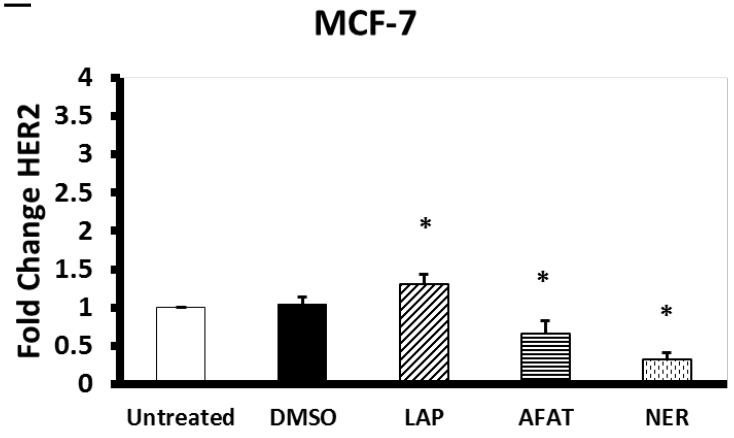

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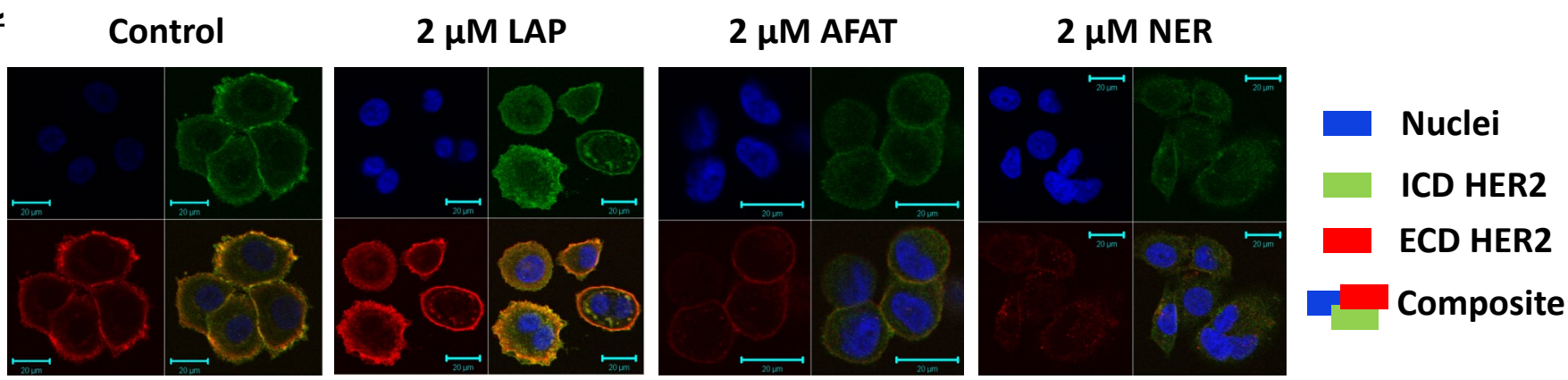


Fig. 2 Effects of lapatinib (LAP), afatinib (AFAT) and neratinib (NER) treatment (2 µM for 48 hours) on total HER2 levels (antibody to extracellular domain) in A) SKBR3 and B) MCF-7 measured by high content analysis. Results expressed as fold change relative to untreated medium controls. The results show three independent experiments ± std. deviation. * $p<0.05$ relative to 0.2% DMSO control. C) Laser scanning confocal microscopy images of HER2 in TKI-treated (48 hours) SKBR3. Antibodies used targeted the extracellular domain (ECD) and intracellular domain (ICD) of HER2. Confocal microscope parameters were kept constant for all images. Scale bar = 20 µm

Figure 3

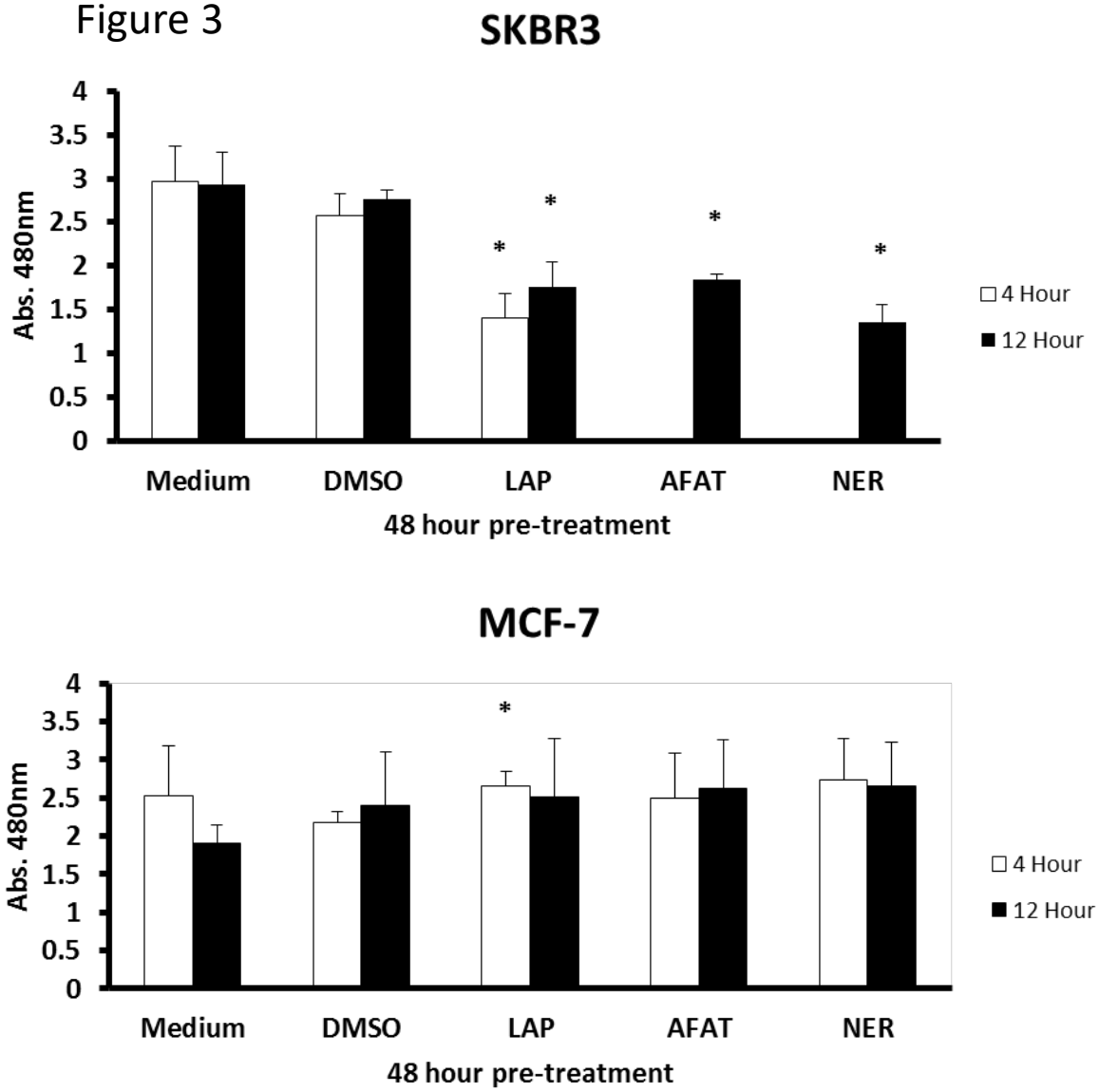


Fig. 3 The effect of lapatinib (LAP), afatinib (AFAT) and neratinib (NER) on maximal LDH values in SKBR3 and MCF-7 in 4 hr. and 12 hr. assays. Target cells were treated with 2 µM lapatinib, afatinib or neratinib for 48 hours before commencement of the assays. 4 hr. values for afatinib and neratinib were not determined in SKBR3. The results show three independent experiments ± std. deviation. * $p<0.05$ relative to 0.2% DMSO control

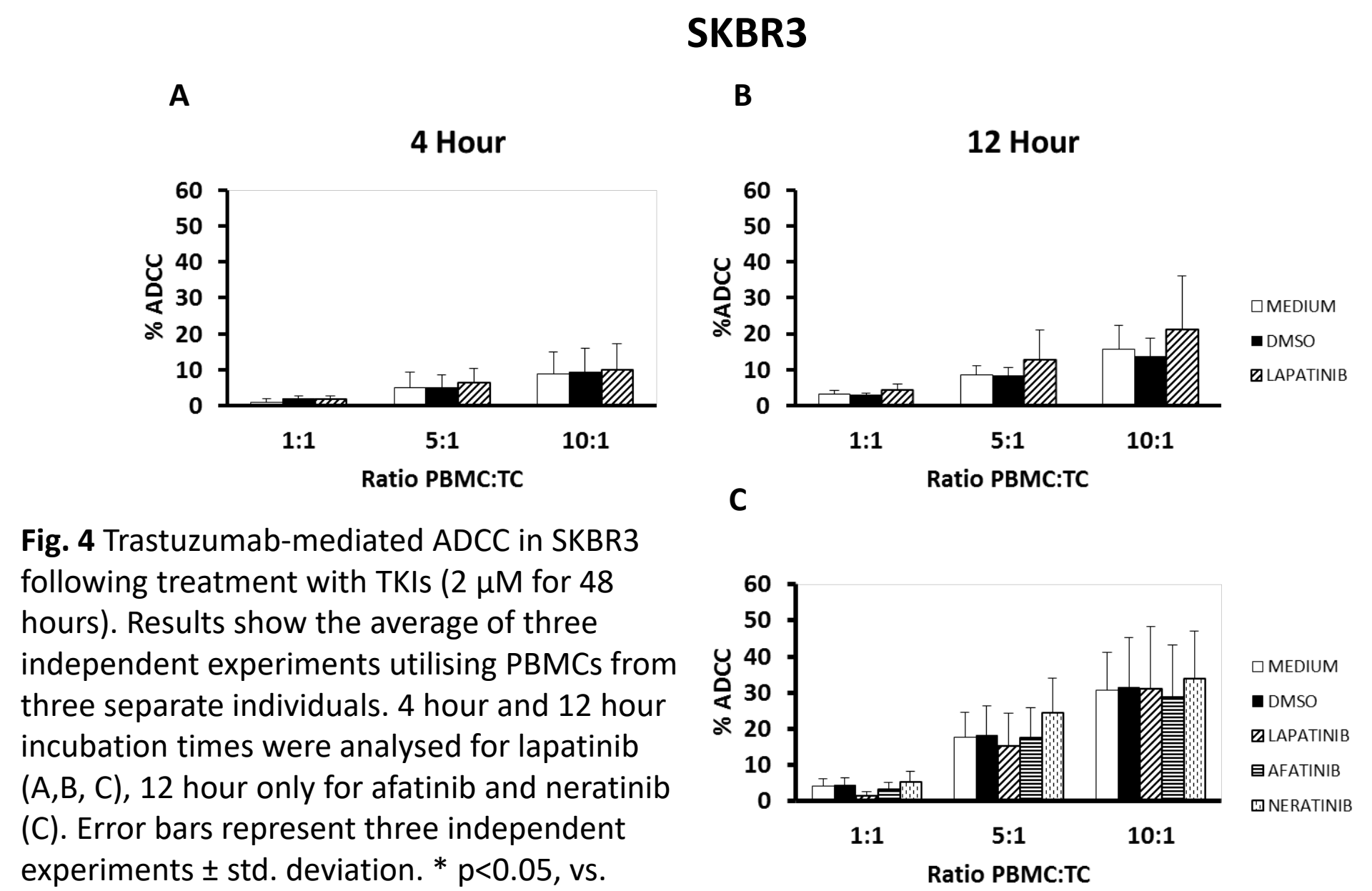


Fig. 4 Trastuzumab-mediated ADCC in SKBR3 following treatment with TKIs (2 µM for 48 hours). Results show the average of three independent experiments utilising PBMCs from three separate individuals. 4 hour and 12 hour incubation times were analysed for lapatinib (A,B, C), 12 hour only for afatinib and neratinib (C). Error bars represent three independent experiments ± std. deviation. * $p<0.05$, vs. DMSO control. Results of individual replicates are shown in Supplementary Figures 3 and 4

Figure 5

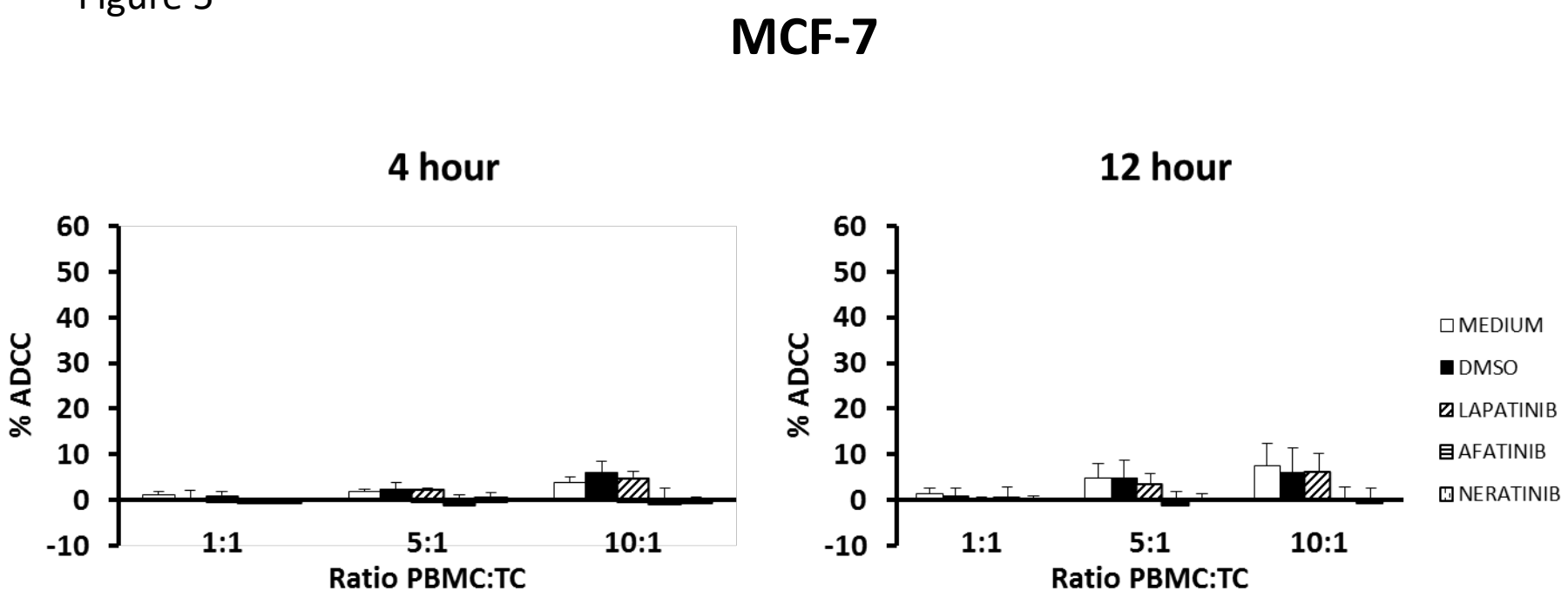


Fig. 5 Trastuzumab-mediated ADCC in MCF-7 following treatment with TKIs (2 µM for 48 hours). Results show the average of three independent experiments utilising PBMCs from three separate individuals. 4 hour and 12 hour incubation times were analysed. Error bars represent three independent experiments ± std. deviation. 12 hour vs. 4 hour, * $p<0.05$

Figure 6

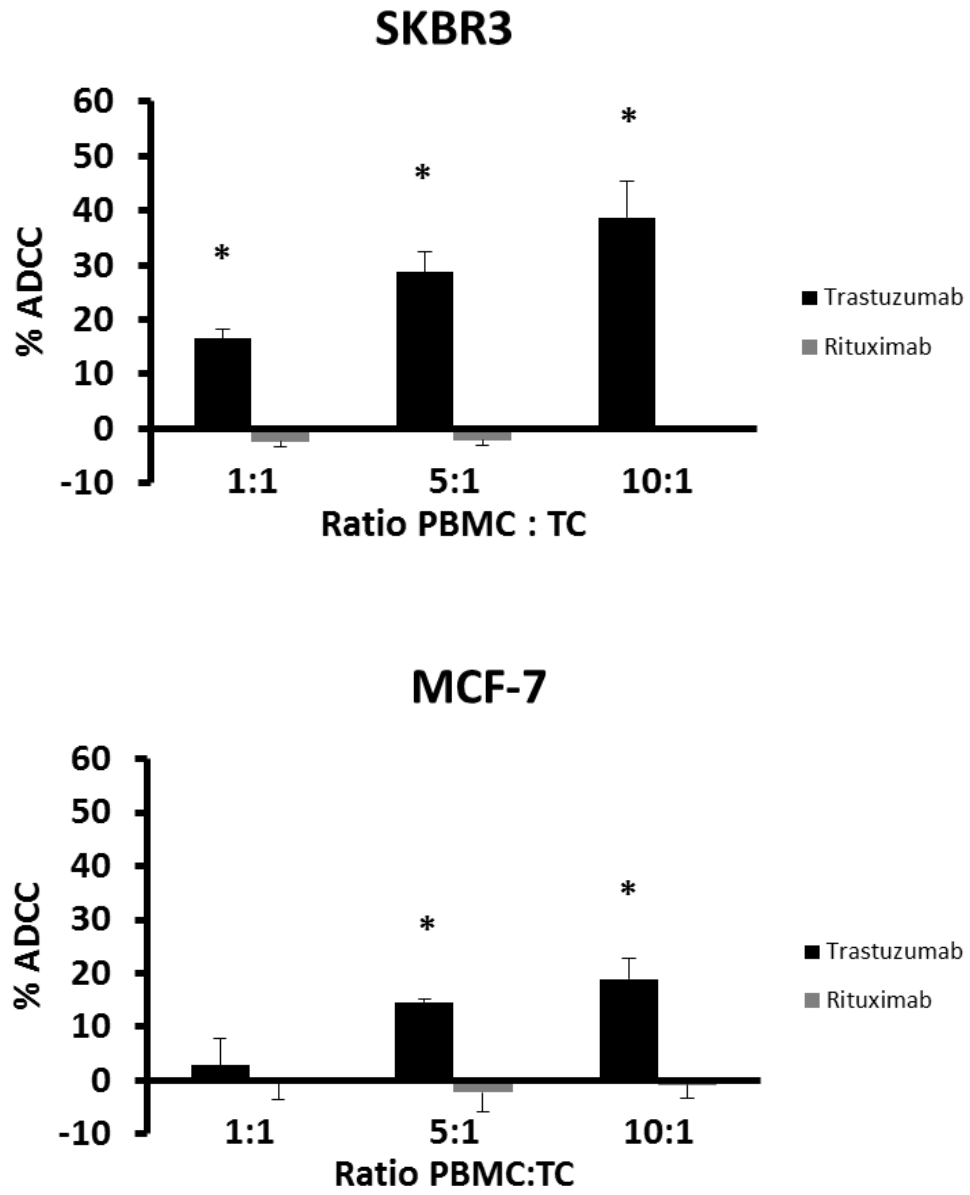
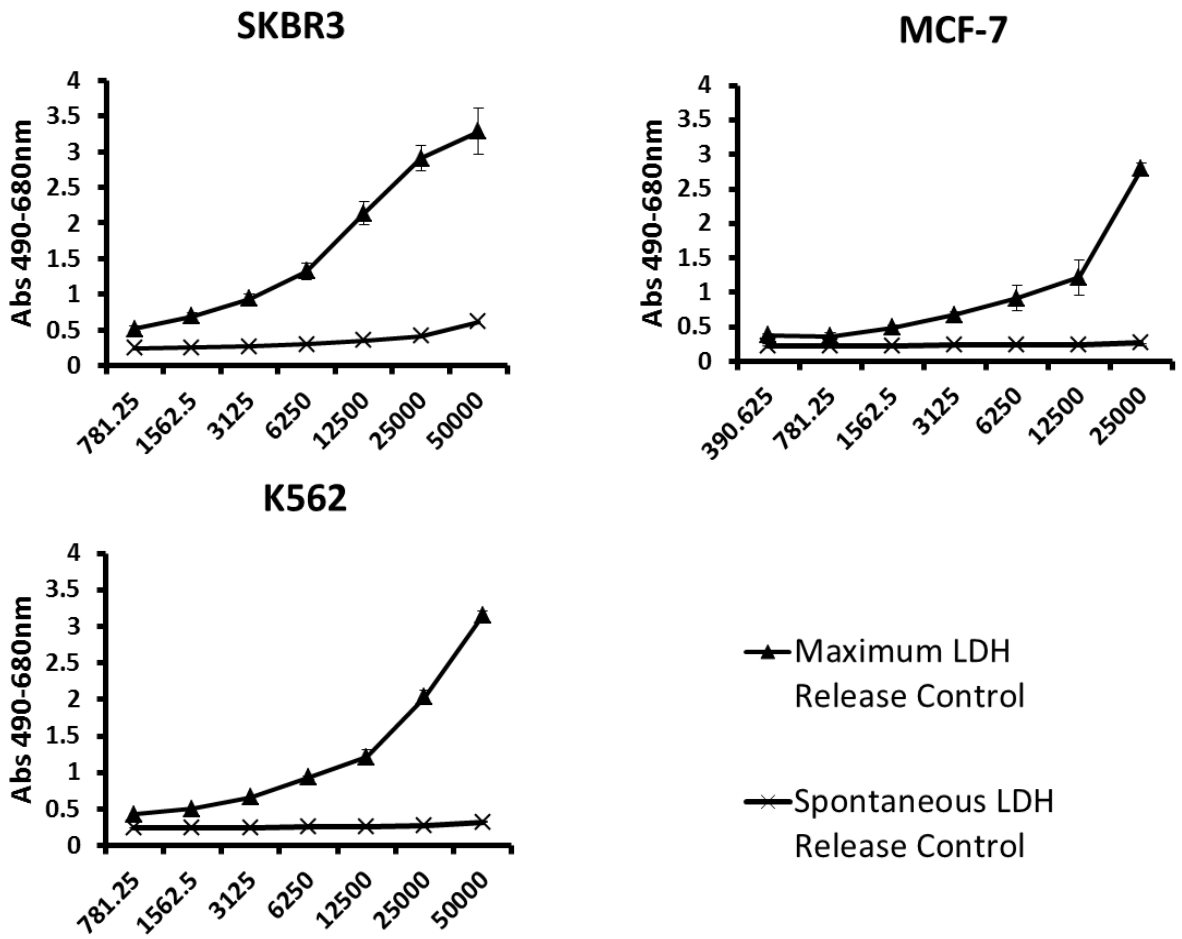
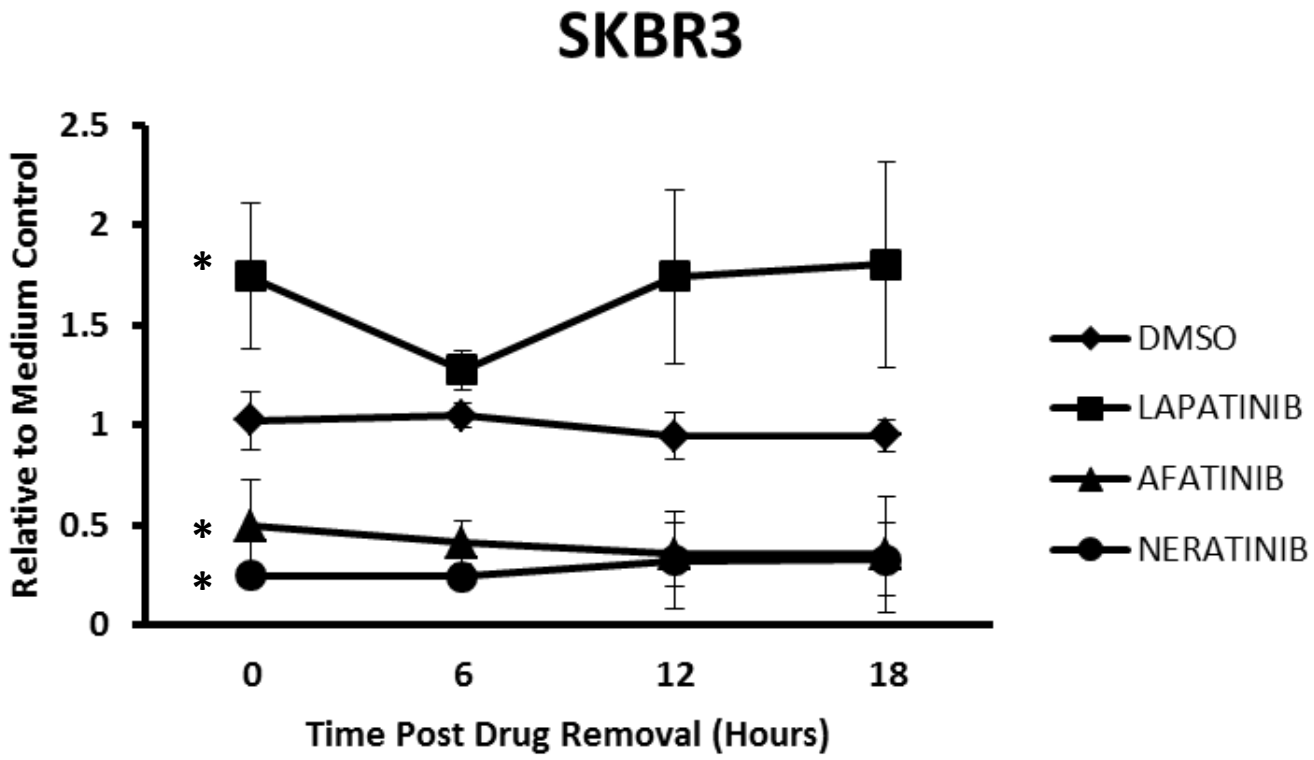


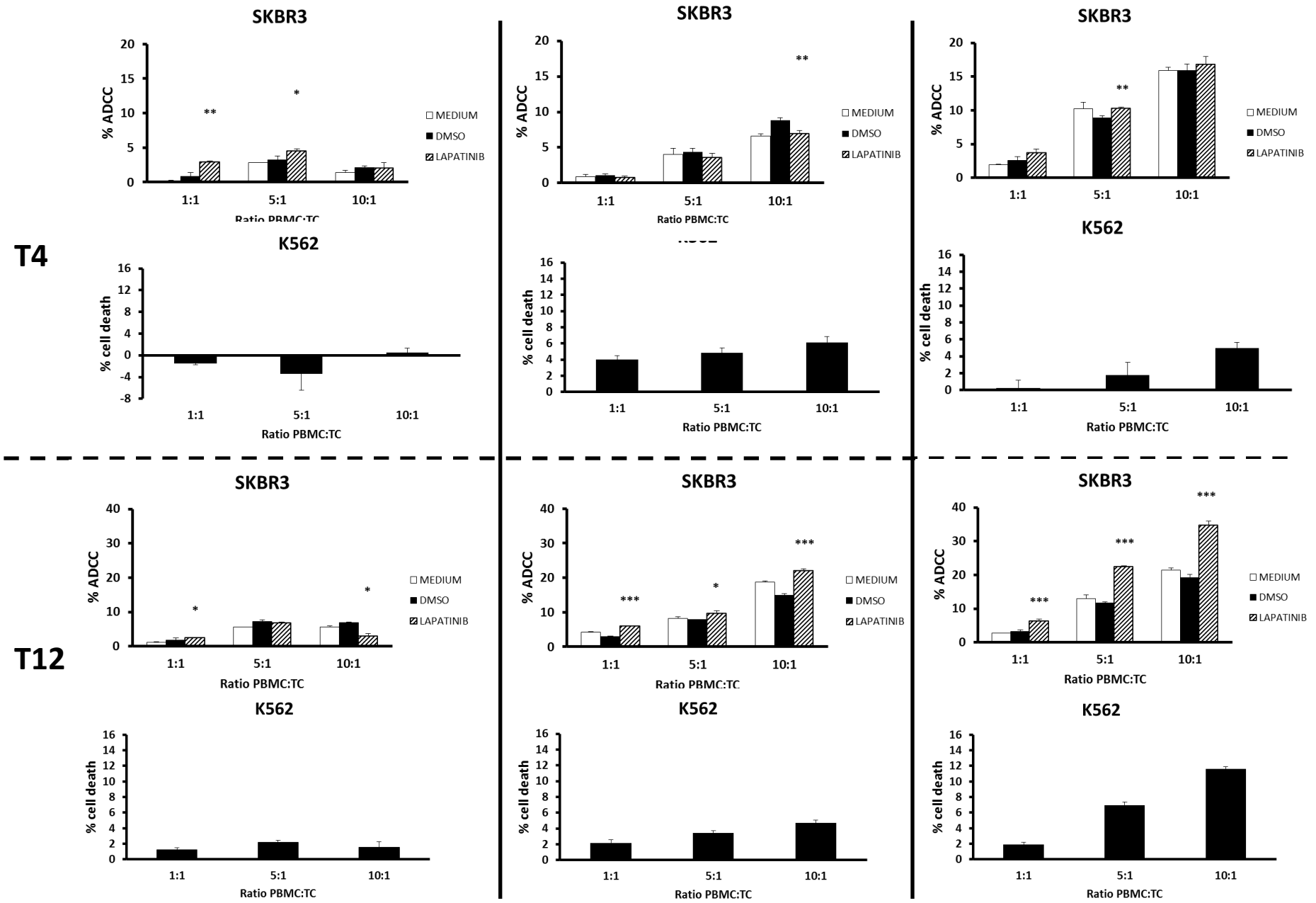
Fig. 6 PBMC-induced trastuzumab-mediated ADCC in A) SKBR3 and B) MCF-7 as determined by flow cytometry. Rituximab was used as a negative control. Assays were carried out at a 12 hr. timepoint. Results are the average of three independent experiments ± std. deviation for each cell line



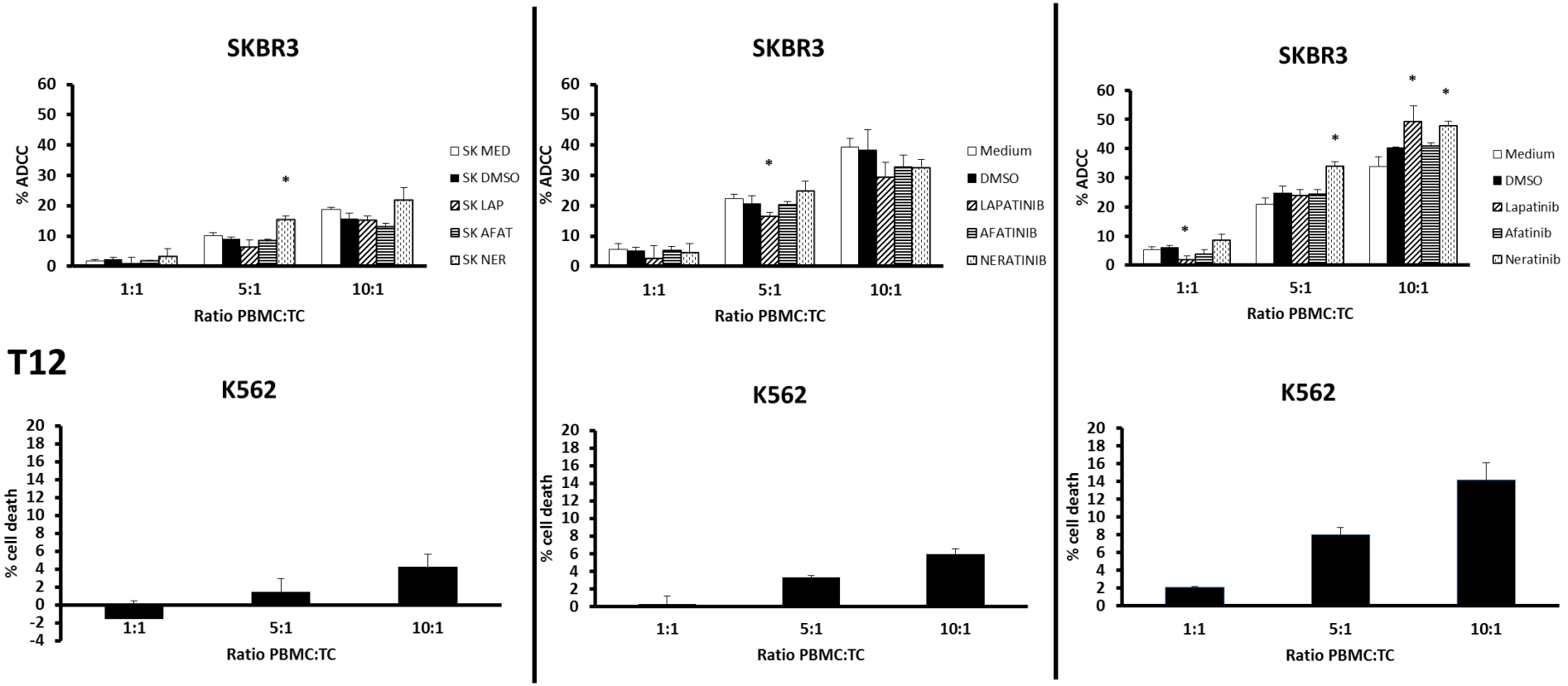
Supplementary Figure 1. LDH assay cell number optimisation for SKBR3, MCF-7 and K562 was carried out in accordance with the manufacturer's protocol. Absorbance following lysis of duplicate wells is plotted against cell number per well (Maximum LDH Release Control). Non-specific cell death is accounted for by the Spontaneous LDH Release Control.



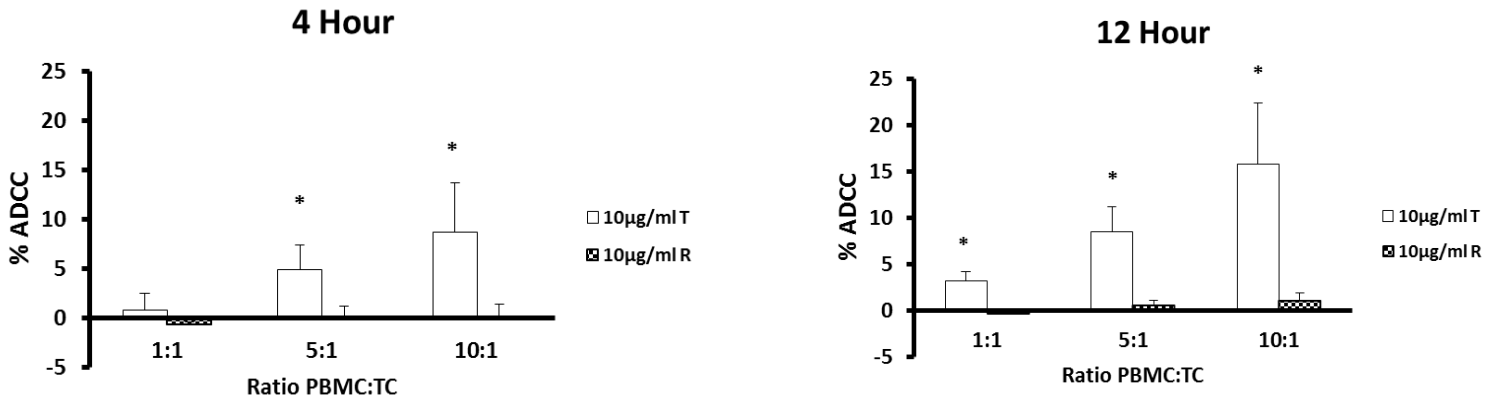
Supplementary Figure 2. Effects of TKI treatment (2 μM for 48 hours) on total HER2 levels following removal of TKIs measured by high content analysis. Results expressed as fold change relative to untreated medium controls. The results show three independent experiments +/- std. deviation. * $p<0.05$ for all timepoints relative to 0.2% DMSO control.


T4
T12
SKBR3
% ADCC
% cell death
Ratio PBMC:TC
1:1
5:1
10:1
MEDIUM
DMSO
LAPATINIB
K562

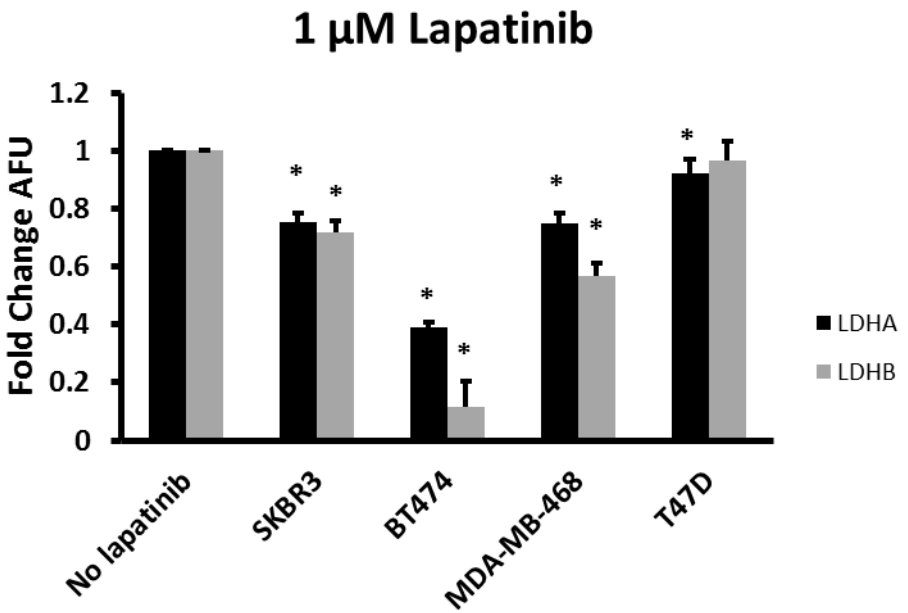
Supplementary Figure 3. T-mediated ADCC in SKBR3 following exposure to lapatinib (2 μM for 48 hours) in three independent experiments utilising PBMCs from three different individuals. 4 hour and 12 hour incubation times were analysed. Direct PBMC cytotoxicity against K562 was determined concomitantly for each PBMC sample. Error bars represent three replicates +/- std. deviation. * $p<0.05$ compared to DMSO control.



Supplementary Figure 4. T-mediated ADCC in SKBR3 following exposure to lapatinib, afatinib and neratinib (2 µM for 48 hours) in three independent experiments utilising PBMCs from three different individuals. 12 hour incubation time was analysed. Direct PBMC cytotoxicity against K562 was determined concomitantly for each PBMC sample. Error bars represent three replicates +/- std. deviation. * $p<0.05$ compared to DMSO control.



Supplementary Figure 5. T-mediated ADCC versus rituximab (R)-mediated ADCC in SKBR3 at 4 and 12 hour timepoints measured by LDH-release. The results show three independent experiments +/- std. deviation. Significant difference between T-ADCC and R-ADCC, * $p<0.05$ compared to rituximab values.



Supplementary Figure 6. cDNA microarray data from E-MEXP-440 showing fold change in LDHA and LDHB gene expression levels following exposure to 1 µM lapatinib for 12 hours. The results show the average of four determinations +/- std. deviation. * $p<0.05$ compared to DMSO controls.