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Cross-Platform Analysis of the Binding and Function of Anti-HER2 Antibody-Drug Conjugates (ADCs)

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Introduction

Antibody-drug conjugates (ADCs) are one of several adaptations made to monoclonal antibodies (mAbs) to improve their anti-tumor activity, with others including fusion to immunotoxins and radionucleotide conjugation.[1,2] ADCs have gained huge traction therapeutically in recent years, with twelve FDA-approved anti-cancer ADC therapeutics to date.[3] Five of these target solid tumors, for example, Trastuzumab emtansine, a treatment for HER2 over-expressing breast cancers and Sacituzumab govitecan, a Trop-2 targeting ADC, used to treat triple-negative breast cancers. The remaining seven approved ADCs target cells such as B cells (via antigens e.g., CD22 or CD19) and are used for treatment of hematological malignancies. ADCs unite both immunotherapeutic and chemotherapeutic interventions to create an efficacious and targeted cancer treatment.[4] They leverage the highly specific nature of therapeutic mAbs as a backbone for delivery of potent cytotoxic drugs. These mAbs have been adapted to include a linker in their constant region, onto which a toxic payload is attached. ADCs can be characterized based on whether the linkers are cleavable (released by proteases) or non-cleavable (degraded in acidic lysosomes).[5] Both types rely on internalization of the ADC into tumor cells to trigger the cytotoxic action of the

payload drug, and therefore work to prevent release of the drug in the blood stream or healthy tissues. The payloads typically used are chemotherapeutic drugs, with mechanisms of action (MoAs) that interfere with processes such as microtubule polymerization or that induce DNA damage.[6] Drugs with these MoAs preferentially kill rapidly proliferating cells, such as cancer cells, adding an extra layer of specificity to ADC cytotoxicity.[7,8]

Aside from the cytotoxic payload delivery to target cells, ADCs can maintain the Fc-mediated immune functions of the mAb on which they were based, for example antibody-dependent AW–cellular cytotoxicity (ADCC) activity. Due to the combination of MoAs that could be exerted by an ADC, it is important that we have a range of suitable *in vitro* assays to characterize their activity towards tumor cells. Traditionally, assays for quantification of ADC binding and function have been limited due to:

1. Low-throughput instrumentation (e.g., traditional flow cytometry).
2. Laborious, time-consuming processes involving protocol optimization, fixation, and repetitive washes.
3. Requirements for large volumes of precious sample and antibody.
4. Complex and lengthy data analysis.
5. The necessity for purified forms of the target protein.

Here we present a combined iQue® Advanced Flow Cytometry and Incucyte® Live-Cell Imaging and Analysis approach for quantifying the live-cell binding and function of two Trastuzumab (anti-HER2) based ADCs: Trastuzumab emtansine (Kadcyla®) and Trastuzumab deruxtecan (Enhertu®). The iQue® measures binding, immune cell marker expression and cytokine release to provide insight into how ADCs interact with both target and immune cells, and the associated changes in phenotype. The Incucyte® Live-Cell Analysis System captures temporal information on mAb target cell killing function by quantifying target and immune cells throughout ADC cytotoxicity and ADCC assays. Live-cell data was complemented with Fcγ Receptor (FcγR) and antigen kinetic protein binding data generated using the Octet® Bio-Layer Interferometry (BLI) System.

Methods

Antibodies

Three anti-HER2-hIgG1 antibodies were characterized: a Trastuzumab biosimilar (Absolute Antibody); Kadcyla® (Trastuzumab emtansine; a therapeutic-grade ADC based on Trastuzumab and the chemotherapy drug emtansine (also known as DM1), Midwinter Solutions) and Enhertu® (Trastuzumab deruxtecan, a therapeutic-grade ADC based on Trastuzumab and the chemotherapy drug deruxtecan (or DXd), Midwinter Solutions). An Anti-β-Gal-hIgG1 mAb (InvivoGen) was used as an isotype control.

iQue® Live-Cell Antibody Binding

Test antibodies (10 µL) were incubated with 20 µL of mixed high HER2 expressing AU565 cells and HER2 negative MDA-MB-468 cells (7.5K/well) in 96- or 384-well plates for 30 minutes (on ice). MDA-MB-468 cells were pre-labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye (1:2000) to distinguish from unlabeled AU565s. Following incubation, plates were washed with PBS + 2% FBS, the media removed, and cells resuspended by shaking on the iQue® plate shaker (2000 RPM). Fluorophore-conjugated secondary antibody (R-Phycoerythrin-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, Jackson ImmunoResearch) was mixed with iQue® Cell Membrane Integrity (R/Red) Dye and added to the plate. After a further 30 minutes on ice, plates were washed and run on the iQue® with a 3-5 second sip time. Binding was quantified using the iQue Forecyt® software as an increase in the median fluorescence intensity (MFI) for the secondary antibody or as the percentage of cells positive for secondary antibody binding above a defined threshold.

Protein Binding Kinetics

Octet® Streptavidin (SA) Biosensors were immobilized with biotinylated HER2 (0.25 µg/mL, ACROBiosystems) for antigen binding studies or biotinylated CD16a (0.325 µg/mL, ACROBiosystems) for FcγR binding analysis. Kinetic binding of serially diluted antibodies to immobilized proteins was measured using the Octet® (BLI) System. All dilutions were performed in Octet® Kinetics Buffer (1X KB). A shake speed of 1000 RPM and temperature of 25°C was used throughout. Resulting traces were fitted using a 1:1 or 2:1 global model for analysis of the HER2 and CD16a antibody binding, respectively.

Antibody Internalization

Incucyte® Live-Cell Analysis

AU565 cells (5K/well) were seeded overnight in 96-well flat bottom plates (Corning®3595). Antibodies were labeled with Incucyte® Human Fabfluor-pH Orange Antibody Labeling Dye, serially diluted, then added to target cells. Phase and fluorescence images (10X) were captured every 15 minutes using the Incucyte®. Images were quantified for Total Orange Area (μm^2 /image).

iQue® Advanced Flow Cytometry

Unlabeled target cells or cells labeled with Incucyte® Nuclight Green Lentivirus were seeded overnight in 96-well flat bottom plates (5K/well) before test antibodies were added at a range of concentrations. After 3-6 days, cells were lifted using Accutase and transferred to V-bottom plates for labeling with iQue® Cell Membrane Integrity (R/Red) Dye. After 30 minutes plates were washed and run on the iQue® platform. The percentage of live target cells was quantified using the iQue Forecyt® software.

ADC Cytotoxicity

Incucyte® Live-Cell Analysis

Target cells labeled with Incucyte® Nuclight Green Lentivirus or Incucyte® Nuclight Orange Lentivirus were seeded (5K/well) in 96-well flat bottom plates overnight. Test antibodies were then added at a range of concentrations. Phase and fluorescence images (10X) were captured using the Incucyte® Live-Cell Analysis System on a 3-hour repeating scan schedule for 3-7 days. Images were quantified for Green and Orange Cell Counts.

iQue® Advanced Flow Cytometry

Unlabeled target cells or cells labeled with Incucyte® Nuclight Green Lentivirus were seeded overnight in 96-well flat bottom plates (5K/well) before test antibodies were added at a range of concentrations. After 3-6 days, cells were lifted using Accutase and transferred to V-bottom plates for labeling with iQue® Cell Membrane Integrity (R/Red) Dye. After 30 minutes plates were washed and run on the iQue® platform. The percentage of live target cells was quantified using the iQue Forecyt® software.

ADCC Assays

Incucyte® Live-Cell Analysis

Target cells labeled with Incucyte® Nuclight Green Lentivirus or Incucyte® Nuclight Orange Lentivirus were seeded at 4K/well in 96-well flat bottom plates overnight before test antibodies were added at a range of concentrations. Pre-isolated natural killer (NK) cells (STEMCELL Technologies) were added (16K/well) alongside IL-12 (10 ng/mL) to improve their longevity. Phase and fluorescence images (20X) were captured using the Incucyte® Live-Cell Analysis System on a 3-hour repeating scan schedule for 5 days. Images were quantified for Green and Orange Cell Counts.

iQue® Advanced Flow Cytometry

Target cells (4K/well) were labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye and seeded in 96-well flat bottom plates overnight. Test antibodies were added at a range of concentrations alongside. NK cells were added (16K/well) alongside IL-12 (10 ng/mL) to improve their longevity. After 24 hours, 10 µL samples of supernatant were transferred to V-bottom plates for analysis of IFNγ and granzyme B cytokine concentrations using 2-plex iQue Qbeads® from the iQue® Human NK Cell Killing Kit. Cells were lifted using Accutase and transferred to V-bottom plates for labeling using the antibody cocktail provided in the kit. The kit contains antibodies for phenotyping of NK cells with markers CD3, CD56, CD16, CD25 and CD69 alongside iQue® Cell Membrane Integrity (B/Green) Dye. The gating and analysis template included with the kit was imported into iQue Forecyt® software for quantification of cytokine concentrations using a standard curve and analysis of NK marker expression.

Results and Discussion

In this application note, we have performed a range of experiments to profile the binding and functional characteristics of three antibodies: Trastuzumab, Kadcyla® and Enhertu®. Trastuzumab is an anti-HER2 mAb therapeutic, while Kadcyla® and Enhertu® are ADCs which have been based on Trastuzumab. Due to the shared Trastuzumab backbone of the ADCs, we predict them to share many functional capabilities, however, as is shown in Figure 1, they also have some key structural differences, which may distinguish their anti-tumor function. For example, the ADCs include different cytotoxic payloads, both unique in terms of the numbers and positions of conjugation sites they contain. The cytotoxic payload in Kadcyla® is emtansine (DM1) and binds lysine residues in the Trastuzumab backbone, with studies suggesting an average of 3.5 conjugations per molecule.[9] Enhertu® is linked to the payload deruxtecan (Dxd) via thioether bonds with cysteine residues, with a target of 8 conjugations per molecule.[10] The ADCs also vary in their linker chemistries, with Kadcyla® containing a non-cleavable amine-to-sulfhydryl crosslinker and Enhertu® containing a valine-citrulline cleavable linker.[11]

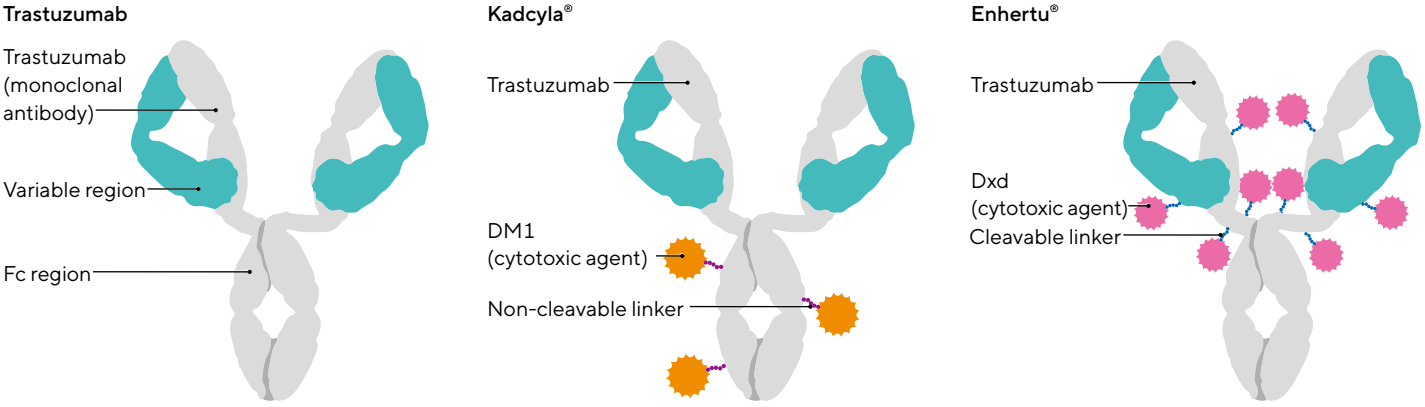
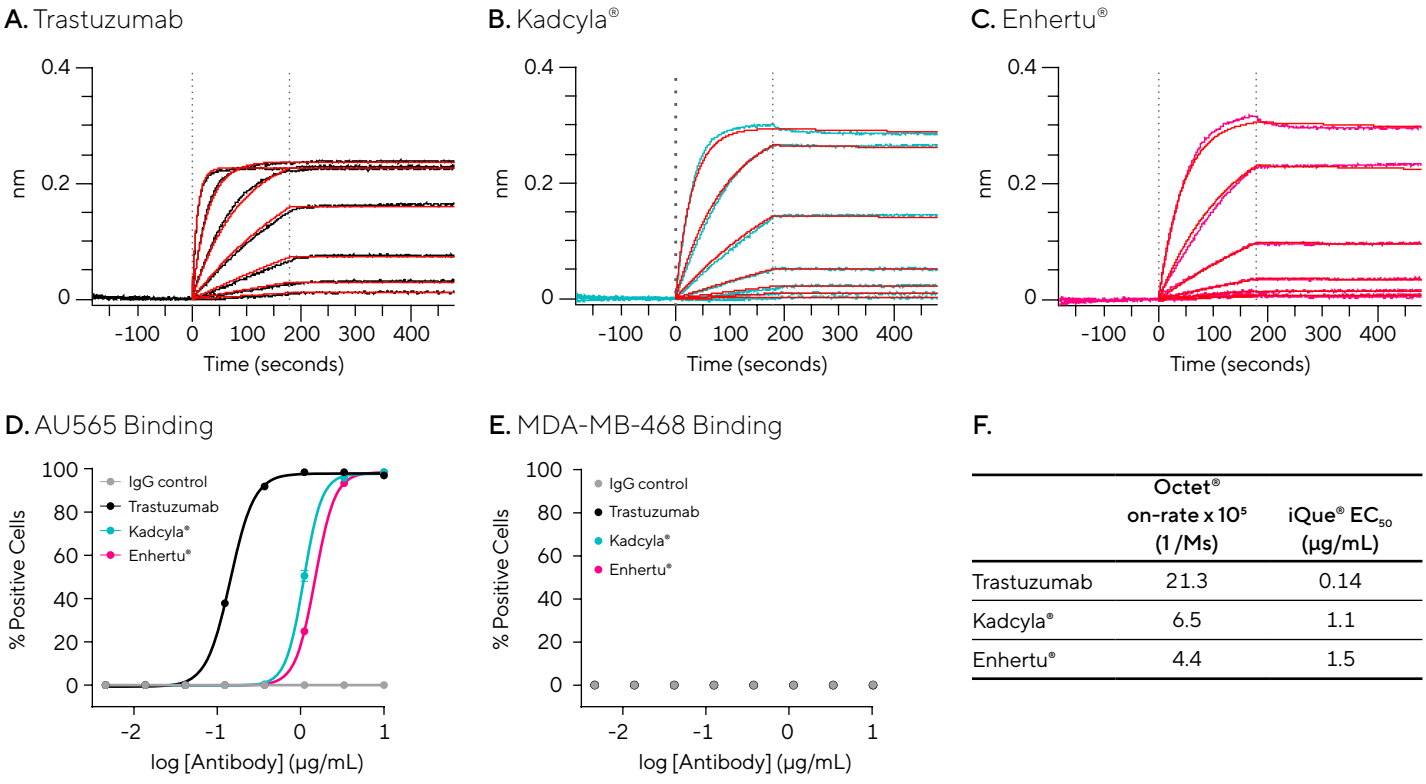


Figure 1. Structures of anti-HER2 antibodies and ADCs
Trastuzumab is an anti-HER2-IgG1 mAb. Kadcyla® is a modified version of Trastuzumab which includes non-cleavable linkers to the chemotherapy drug emtansine (DM1). Enhertu® is also an adapted version of Trastuzumab, with cleavable linkers to attach the cytotoxic payload, deruxtecan (Dxd).

Antigen Binding Characterization

It is critical during development of ADCs from therapeutic mAbs, that the highly specific and typically high affinity antigen binding is not disrupted by the inclusion of the cytotoxic payload. To explore the target binding of the three anti-HER2 ADCs, we used two approaches, which together generated complementary data on the antigen binding characteristics of the antibodies. The first was analysis of antibody binding kinetics using the Octet® BLI System, and the second was measurement of antibody binding to live cells using the iQue® Advanced Flow Cytometry Platform. The Octet® assay uses solubilized protein to measure antibody on-rates (k_a), off-rates (k_d) and affinity (K_D) values which reveal important information about how drugs may act on receptors *in vivo*. Conversely, the iQue® assay quantifies binding to the target as it is found on live, antigen expressing cells. This can be multiplexed with measurement of binding to antigen negative cells, allowing confirmation that there is a lack of non-specific, off-target binding. The Octet® response curves in Figures 2A-C and on-rate analysis in 2F show that Trastuzumab had the fastest on-rate binding to HER2 at 21.3 x 10^5 /Ms compared to 6.5 x 10^5 /Ms for Kadcyla® and 4.4 x 10^5 /Ms for Enhertu®. The off-rates for this reaction were very slow in these conditions, and therefore we have focused on the on-rate as a measure of binding for this assay. The maximal levels of response (nm) for Kadcyla® and Enhertu® were slightly higher than for Trastuzumab, which is expected due to the slightly higher molecular weights of the ADCs with the addition of the drug groups.

The live cell binding curves generated using the iQue® (Figures 2D and E) and EC_{50} values for binding potency (2F) show a similar pattern to the kinetic binding data, in that the potency of binding of Trastuzumab to high HER2 expressing AU565 cells is much higher with an EC_{50} of 0.14 µg/mL, while the EC_{50} values for Kadcyla® and Enhertu® are comparable at 1.1 and 1.5 µg/mL, respectively. None of the antibodies bound to the HER2 negative MDA-MB-468 cells, exemplifying their high specificity.



F.

	Octet® on-rate x 10^5 (1/Ms)	iQue® EC_{50} (µg/mL)
Trastuzumab	21.3	0.14
Kadcyla®	6.5	1.1
Enhertu®	4.4	1.5

Figure 2. Potency and on-rate for binding of Trastuzumab to HER2 was greater than the ADCs
(A-C) Binding of Trastuzumab, Kadcyla® and Enhertu® to Streptavidin biosensor immobilized HER2 protein (0.25 µg/mL) as measured by Octet®. Antibodies were serially diluted from 50 – 0.07 nM in 1X KB. (D, E) Curve showing % of AU565 and MDA-MB-468 cells above a defined MFI threshold for fluorophore-conjugated secondary antibody binding to test antibodies as measured using iQue® direct live cell binding assay. (F) Table comparing on-rates from the Octet® assay and binding potency from the iQue® for each antibody.

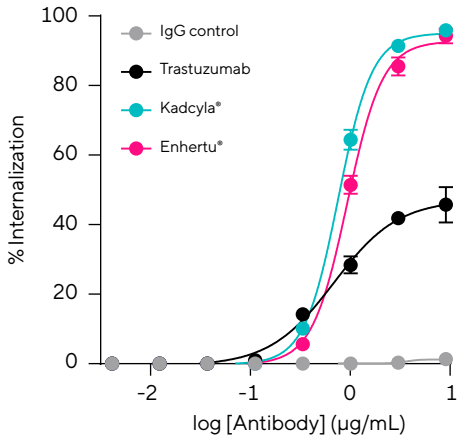
Antigen Characterization

Internalization of ADCs into antigen-expressing target cells is essential for the delivery of the cytotoxic payload. Internalization is followed by release of the cytotoxic payload, either through proteolytic degradation of the linker or by metabolism in the acidic lysosome. This directs the action of the payload specifically to antigen expressing cells, reducing off-site toxicity.

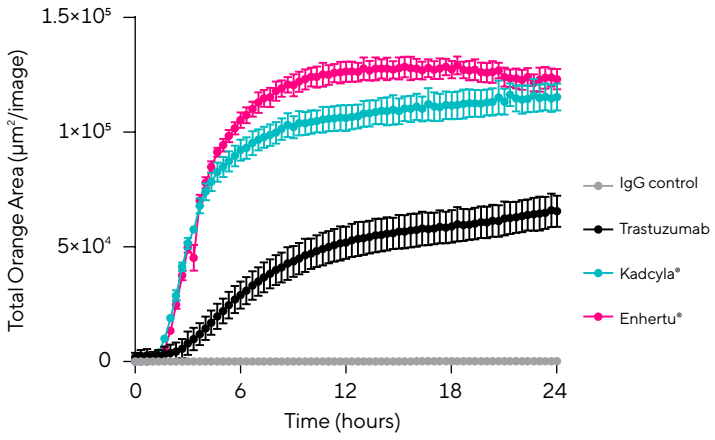
To measure the internalization of the anti-HER2 antibodies, experiments were performed on both the iQue® and Incucyte® platforms. In the iQue® assay, AU565 cells were incubated with antibodies that had been labeled using a pH-sensitive probe from the iQue® Human Antibody Internalization Kit. This probe fluoresces upon internalization into acidic lysosomal or endosomal pathways meaning internalization can be quantified due to an increase in MFI. The data in Figure 3 shows highly comparable internalization after 6 hours of Kadcyla® and Enhertu®, with EC_{50} values of 0.75 and 0.93 µg/mL, respectively. The EC_{50} for internalization of Trastuzumab was also comparable at 0.68 µg/mL, however the maximal level of internalization for Trastuzumab was considerably lower, with the highest concentration yielding 46% of cells above the defined MFI threshold for internalization, compared to 94% and 96% for the ADCs.

The Incucyte® Antibody Internalization Assay also utilizes a fluorescent probe to label antibodies, namely the Incucyte® Human Fabfluor-pH Orange Dye. The Incucyte® assay allowed us to verify the results from the iQue® and to observe how internalization progressed over time. Both the time course and concentration response curves at 6 hours generated using the Incucyte® corroborated iQue® data as they showed highly similar internalization of the ADCs and roughly 50% lower internalization of Trastuzumab.

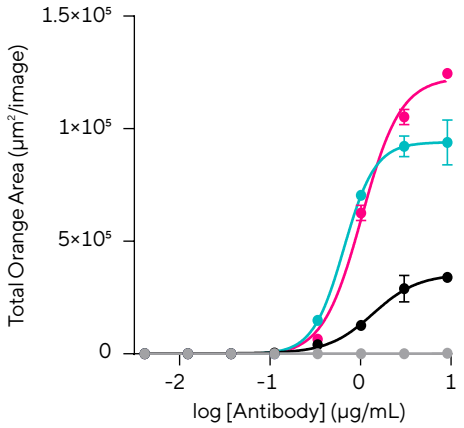
A. iQue® (6 hours)



B. Incucyte® Time Course (3 µg/mL)



C. Incucyte® (6 hours)



D.

	iQue® EC_{50} (µg/mL)	Incucyte® EC_{50} (µg/mL)
Trastuzumab	0.68	1.4
Kadcyla®	0.75	0.65
Enhertu®	0.93	1.0

Figure 3. Internalization of ADCs was greater than Trastuzumab
(A) Antibodies labeled with the pH sensitive probe from the iQue® Human Antibody Internalization Kit were added to AU565 cells alongside a membrane integrity dye. The percentage of live cells above a defined MFI threshold for internalization after 6 hours is plotted. (B) Antibodies labeled with Incucyte® Human Fabfluor-pH Orange Dye were added to AU565 cells and images captured using the Incucyte® system. Total Orange Area was quantified over time. (C) Concentration response curve taken from the data in (B) at 6 hours. (D) Table of EC_{50} values for iQue® and Incucyte® internalization at 6 hours.

ADC Cytotoxicity

ADC cytotoxicity towards cell monocultures was tested using the Incucyte® and iQue® platforms (Figure 4). The Incucyte® time course data (Figures 4C and D) enables the selection of an appropriate endpoint to lift the cells, label with membrane integrity dye and transfer to the iQue® for cell count analysis. Cells expressing an Incucyte® Nuclight Green Lentivirus (NucGreen) were used, as these can easily be masked for quantification from Incucyte® images as demonstrated in Figure 4A. For the iQue®, a further selection is applied after gating the NucGreen labeled cells, to exclude any that are positive for labeling with the membrane integrity exclusion dye (Figure 4B).

Both instruments presented highly comparable quantification of death of high HER2 expressing AU565 cells after 96 hours, with a 2-fold lower EC_{50} for Kadcyla® activity (Incucyte® = 0.059 µg/mL, iQue® = 0.075 µg/mL) compared to Enhertu® (Incucyte® = 0.13 µg/mL, iQue® = 0.16 µg/mL). Trastuzumab induced minimal effect on the growth of AU565 cells, highlighting that without the addition of the cytotoxic payload, Trastuzumab activity is reliant on the presence of immune cells to act via the FcγRs. Death of the HER2 negative MDA-MB-468 cells was minimal, again reinforcing the high specificity of the ADCs.

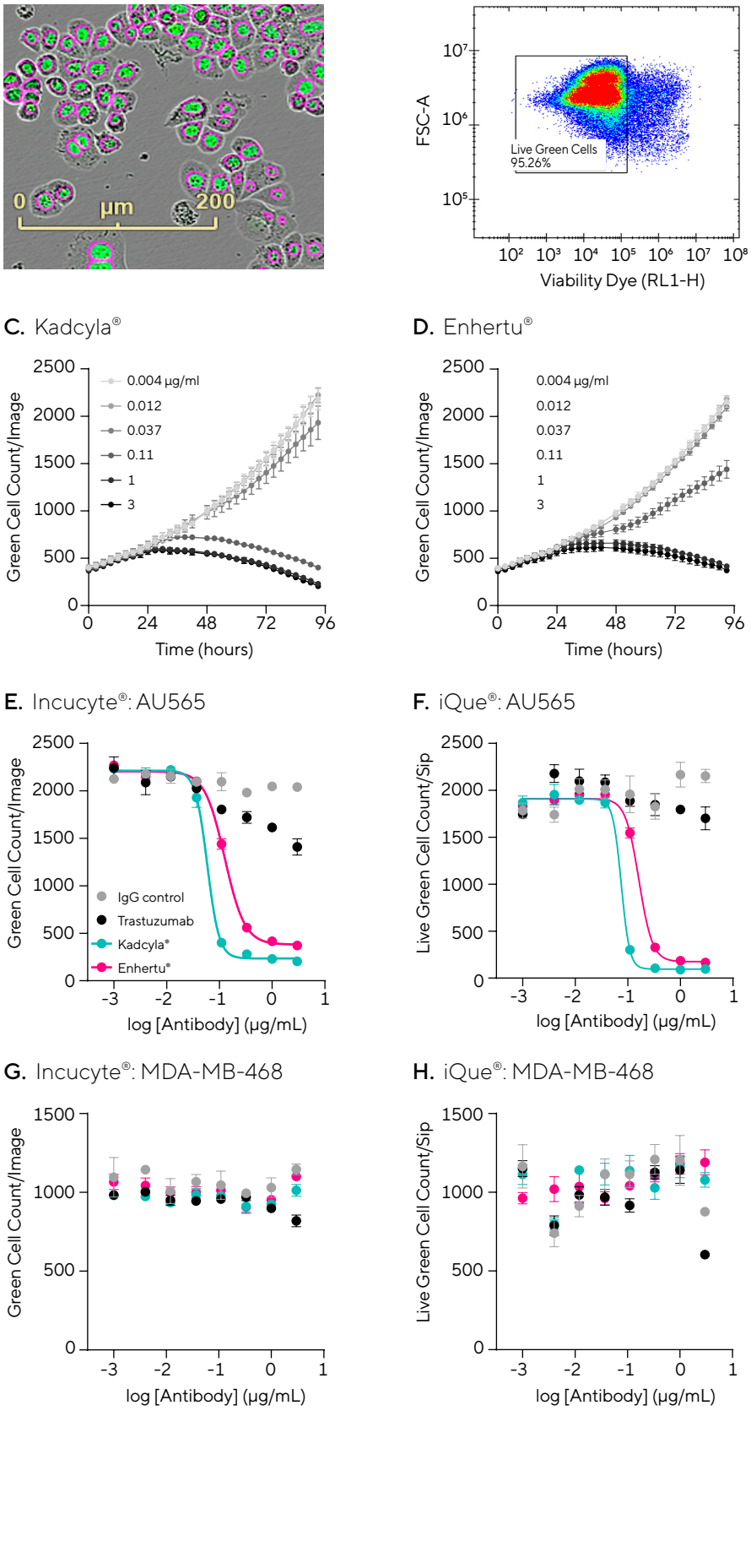


Figure 4. anti-HER2 ADCs induce death of high HER2 expressing cells in monoculture
Incucyte® Nuclight Green Lentivirus labeled cells (high HER2 expressing AU565s or HER2 negative MDA-MB-468s) were seeded with a range of concentrations of antibody. (A) Incucyte® images were captured and quantified for Green Cell Counts using the pink outline mask as shown. (B) After 96 hours, cells were lifted, labeled with viability dye and analyzed using the iQue®. Live green cell counts were gated in iQue® Forecyt as in plot. (C, D) Incucyte® time course for growth of AU565 cells in the presence of Kadcyla® and Enhertu®. Curves show Incucyte® and iQue® counts of (E, F) AU565 and (G, H) MDA-MB-468 cells.

Enhertu® Bystander Killing

In 2022, Enhertu® was the first HER2-directed therapy to be approved for treatment of patients with low-HER2 expressing breast cancer.[12] Its success against these cancers has been largely attributed to a unique 'bystander' activity. This activity is thought to be facilitated by the high membrane permeability of the Dxd payload, meaning that once the ADC has killed a HER2 expressing cell, the payload can then be released and diffuse easily into neighboring cells, regardless of their HER2 expression, resulting in their death.[13]

To explore this *in vitro*, we set up co-culture assays containing various ratios of high HER2 expressing and HER2 negative cells, alongside the antibodies of interest (1 µg/mL). Figures 5A and 5B show the counts (normalized to time = 0) of Incucyte® Nuclight Orange Lentivirus (NucOrange) labeled HER2 negative MDA-MB-468 cells that were co-cultured with high HER2 expressing AU565 cells, at MDA:AU565 ratios of 1:4, 2:3, 3:2 and 4:1. In the presence of Enhertu®, the data shows that the higher the ratio of AU565 cells present in the

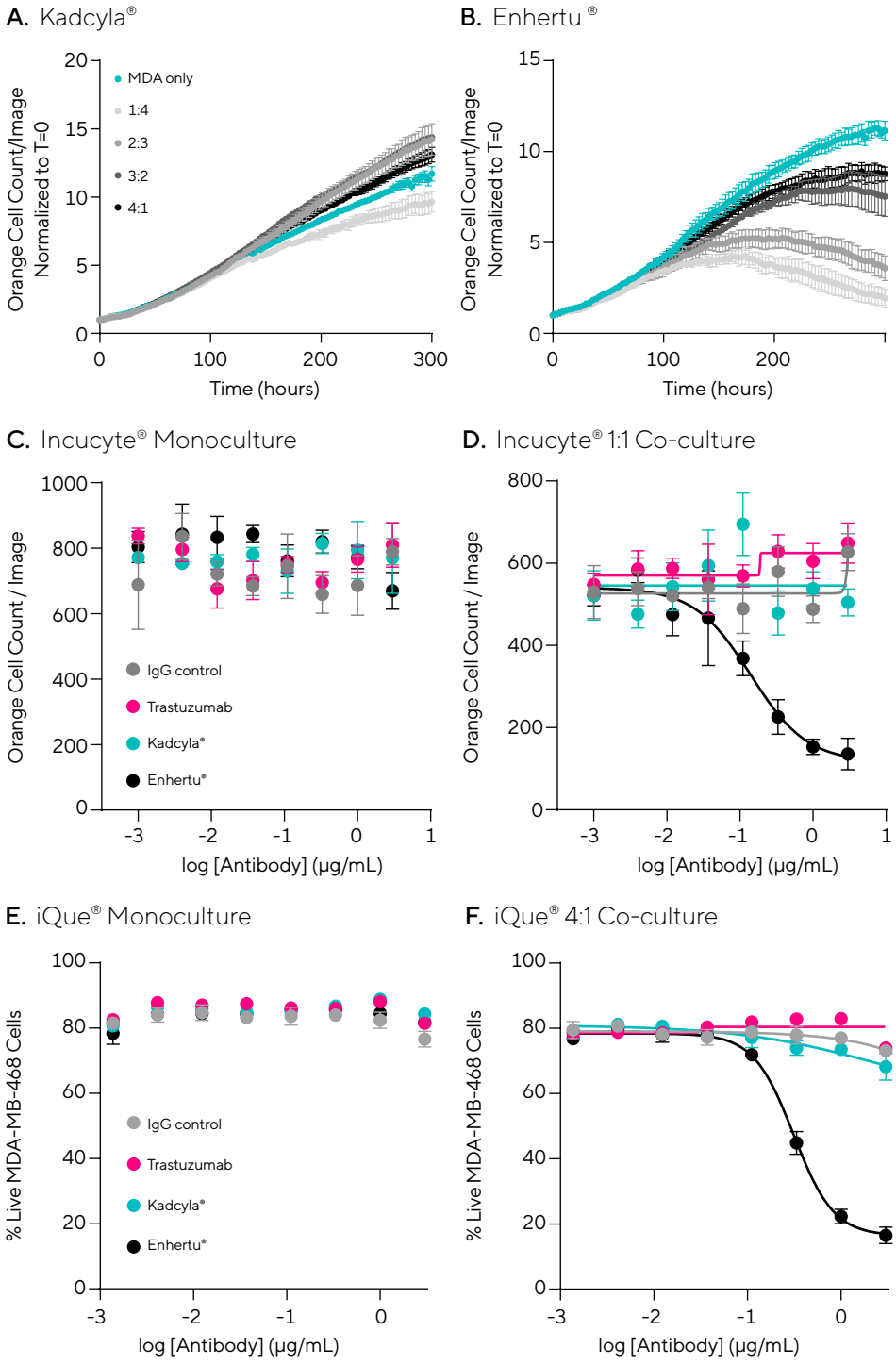


Figure 5. Enhertu® induced killing of HER2 negative bystander cells in a co-culture ratio and concentration dependent manner
(A,B) Incucyte® time course data shows Nuclight Orange MDA-MB-468 counts (normalized to time (T) = 0) in the presence of Kadcyla or Enhertu (1 µg/mL) with various ratios of MDA-MB-468 and AU565 cells (Nuclight green labeled). (C,D) Concentration response curves show effects of antibodies on MDA-MB-468 counts (day 12) in monoculture or in a 1:1 co-culture with AU565 cells (Nuclight green labeled) as quantified from Incucyte® images. (E,F) Percent viability of Nuclight green labeled MDA-MB-468s as measured by iQue® on day 4 of monoculture or co-culture with unlabeled AU565 cells.

culture, the greater the level and rate of death of the MDA-MB-468 cells. Kadcyla® did not increase death of the MDA-MB-468 cells in the same conditions, and no ADC cytotoxicity was observed in the MDA-MB-468 monoculture. These data support the unique bystander activity of Enhertu® and display its dependence on the presence of HER2 expressing cells for release of the cytotoxic payload.

To investigate the effect of concentration on the bystander effect, MDA-MB-468 cells were incubated with a range of concentrations of antibody, either in monoculture or co-culture with AU565 cells. For Incucyte® assays, the NucOrange labeled MDA-MB-468 cells were combined at a 1:1 ratio with NucGreen labeled AU565 cells, and the counts of each cell type quantified from images using a basic analyzer mask. Day 12 orange cell count data in Figure 5C and 5D demonstrate that only the Enhertu®, in the co-culture conditions, induced a concentration dependent decrease in HER2 negative cell counts.

Due to the detection channels available in the iQue® instrument (VBR), and the relative ease of distinguishing green labeled and unlabeled adherent cell types using the flow cytometry assay, the co-cultures for iQue® assays instead contained NucGreen labeled MDA-MB-468 cells alongside unlabeled AU565 cells. Cells were labeled with a viability dye at the assay endpoint to aid identification of live and dead populations. Since earlier experiments demonstrated faster killing in the 1:4 MDA-MB-468 to AU565 co-culture, this ratio was used for antibody comparison by iQue®, meaning a shorter assay endpoint of 4 days could be taken for the concentration response curve. Despite differences in the timepoints and ratios used for each instrument, the EC_{50} values for Enhertu® bystander activity were similar at 0.14 and 0.31 µg/mL for the Incucyte® and iQue®, respectively.

ADCC Activity

In addition to induction of cytotoxicity through action of the payload, ADCs generally maintain other Fc effector functions of the original mAb, including ADCC activity. To quantify the ADCC activity of the anti-HER2 antibodies, NucOrange MDA-MB-468 and NucGreen AU565 cells were used in either single or dual target cell ADCC assays with isolated NK cell effectors. Incucyte® images were quantified for 5 days and the resulting time courses in the presence of 3 µg/mL of antibody are displayed in Figure 6.

In the single target cell assays, the ADCs induced a high level of death of the AU565 cells, with a slightly elevated level of killing induced by the Kadcyla® than the Enhertu®, similarly to what was seen in the absence of NK cells. Trastuzumab induced a high level of killing in the presence of NK cells, which was not seen in the monoculture assay, reinforcing the need for effector cells to exert significant cytotoxicity (Figure 4). The bar charts in Figures 6C and 6D combined with Incucyte® time course data (Figure 4C and D, Figure 6B), demonstrate that at time points later than 24 hours, the progression of killing is faster in the presence of NK cells than with target cells alone, highlighting the dual killing mechanism of ADC toxicity with ADCC.

Killing of AU565 cells in the dual target cell assay was similar to the single target ADCC assay (Figures 6E and F). However, a difference was observed in the action of the antibodies towards the MDA-MB-468 cells, depending on whether AU565 cells were also present. This was evidenced by a reduction in MDA-MB-468 count for all three antibodies relative to the control in the dual target ADCC, but not the single target assay. This may suggest that once the NK cells have become activated during targeted ADCC of HER2 expressing cells, that the NKs can then induce death of surrounding cells regardless of HER2 expression. This may be due to the action of cytokines, such as proteolytic Granzyme B, that are released into the supernatant during NK killing.

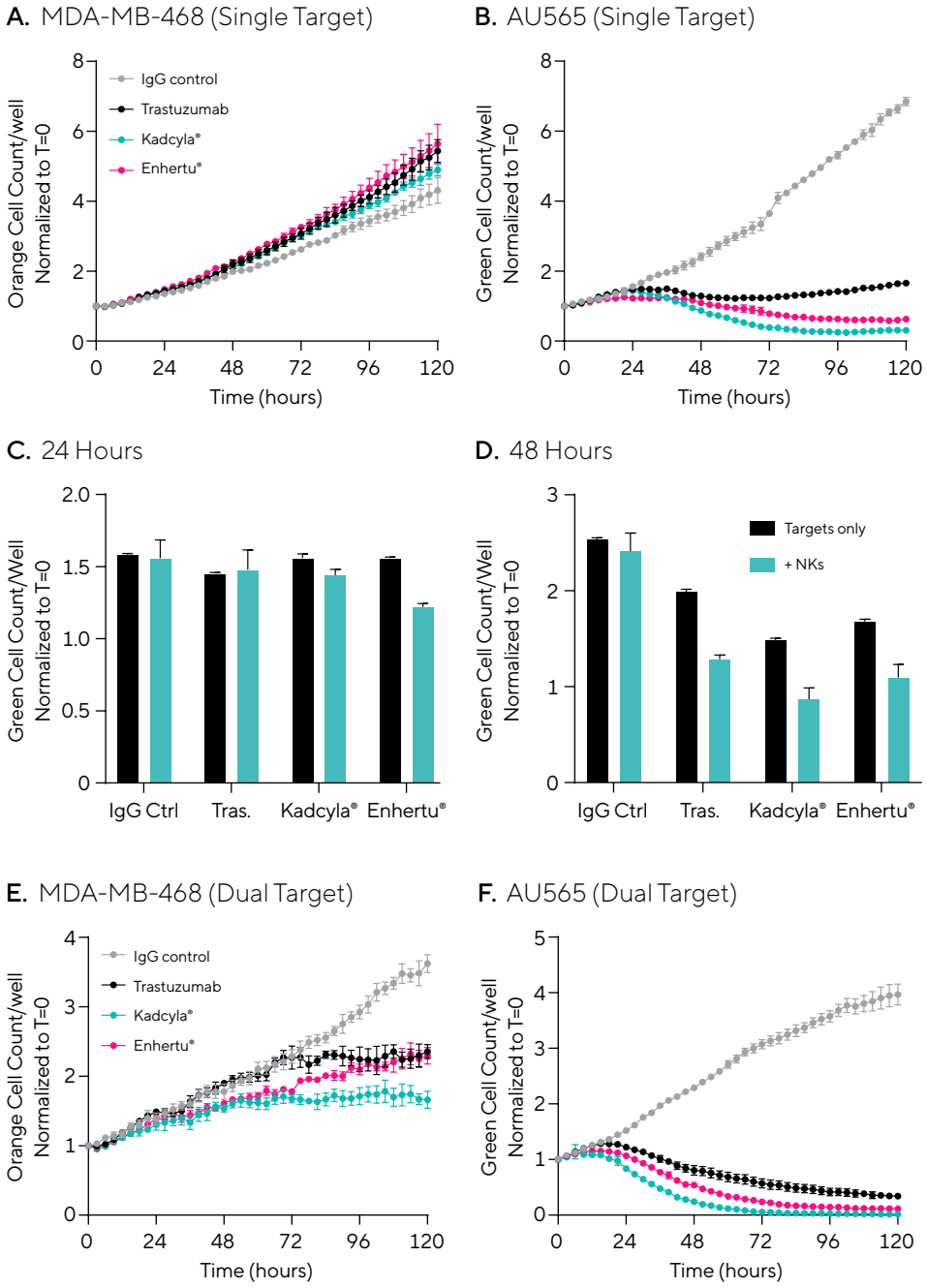
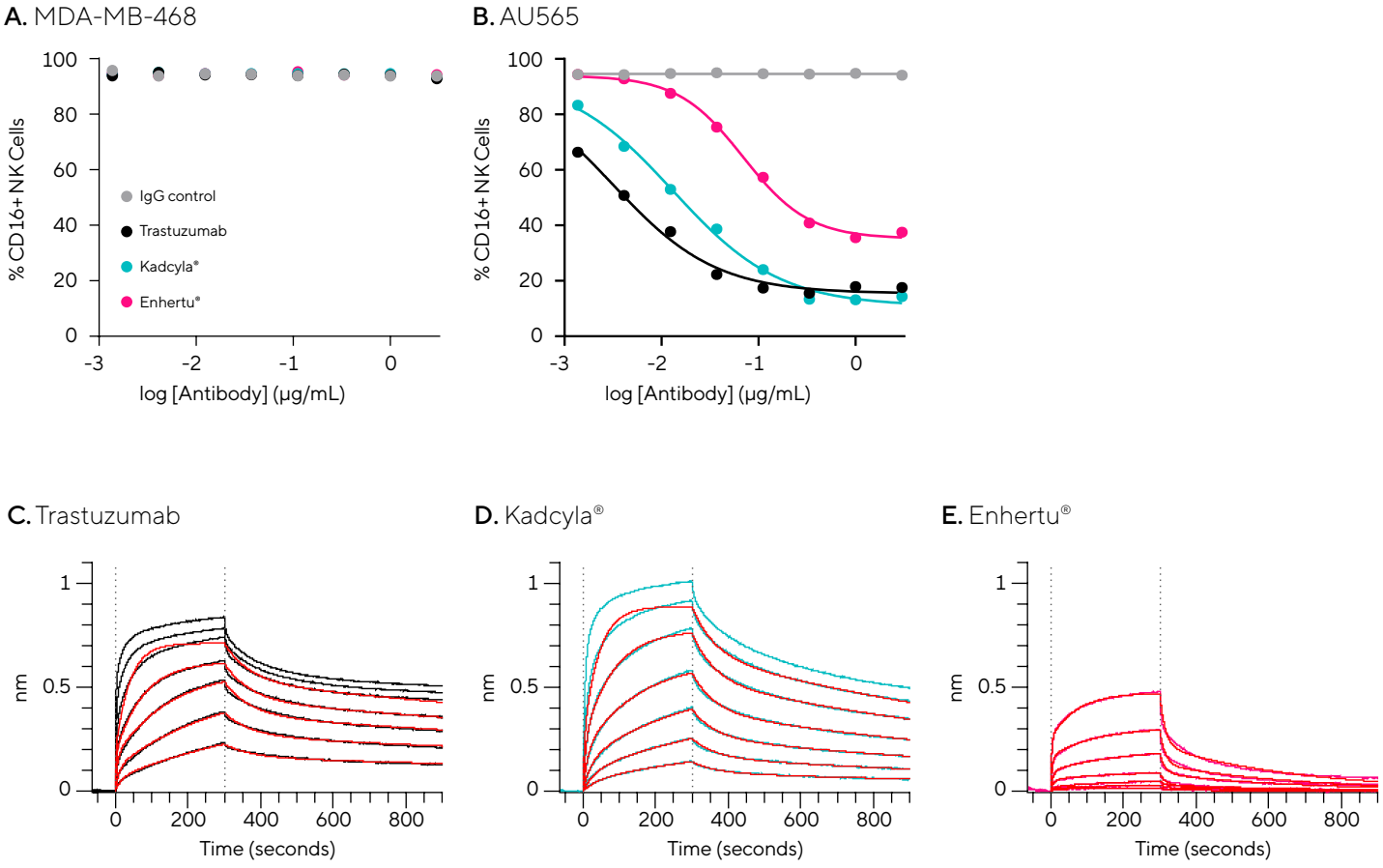


Figure 6. Antibodies induced NK killing of HER2 negative targets when HER2 positive cells were present
(A,B) Incucyte® time course data shows growth of Nuclight Orange MDA-MB-468 and Nuclight Green AU565 cells co-cultured with NK cells and antibody (3 µg/mL) over 5 days. (C,D) Comparison of AU565 cell counts (normalized to T=0) at 24 and 48 hours in the presence and absence of NK cells and antibody (3 µg/mL). (E,F) Incucyte® quantification of cell counts over time in a mixed target ADCC assay containing Nuclight Orange MDA-MB-468, Nuclight Green AU565 and NK cells.

ADCC Activity

To further characterize the ADCC activity of the antibodies, the iQue® NK killing kit was used to measure the marker expression and cytokine release induced during ADCC. MDA-MB-468 or AU565 cells were labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye and seeded in single target ADCC assays with NK cell effectors. The antibody cocktail in the kit was used to label cells, to distinguish the CD3 negative/ CD56 positive NK cells and to determine their CD16 expression. CD16 is the key FcγR associated with ADCC, and we expect its expression on NK cells to decrease with increasing ADCC activity. The results in Figure 7B show a clear decrease in CD16 expression with increasing concentration of all three anti-HER2 antibodies, with the highest potency response observed with Trastuzumab (EC_{50} = 0.0031 µg/mL), then Kadcyla® (EC_{50} = 0.013 µg/mL). Enhertu® displayed the lowest level of ADCC induction, with the lowest potency response (EC_{50} = 0.069 µg/mL) and the highest minima for CD16 expression of 35 ± 1.8%, compared to 15.4 ± 1.9% and 10.8 ± 2.3% for Trastuzumab and Kadcyla, respectively. No impact on CD16 expression was observed with the HER2 negative MDA-MB-468 cells (Figure 7A).



F.

Antibody	K_{D1} (nM)	K_{D2} (nM)	$K_{a1} \times 10^{-5}$ (1/Ms)	$K_{d1} \times 10^{3}$ (1/s)	$K_{a2} \times 10^{-5}$ (1/Ms)	$K_{d2} \times 10^{3}$ (1/Ms)
Trastuzumab	3.37	5.26	1.03	0.35	26.6	14.0
Kadcyla®	15.4	33.3	0.47	0.72	4.79	16.0
Enhertu®	188	229	3.46	65.2	0.11	2.62

Figure 7. Loss of expression of CD16 on NK cells during ADCC correlated with antibody binding affinity to the FcγR
(A, B) CD16 expression on NK cells during ADCC was quantified after 24 hours with either MDA-MB-468 or AU565 target cells using the iQue® NK Killing Kit. (C-E) Biotinylated CD16a was immobilized onto Streptavidin biosensors and binding to Trastuzumab, Kadcyla® and Enhertu® (2-fold serial dilution from 1 – 0.016 µM) was quantified using Octet®. Red line shows 2:1 fit model with the highest concentrations of Trastuzumab and Kadcyla® excluded. (F) Kinetic binding parameters as measured from traces in (C-E).

The Octet® BLI system was used to provide further insight into the impact of the antibodies on CD16. For this assay, biotinylated CD16a was immobilized onto Streptavidin biosensors and the kinetics of binding of a range of concentrations of each antibody was measured. Traces were fit with a 2:1 heterogenous binding model, as indicated by the red lines in Figures 7C-E. The top concentrations of Trastuzumab and Kadcyla® were excluded from the kinetic analysis due to poor fitting with the model, likely due to artifacts from non-specific binding or aggregation at high antibody concentrations. The K_D values indicated highest affinity CD16a binding by the Trastuzumab antibody (K_{D1} = 3.37 nM and K_{D2} = 5.26 nM). Kadcyla® binding affinity was 5 and 6-fold lower with a K_{D1} of 15.4 nM and K_{D2} of 33.3 nM, while the Enhertu® displayed considerably inferior binding with a 55-fold lower K_{D1} (188 nM) and 44-fold lower K_{D2} (229 nM) than Trastuzumab. The higher K_{D1} for Enhertu® is largely due to the much higher rate of dissociation (k_{d1}) compared to the other two antibodies, while the difference in K_{D2} is mostly driven by the vastly lower rate of association (k_{a2}). Overall, the relative order of affinity for CD16 binding for each antibody is in line with the impact on CD16 expression on NK cells measured in the live-cell ADCC assay.

NK Activation and Cytokine Release

Another measurement provided by analysis using the iQue® NK killing kit is quantification of NK cell activation markers CD69 and CD25, expression of which we would expect to increase as the NKs conduct tumor cell killing via ADCC. Figure 8A and B show the level of activation marker expression on CD3-CD56+ NK cells during ADCC stimulated by the anti-HER2 antibodies. Like the CD16 expression data, these results indicated that Trastuzumab and Kadcyla® induced the highest levels of ADCC activity, while Enhertu® demonstrated a lower potency NK activation response.

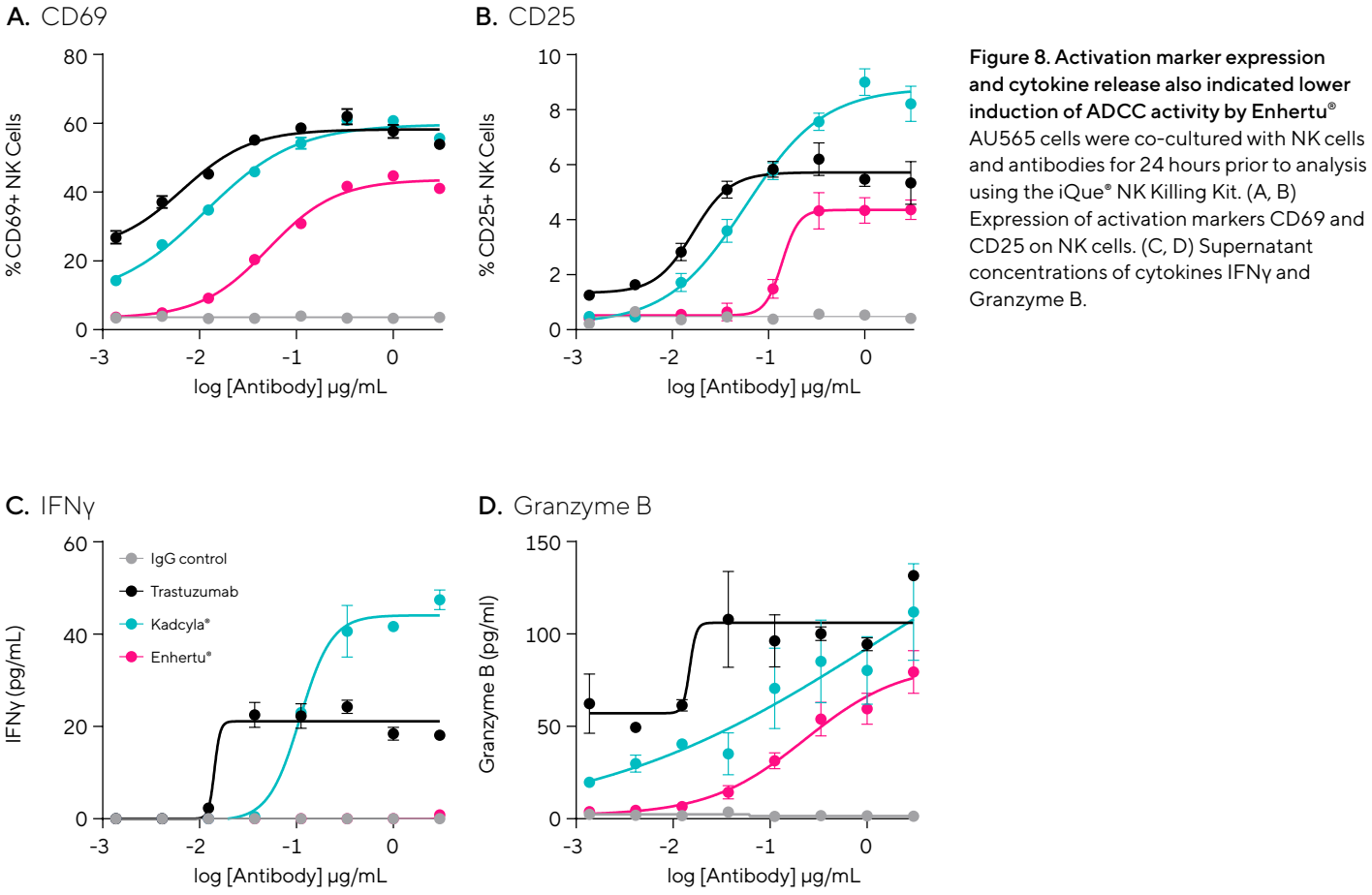


Figure 8. Activation marker expression and cytokine release also indicated lower induction of ADCC activity by Enhertu® AU565 cells were co-cultured with NK cells and antibodies for 24 hours prior to analysis using the iQue® NK Killing Kit. (A, B) Expression of activation markers CD69 and CD25 on NK cells. (C, D) Supernatant concentrations of cytokines IFNγ and Granzyme B.

After the 24-hour incubation of the cells and NK cells, and prior to cell lifting for phenotype analysis, supernatant samples were removed for analysis of IFNγ and Granzyme B cytokines using Qbeads from the iQue® NK Killing Kit. IFNγ is a pro-inflammatory cytokine indicative of NK activation, while Granzyme B is a pro-apoptotic protease directly involved in the NK killing mechanism. These results were also in line with the CD16 and activation marker expression data, supporting the suggestion that higher ADCC is induced by Trastuzumab and Kadcyla® than Enhertu®. The higher ADCC activity observed by Trastuzumab despite lower induction of cell death (Figure 6) implies that the ADCs induce cytotoxicity via the combined action of the ADC payload and ADCC Fc function, particularly by later timepoints in the ADCC assays.

Conclusions

These data show how iQue® Advanced Flow Cytometry and Incucyte® Live-Cell Imaging and Analysis can be combined to build a comprehensive *in vitro* functional profile of ADCs. This can be used to identify differences in the MoAs adopted by ADCs, which can be linked back to structural variations that may improve the efficacy of novel drugs. Accompanying functional studies with kinetic analysis of target and FcγR binding using the Octet® BLI system provided more in-depth characterization of drug candidates. The experiments in this application note illustrate the advantages of these techniques, including:

1. Simple, pre-optimized workflows allow for data generation across multiple platforms with ease, facilitating broader profiling of many different antibody characteristics.
2. Measuring antibody and antigen interactions using both iQue® and Octet® provides complementary results which assess antibody binding both to the antigen as it is found on live cells as well as kinetic analysis of protein interactions.
3. Short-term iQue® ADCC phenotyping can be combined with temporal Incucyte® analysis to allow both the fast changes in ADCC-associated NK marker expression and cytokines, as well as the resulting, longer-term impact on cell death to be observed.
4. All three systems come with inbuilt data analysis software, which streamlines data processing and speeds the time to actionable results.

Together, these advantages create a powerful workflow for ADC characterization that can uncover differences in binding and functional MoAs, which can inform and enhance antibody discovery processes.

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