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Application Note

May 1, 2023

Keywords or phrases:
Antibody-drug Conjugates, ADCs, Monoclonal Antibodies, mAbs, Biosimilars, Trastuzumab, Octet®, iQue®, Kadcyla®, Enhertu®, Antibody Characterization, Biosensors, Antibody-dependent Cellular Cytotoxicity, ADCC

Characterization of Trastuzumab Antibody-Drug Conjugates Using Bio-layer Interferometry and Advanced Flow Cytometry

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Introduction

While monoclonal antibodies (mAbs) remain the target for new biotherapeutics for various diseases, there is an increasing trend toward new modalities such as bispecific antibodies, fusion proteins, peptides, and antibody-drug conjugates (ADCs). New modalities are often a modification of the mAb leading to improved efficacy through different mechanisms of action. ADCs are among the fastest growing class of biologics and are developed as a combination of mAb and highly potent cytotoxic molecules (drugs). The antibody, in addition to its own biological specificity through its paratope, has the function of a targeted transporting tool of the drug to the target cell.[1] The conjugated molecules or particles typically have cytotoxic activity and thus increase the efficiency of the antibody by an additional mechanism, leading to increased death of cells carrying the target antigen.

Conjugation of these drugs to antibodies, however, increases structural complexity which in turn triggers the need for improved characterization methods. Commonly used analytical technologies for characterization of ADCs, such as liquid chromatography, electrophoresis, and mass spectrometry, are useful in enabling the optimization of ADCs but need to be complemented with technologies that can add biophysical and functional information.[2]

In this application note, we demonstrate the use of the Sartorius Octet® Bio-Layer Interferometry (BLI) platform in combination with the iQue® Advanced Flow Cytometry Platform to characterize and compare a biosimilar trastuzumab to the trastuzumab-containing Reference Medicinal Products (RMPs) ADCs Kadcyla® and Enhertu®.

Antibody-Drug Conjugates

An ADC is composed of three main components: a monoclonal or bispecific antibody, a chemical payload, and a linker.[3] In these studies, two ADCs were studied in addition to the mAb drug trastuzumab as the primary antibody for the ADCs. Kadcyla® (trastuzumab emtansine), one of the first ADCs to be approved by the FDA, and Enhertu® (trastuzumab deruxtecan) are derivatives of trastuzumab where the antibody is conjugated to myatansinoid (DM1) and deruxtecan (DXd), respectively. While the antibody in Kadcyla® is linked to DM1 via a stable thioether linker MCC (4-[N-maleimidomethyl] cyclohexane-1-carboxylate), the antibody in Enhertu® is linked to DXd via a stable tetrapeptide linker. The choice of linker can impact the structure of the complex which may in turn affect the binding affinity of the ADC to target antigen. We show here that target binding studies on the Octet® BLI system can be used in conjunction with functional analysis on the iQue® platform to verify the effect of structure on the function of the ADC.

Octet® BLI System in Kinetics Characterization Studies

Analysis of biomolecular interactions on the Octet® BLI system starts with immobilization of a ligand onto the surface of the biosensor. Biosensors come ready to use with standard binding agents such as streptavidin or amine-reactive groups, allowing for irreversible attachment of proteins to create custom biosensor surfaces. Alternatively, capture agents such as anti-human IgG Fc capture or Ni-NTA provide a means for highly specific capture of antibodies or recombinant proteins even from unpurified samples. The most important consideration for biosensor selection is choosing a format that best maintains the structure and activity of the immobilized or captured ligand. Direct immobilization of a target protein to a biosensor can be accomplished by covalent bond to free lysine residues via an Amine Reactive biosensor (AR2G) or via biotin interaction with a Streptavidin biosensor. Direct immobilization results in stable, non-reversible coupling of a molecule to the biosensor with minimal dissociation from the biosensor.[4]

Compared to Streptavidin biosensors, which require the use of a biotinylated ligand, capture biosensors are pre-immobilized with a high affinity capture antibody or protein which binds to the protein ligand via a known motif or tag, enabling favorable orientation on the surface and improved homogeneity. For example, anti-human and anti-mouse IgG Fc capture surfaces bind an antibody ligand via the Fc region, orienting the captured antibody so that the Fv region is readily available for analyte binding. Generally, the choice of the appropriate orientation is dependent on many factors including protein size, valency, activity in solution, propensity to display avidity effects. In these studies, two different assay orientations were evaluated to probe potential ADCs structural or conformational influence upon conjugation of the antibody to the small molecule.

A key consideration in Octet® kinetics data is ligand loading density. The optimal density of immobilized ligand on the biosensor surface is critical to obtaining quality kinetic data. An excess of ligand bound to the biosensor can lead to data artifacts due to crowding, steric hindrance, and aggregation on the surface. Over-saturation of the biosensor may also promote weaker non-specific interactions at higher analyte concentrations, or analyte 'walking' or 'rebinding' effects at lower analyte concentrations. These artifacts may significantly impact observed binding kinetics. If too little ligand is immobilized, however, the signal in the analyte association step may be too low to detect. When performing the loading step in a kinetic assay, slow loading for a longer time is preferable to rapid ligand immobilization. Ideally, the binding curve in the loading step will show a gradual increase in signal and should not be allowed to reach saturation.

Materials and Methods

- **Instrument**
  The Octet® R8 system was used for all binding studies while the iQue® platform was used for functional assays.

- **Biosensors**
  The studies were carried out using Streptavidin (SA) biosensors for the immobilization of biotinylated HER2 protein (ACRObiosystems) and biotinylated Fc Receptors (CD16a V176 and CD64, ACRObiosystems). For target binding, an alternative orientation where the ADC was captured onto the biosensor was performed using anti-human capture (AHC2) biosensors.

- **Buffer System**
  Sartorius 1X Kinetic Buffer (1X KB) was used for sample dilutions and as the assay buffer for all studies.

- **Samples**
  Research-grade trastuzumab (anti-HER2-hIgG1) was purchased from Absolute Antibody while pharmaceutical grade Kadcyla® (trastuzumab emtansine; based on trastuzumab and the small molecule drug emtansine) and Enhertu® (trastuzumab deruxtecan; based on trastuzumab and the small molecule payload (deruxtecan) was purchased from WEP Clinical.

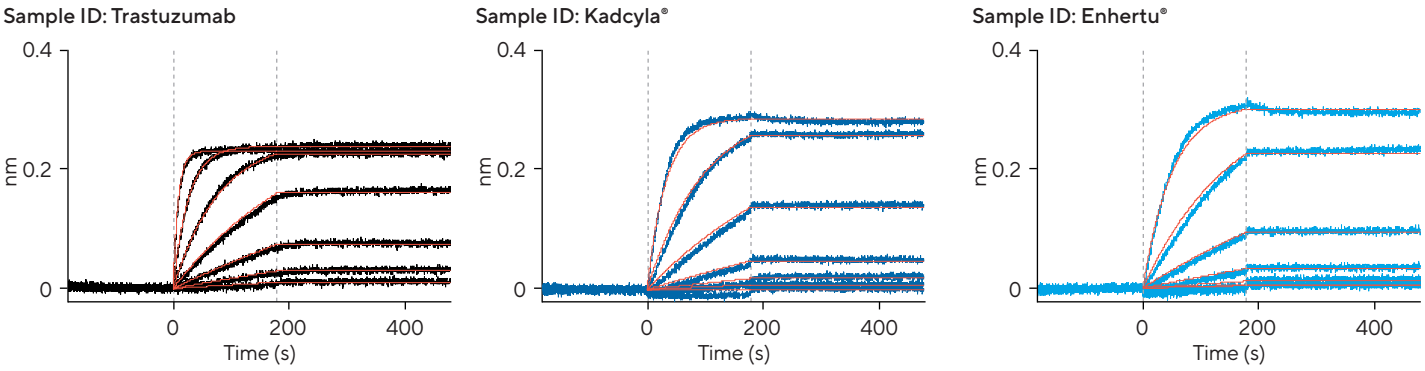
- **ADCC Method**
  Target cells (4 K/well) were labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye and seeded in 96-well flat bottom plates overnight. Target cells were AU565 or MDA-MB-468 cells, which are from high HER2 expressing and HER2 negative breast cancer cell lines, respectively. Test antibodies (IgG control, trastuzumab, Kadcyla® and Enhertu®) were added at a range of concentrations alongside NK cells (20 K/well). 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.

Results and Discussions

ADCs Binding to HER2 Receptor Protein
HER2-directed ADCs bind to HER2 receptor proteins on the cell surface before they are internalized through endocytosis. The drug induces cytotoxic effect after its release when the ADCs linkage is cleaved by lysosomal enzymes.

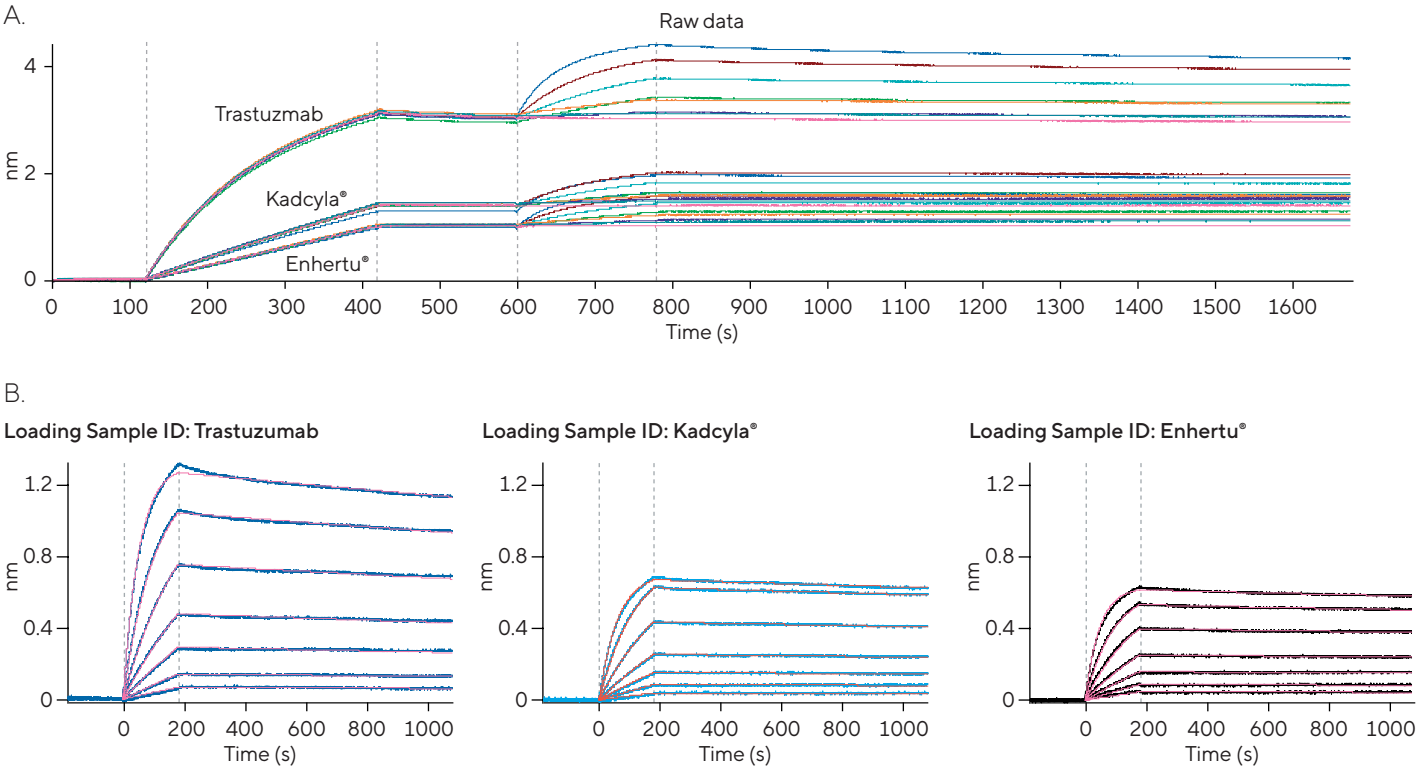
To investigate the effect of structure on the binding affinity of each ADC to the HER2 receptor, we used two assay formats; in the first assay format, biotinylated HER2 protein was immobilized onto a Streptavidin biosensor then dipped into a serially diluted sample of each ADC to monitor dose response binding. In the second format, each ADC was captured onto an anti-human capture (AHC2) biosensor at an equivalent concentration (5 µg/ml). Loaded biosensors were then dipped into a 2-fold diluted sample of HER2 protein (75–1.17 nM). The dose response binding curves for each assay was fit to a 1:1 Langmuir model in the Octet® Data Analysis Studio. The response curves for Kadcyla® and Enhertu® exhibit slightly higher response than is observed for trastuzumab (as would be expected since both have slightly higher molecular weight than trastuzumab). In this assay format, trastuzumab exhibits a faster on-rate (k_a) than the ADCs as can be observed from a steeper association slope (Figure 1). The off-rate (k_d) was observed to be too slow to generate meaningful data and as a result, K_D values were not extracted for this assay format. In the second format, with an equivalent mass used to capture each ADC, the response signals were observed to be different. This is consistent with differences in molar concentrations, given that the ADCs are higher in molecular weight than trastuzumab. As a result, the response from the capture of trastuzumab is higher than the ADCs (Figure 2A); it is also likely that the conjugated drug structurally inhibits the antibody–AHC2 capture site hence the lower response. This in turn results in a higher response from the binding of HER2 to trastuzumab than to the ADCs (Figure 2B). However, a 1:1 Langmuir model fit of the resulting binding curves reveal relatively similar affinity constants with Enhertu® binding slightly tighter than Kadcyla® which in turn binds slightly tighter than trastuzumab suggesting that both ADCs exhibit slight improvement in binding affinity over trastuzumab (Table 1).

Figure 1: *Binding of Trastuzumab, Kadcyla® and Enhertu® to Streptavidin Biosensor Immobilized with Biotinylated HER2 Protein*



Note. HER2 protein was immobilized at 0.325 µg/ml while the ADCs were serially diluted from 75–1.17 nM in 1X KB.

Figure 2: *Binding of HER2 Protein to AHC2 Captured Trastuzumab, Kadcyla® and Enhertu®*



Note. The ADCs were captured at 5 µg/ml while HER2 protein was serially diluted from 75–1.17 nM in 1X KB. Raw curves (A) show differences in immobilization response from the three ADCs attributed to differences in structure that in turn results into differences in HER2 binding response (B).

Table 1: *Affinity Constants and Kinetic Rates of Binding for HER2 Protein to Immobilized ADCs*

	K_D (M)	k_a (1/Ms)	k_d (1/s)
Trastuzumab	4.87E-10	2.60E+05	1.27E-04
Kadcyla®	3.48E-10	2.19E+05	7.64E-05
Enhertu®	2.18E-10	2.83E+05	6.18E-05

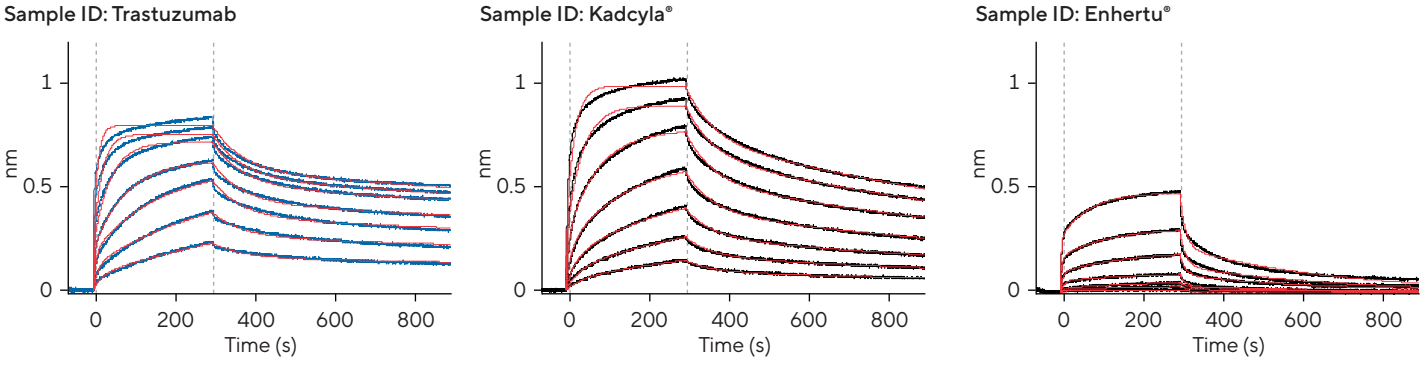
Measuring ADC FcγR-IgG Interactions on the Octet®

Several major mechanisms of action of therapeutic mAbs are initiated via binding to Fc gamma receptors (FcγRs) expressed on the surface of immune effector cells. The ability of therapeutic mAbs and their derivatives to bind FcγRs can greatly impact their safety and efficacy, and as such, efforts to analyze and enhance Fc interactions with FcγRs are an integral part of biotherapeutic development processes. There are three classes of FcγRs based on genetic similarity: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). FcγRI binds monomeric IgG as well as immune complexes. Binding can be affected by genetic polymorphisms of the receptors as well as glycosylation patterns in the Fc region of the antibody. Here we present an analysis of any potential FcR binding differences between the two ADCs relative to trastuzumab. Since the base antibody is the same between the three molecules, we speculate that any differences observed in binding kinetics must be due to potential conformation differences as a result of the conjugated small molecule drug that may impact the receptor binding region.

The first step of an FcR binding analysis on Octet® BLI is the selection of an appropriate biosensor surface. Biosensor selection is premised on the choice of the appropriate molecule between the ADC and receptor to immobilize. Since FcγR-IgG interactions are often relatively low affinity, concentrations of analyte for association may need to be quite high, often in the micromolar range. In contrast, the ligand molecule immobilization step is typically performed at lower concentrations.[5]

To immobilize FcγRs on the surface of the biosensor, Ni-NTA or Streptavidin-based biosensors can be used with His-tagged or biotinylated proteins, respectively. An alternative approach is to capture the antibody-based drug on the biosensor using an Anti-human Fab–CH1 (e.g., FAB2G) biosensor through the Fab region of the ADC. Each of these orientations may have advantages and disadvantages, however since our goal is to probe the effect of steric hindrance or conformation on receptor binding, the receptor immobilization format was used. Biotinylated CD16a V176 and CD64 were each immobilized onto Streptavidin biosensors to achieve low loading levels. To ensure optimal and consistent loading, 0.325 µg/ml of the FcR was used, while the Octet® Discovery Software was set to a threshold of 0.3 nm for each biosensor. Both assays were initially run at 25 °C, however, since the binding of CD64 was observed to exhibit a relatively slow off-rate, the assay was later repeated at 37 °C. The binding of CD16a V176 to the ADCs was fit to a 2:1 fit model while CD64 binding was fit to a 1:1 model. The results indicate that CD16a V176 has higher affinity for Kadcyla® than trastuzumab primarily evidenced through a faster binding rate (Table 2). Interestingly, Enhertu® exhibits significantly weaker CD16a V176 affinity than the other two drugs suggesting that it has the most structurally hindered Fc binding region (Figure 3). On the other hand, CD64 binds to all three drugs with relatively similar affinities, with only slightly higher affinity binding of trastuzumab observed (Figure 4, Table 3).

Figure 3: *Biotinylated CD16a V176 was Immobilized onto Streptavidin Biosensors*



Note. Trastuzumab, Kadcyla® and Enhertu® were kept in solution as the analyte and assessed in a dose response manner with 2-fold serial dilution starting from 1–0.016 µM.

Table 2: *Affinity Constants and Kinetics Rates of Binding for ADCs Binding to Immobilized Biotinylated CD16a V176*

	K_D (M)	K_D2	k_a (1/Ms)	k_a2	k_d (1/s)	k_d2
Trastuzumab	1.379E-08	3.001E-08	4.882E04	4.971E05	6.732E-04	1.492E-02
Kadcyla®	2.526E-09	5.698E-09	9.444E04	2.301E06	2.386E-04	1.311E-02
Enhertu®	1.934E-07	2.295E-07	3.654E05	1.161E04	7.067E-02	2.664E-03

Figure 4: *Biotinylated CD64 was Immobilized onto Streptavidin Biosensors*

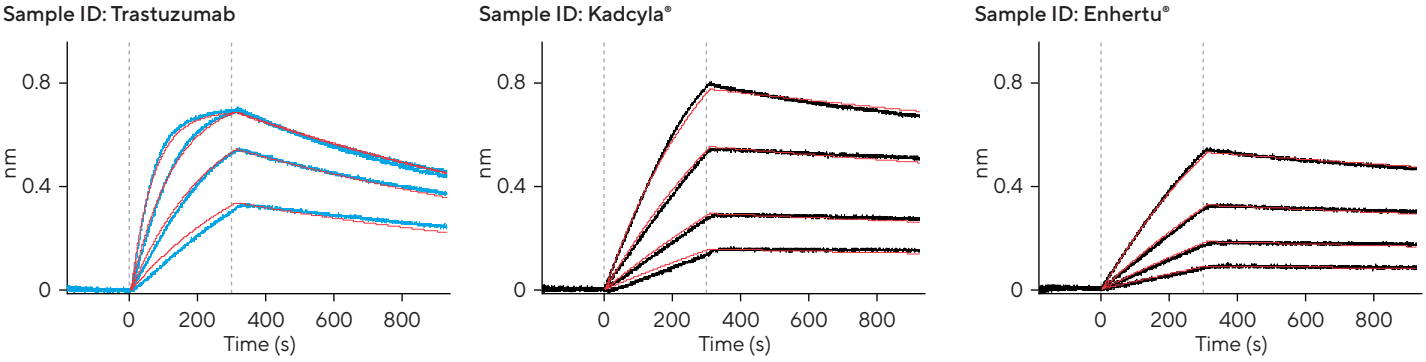


Table 3: *Affinity Constants and Kinetic Rates of ADCs Binding to Immobilized Biotinylated CD64*

	K_D (M)	k_a (1/Ms)	k_d (1/s)
Trastuzumab	8.810E-10	7.649E05	6.739E-04
Kadcyla®	1.265E-09	1.488E05	1.882E-04
Enhertu®	2.161E-09	8.462E04	1.829E-04

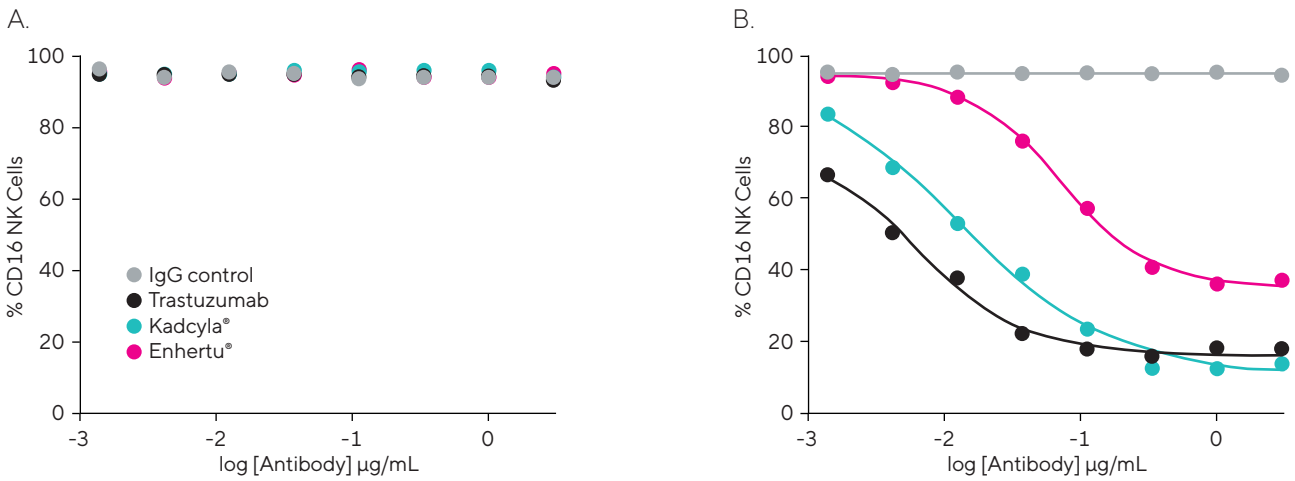
Antibody-Dependent Cellular Cytotoxicity Studies on the iQue® Advanced Flow Cytometer

Antibody-dependent cellular cytotoxicity (ADCC) involves the specific binding of an antibody to target cells and engaging of natural killer (NK) cells through FcR recruitment/binding resulting in cell lysis. CD16 is used as an indicator of ADCC, with an antibody concentration dependent decrease in CD16 expression linked to increased ADCC activity. Expression of markers CD69 and CD25 will increase upon enhanced activation status of NK cells, as observed during tumor killing. Activation status of NK cells can be further reinforced by measuring the release of cytokine IFNγ, while secretion of Granzyme B, a pro-apoptotic protease, is a direct indicator of the killing mechanism adopted by NK cells during ADCC.

To investigate the ADCC function of the two ADCs and trastuzumab to complement Octet® BLI binding interaction data, we used the iQue® Advanced Flow Cytometry Platform with the iQue® Human NK Cell Mediated Killing 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 and Qbeads® for quantification of supernatant cytokines. A gating and analysis template is included with the kit and was imported into iQue® Forecyt software for quantification of cell marker expression (using a standard curve).

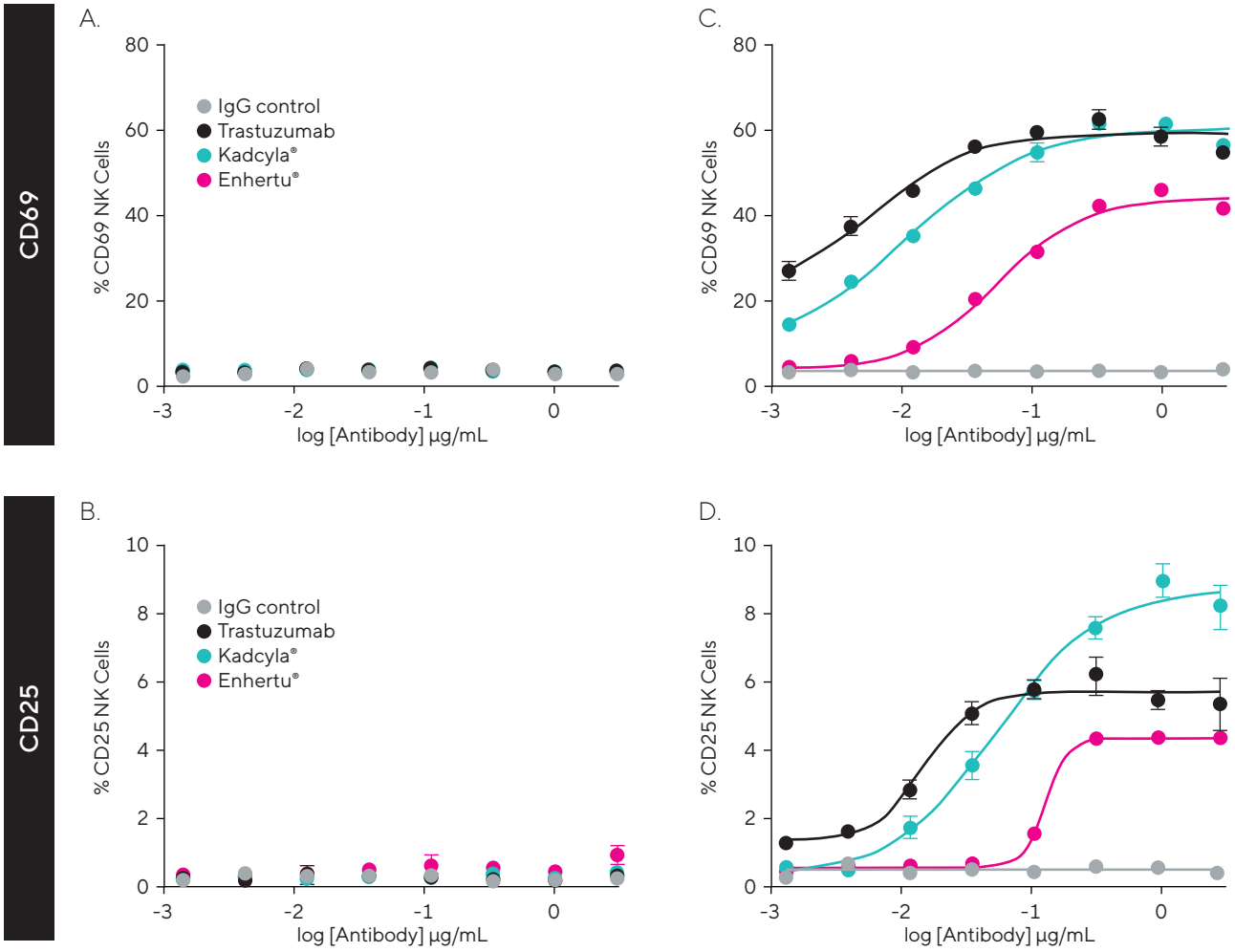
Target cell death via ADCC was confirmed over time (data not shown). Marker expression data shows decreased CD16 expression (Figure 5B) and an increase in activation marker (CD25 and CD69) expression (Figure 6) at 24 h post addition of NK cells. The activation is greater with Kadcyla® and trastuzumab than with Enhertu®. Cytokine release is also greater in the presence of trastuzumab and Kadcyla® (figure not shown) suggesting that these two drugs are more similar in ADCC functional characteristics than Enhertu®.

Figure 5: *CD16 Expression on NK Cells Following 24-Hour Incubation with Test Antibodies and MDA-MB-468 (HER2 Negative) Target Cells and AU565 (High HER2 Expressing) Cells.*



Note. Loss in CD16 expression with increasing ADC concentration was specific to the AU565 cells, with Kadcyla® and trastuzumab exhibiting greater ADCC induction than Enhertu® (Figure B). The HER2 negative cells (Figure A) exhibited no change.

Figure 6: *CD69 and CD25 Expression on NK Cells Following 24-Hour Incubation with Test Antibodies and MDA-MB-468 (HER2 Negative) Target Cells and AU565 (High HER2 Expressing) Cells.*



Note. Expression of CD69 (Figure C) and CD25 (Figure D) activation markers increase with enhanced activation of NK cells during cell death. Figures A and B are the respective negative controls.

Summary

Biophysical characterization of biologics is a critical aspect of drug development. This characterization can be multifaceted depending on the molecule in question but should include at a minimum an understanding of the mechanism of binding of the candidate to its target. For ADCs, it is important to verify that conjugation of the small molecule drug using linkers has not disrupted binding of the primary antibody. The linkers could impact the structure of the ADC, which may in turn affect the candidate drug's binding and activity. While binding characteristics can be rapidly investigated on the label-free Octet® BLI system, a select group of functional activities including ADCC can be evaluated by flow-cytometry using appropriate cell-based kits. This case study uses Kadcyla® and Enhertu®, to highlight the utility of the Octet® BLI system as a tool for the binding characterization of ADCs to the target receptor (HER2 protein) and to FcR molecules (CD16a V176 and CD64). Since the two ADCs are derived from the same backbone mAb (trastuzumab), we expected relatively minor differences in binding to its antigen that were observed. We also show that with the use of different Octet® biosensor chemistries and assay designs we can investigate any potential structural impediments to binding. In the same manner, an appropriate assay orientation can be selected to investigate FcR binding differences. Interestingly, in this case study, while the HER2 protein binds to the ADCs with similar binding affinities, CD64 exhibits a higher affinity for tratuzumab (primarily through a faster on-rate) than either Kadcyla® or Enhertu®. The most significant binding differences were observed with CD16a V176 analysis. Here, we saw significantly weaker interactions between the FcR and Enhertu® than with the other two drugs, with Kadcyla® exhibiting that strongest affinity for CD16a V176. These results are further validated in iQue® cell-based assays using the iQue® Human NK Cell Killing Kit. This showed a smaller decrease in CD16 marker expression with Enhertu® than with either trastuzumab or Kadcyla®; a clear indication of lower ADCC activity with this drug. In addition to ADCC activity, the iQue® also quantifies other critical activation markers and cytokines (CD25, CD69, IFNγ and Granzyme B), which, when combined with Octet® binding affinity data, can provide researchers with useful biophysical and functional information during ADCs development.

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