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Application Note

March 20, 2022

Keywords or phrases:
ADC, Kadcyla®, Live-Cell Analysis, Herceptin, Trastuzumab, DM1, HER2

Pharmacological Characterization of the Antibody Drug Conjugate, Kadcyla®, Using Live-Cell Analysis

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Introduction

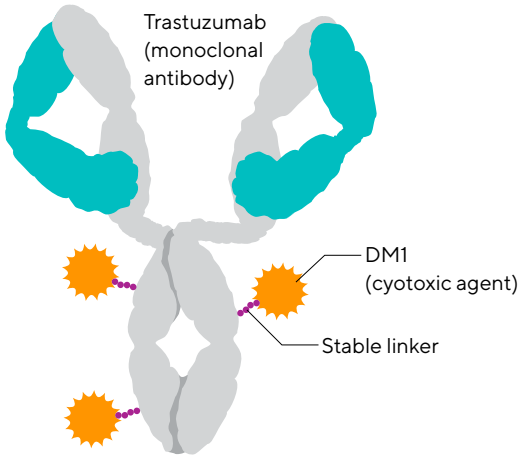
In recent years, increasing development in antibody therapeutics has led to the discovery of new treatments for multiple diseases. Of particular interest in the cancer therapy field is the development of antibody-drug conjugate (ADC) complexes. ADCs exploit the specificity of monoclonal antibodies (mAbs) to achieve targeted cell death by delivering a cytotoxic drug directly to tumor cells expressing a characteristic marker or antigen.

A few examples of clinically relevant ADCs are Kadcyla®, Mylotarg®, Blenrep® and Zynlonta™, which have been used in the treatment of breast, leukemia, multiple myeloma and lymphoma cancers.[1-4] Kadcyla®, clinically used against some forms of breast cancer, is a combination ADC of trastuzumab (Herceptin®), an antibody targeting the human epidermal growth factor receptor 2 (HER2), and emtansine (DM1), a cytotoxic agent (Figure 1).

There are multiple mechanisms of action (MoA) for Kadcyla®: (1) the selective delivery of DM1 to HER2 positive cancer cells, (2) the trastuzumab antibody-mediated inhibition of HER2 signalling, (3) the inhibition of HER2 extracellular domain shedding and (4) the induction of antibody-dependent cell-mediated cytotoxicity (ADCC).[5] The first MoA can be described in two key stages: the effective binding of the antibody to HER2 resident on the target cell surface, and the internalization of the entire molecule releasing cytotoxic DM1 within the cell, leading to targeted cell death. The cytotoxic component of the ADC is completely reliant on the binding affinity and specificity of the antibody component. For this reason, it is important

to analyze the pharmacological effects of Kadcyla®, through binding affinity, internalization, and cytotoxicity studies to characterize its efficacy as a clinical treatment agent. This document exemplifies how the various biological activities of Kadcyla® can be characterized using the Incucyte® Live-Cell Analysis System in combination with multiple functional assessments.

Figure 1: *Kadcyla® Molecule*



Methods

The internalization of Kadcyla®, along with its effects on cell health and cell cycle, were fully characterized using Incucyte® live-cell imaging and analysis; trastuzumab was included for comparison. For all studies Kadcyla® was obtained from Midwinter Solutions and trastuzumab was purchased as a research grade biosimilar (Absolute Antibody).

The internalization of both Kadcyla® and trastuzumab was assessed using the Incucyte® Human Fabfluor-pH Antibody Labeling Dye. This reagent comprises a pre-conjugated antigen-binding fragment (Fab) against the Fc region of IgG, labeled with a pH-sensitive dye and enables easy conjugation to an antibody of interest. Upon binding to the specific epitope, the complex is internalized in the cell where it enters the low pH environment of the lysosome. The change in pH causes the dye to fluoresce, which can be visualized and quantified on the Incucyte® Live-Cell Analysis System. Test antibodies (Kadcyla®, trastuzumab, or an isotype control IgG) and Incucyte® Fabfluor-pH Orange Dye were incubated together (15 min, 37 °C, 12 µg/mL) at a 1:3 molar ratio, which resulted in the formation of an antibody-Fabfluor-pH complex. AU565 (HER2+) or MDA-MB-231 cells (HER2 low) were seeded overnight in 96-well plates before the serially diluted antibody complex (1 in 2) was added to wells (final assay concentration 6 µg/mL to 3 ng/mL) and imaged using the Incucyte® SX5 Live-Cell Analysis System.

Cell health was assessed using AU565 cells stably expressing a nuclear restricted green fluorescent protein (Incucyte® Nuclight Green Lentivirus). Cells were seeded overnight in 96-well plates before Kadcyla® or trastuzumab (6 µg/mL to 3 ng/mL) were added to the well. Isotype control IgG (3 µg/mL) and the cytotoxic drug camptothecin (1 µM) were included as negative and positive controls for the assay. Images were collected by the Incucyte® and Green Nuclear Count used as a readout for cell numbers in the well.

Effects on cell cycle were assessed using AU565 cells stably expressing Incucyte® Cell Cycle Lentivirus. This indicator has been developed to distinguish between cells in the G1 and S | G2 | M cell cycle phase without altering cell function. Cells fluoresce green during S | G2 | M and red or orange (depending on construct used) during G1; cells are colorless during the transition from M to G1 and yellow (expressing green and red, or green and orange, simultaneously) in transition from G1 to S phase. Cells were seeded overnight in 96-well plates before Kadcyla® (6 µg/mL to 3 ng/mL) was added to the wells. An IgG isotype control (3 µg/mL) was added as a negative control.

ADCC was assessed as a co-culture of AU565 Nuclight Green cells and pre-isolated natural killer cells (StemCell Tech) using a 1:5 target to effector cell ratio in the presence of Kadcyla® (6 µg/mL to 3 ng/mL). Incucyte® Annexin V NIR Dye was also added for quantification of target cell apoptosis. Images were collected by Incucyte® and ADCC was quantified as the loss of target cell green nuclei over time. Replicate plates using AU565 wild type cells were set up for further analysis using the iQue® Advanced Flow Cytometry Platform utilizing the iQue® Human NK Cell Killing Kit to assess activation marker expression on the NK cells and secreted cytokine levels (Figure 2).

Characterization of Kadcyla® in HER2 Positive Monocultures

Pharmacological, Kinetic Quantification of Antibody Internalization

As Kadcyla® and trastuzumab share the same epitope recognition, specificity, and base antibody construction, it is expected they should have similar internalization profiles when mixed with HER2 positive target cells. This was investigated using an antibody–Fabfluor-pH sensitive complex, which was incubated with HER2 positive breast carcinoma AU565 cells. The images in Figure 3A show that after incubation (12 h) of cells with the labeled antibody, an orange fluorescence can be seen for both Kadcyla® and trastuzumab treatments but not in IgG isotype control-treated cells. Quantification of the response using orange fluorescence area normalized for cell phase area

demonstrates that Kadcyla® is rapidly internalized in AU565 cells, reaching a plateau in < 6 h for the maximal concentration tested (6 µg/mL, Figure 3B). A similar internalization profile was measured for trastuzumab in AU565 cells. Results show a concentration-related effect producing similar EC_{50} values after 24 h (Kadcyla® 0.38 µg/mL ≡ 2.48 nM, trastuzumab 0.22 µg/mL ≡ 1.42 nM, Figure 3C). Demonstrating the specificity of the response, minimal internalization was detected for either antibody complex when incubated with MDA-MB-231 cells (low HER2 expressing by flow, Figure 3D).

Figure 2: *iQue® Human NK Cell Killing Kit*

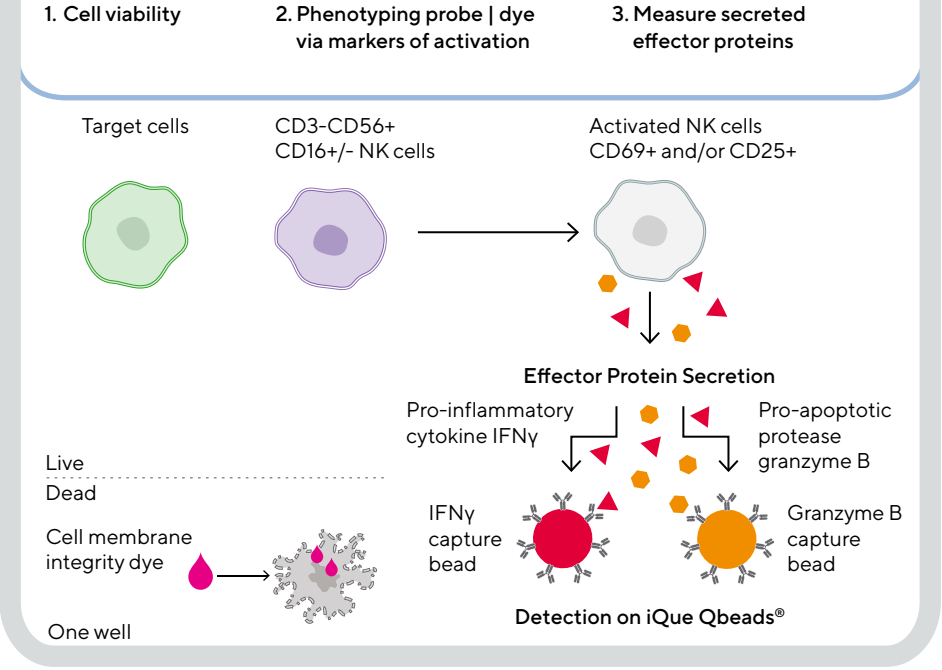
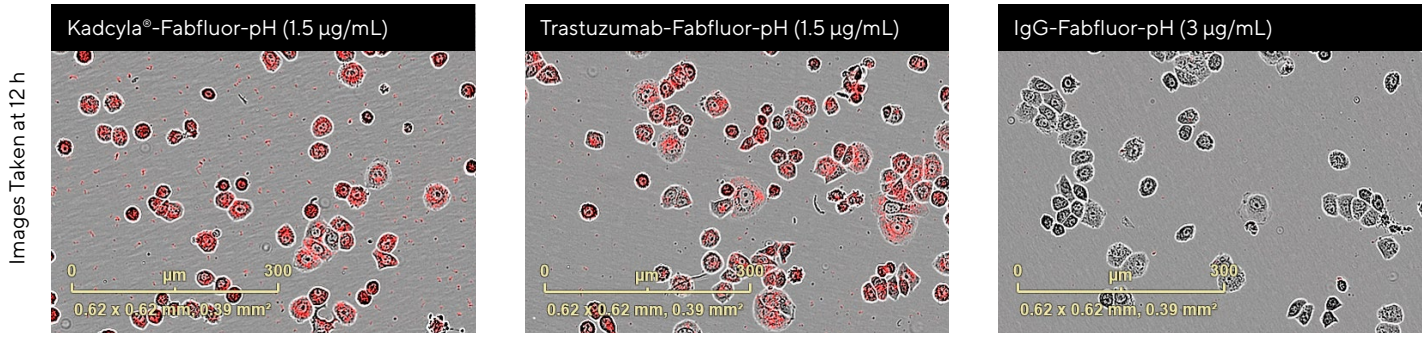
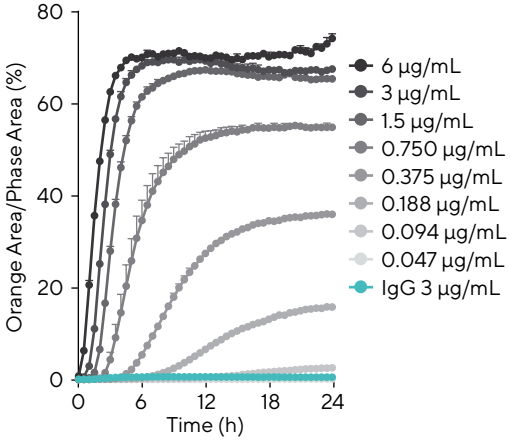


Figure 3: *Internalization Profiles*

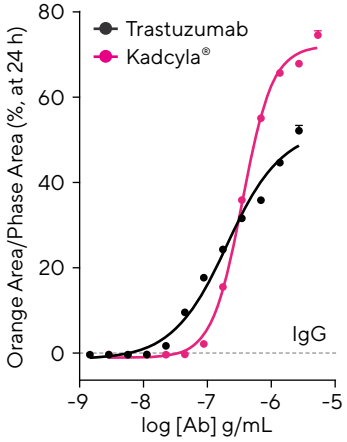
A. Internalization of Kadcyla® and Trastuzumab



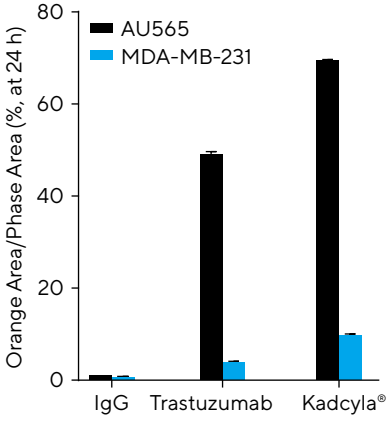
B. Internalization Kinetics



C. Potency



D. Cell Type Dependence



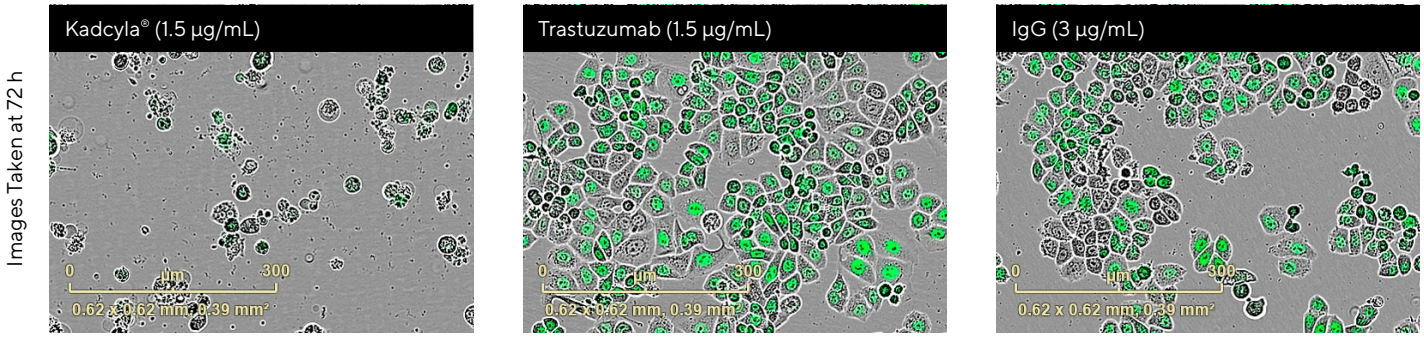
Note. AU565 cells were treated with either Kadcyla®, trastuzumab or isotype IgG complexed to Incucyte® Fabfluor-pH Orange. Images (A) show a fluorescent signal in cells treated with either Kadcyla® or trastuzumab but not IgG. The time course (B) shows a rapid concentration related internalization signal for Kadcyla®, which has a similar potency to trastuzumab (C). No internalization is seen with the isotype control (teal [B], dotted line [C]). The bar graph (D) demonstrates the specificity of the signal with no signal measured in HER2 low MDA-MB-231 cells. All data shown as the mean ± SEM for 3 wells.

Quantification of Cell Health

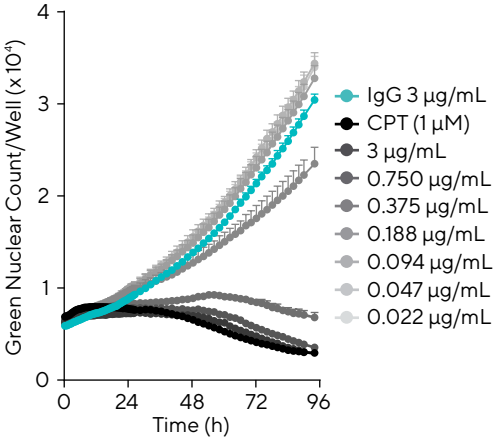
The DM1 payload attached to Kadcyla® is a tubulin inhibitor that inhibits the assembly of microtubles causing disruption of the cell cycle resulting in mitotic arrest and then death. Upon internalization of Kadcyla® into lysosomes, the DM1 is cleaved and released into the cells where it can take effect. To measure this effect, AU565 cells transduced with Incucyte® Nuclight Green Lentivirus to express a nuclear restricted green fluorescent protein were treated with either Kadcyla® or trastuzumab and imaged using Incucyte® Live-Cell Analysis System. The images below (Figure 4A) clearly show that after 72 h, cells treated with Kadcyla® (1.5 µg/mL) appear unhealthy and are no longer proliferating while cells treated with either trastuzumab (1.5 µg/mL) or an isotype control IgG (3 µg/mL) look healthy and continue to proliferate, as expected. Analysis of the images using the integrated Incucyte® Basic Analyzer software enables quantification of the number of green nuclei present in the well as an indicator of cell proliferation and health. Those cells treated with Kadcyla® demonstrate a rapid concentration-dependent decrease in nuclei numbers over time (Figure 4B) indicating poor cell health. No effect on cell number was measured in the presence of trastuzumab (Figure 4C) or the isotype control IgG. Camptothecin (1 µM), a cytotoxic control compound, was included to act as positive control for maximum cell death. The potency of the effect of Kadcyla® on cell health was quantified to be 0.24 µg/mL, similar to the potency of internalization and shows a comparable maximal effect to camptothecin. Similar quantification of cell apoptosis with Kadcyla® was measured using the cell heath indicator Incucyte® Annexin V Dye (0.30 µg/mL, data not shown).

Figure 4: *Effects on proliferation*

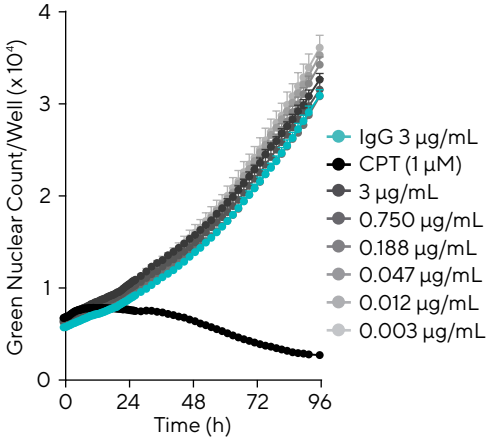
A. Viability of AU565 Nuclight Green Cells



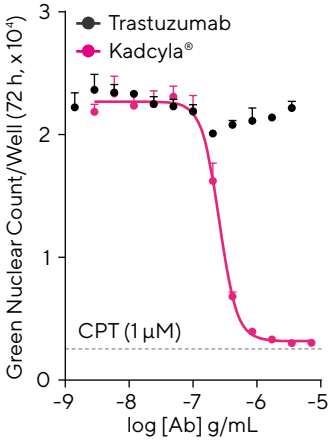
B. Kadcyla®



C. Trastuzumab



D. Cell Viability



Note. Nuclight Green-expressing AU565 cells were treated with either Kadcyla®, trastuzumab, isotype IgG or camptothecin (CPT). Images (A) show the poor health of cells treated with Kadcyla® and lack of effect of trastuzumab or IgG. The time course (B) shows a rapid concentration related decrease in cell numbers after Kadcyla® treatment while trastuzumab (C) has no effect. The isotype control (teal line) has no effect on cell proliferation while CPT (black line) causes maximal cell death. Potency of effect of Kadcyla® (D) is quantified after 72 h. All data shown as the mean ± SEM for 3 wells.

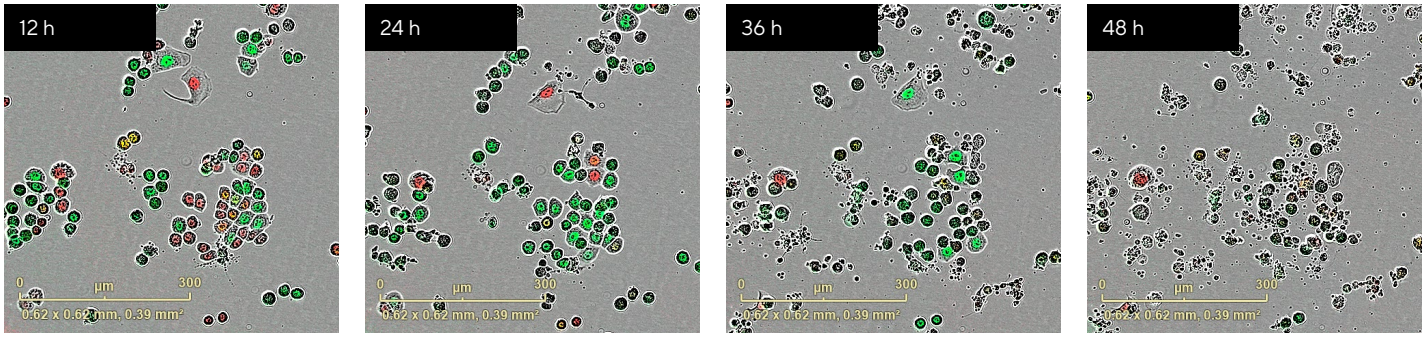
Effect of Kadcyla® on Cell Cycle

The effect of DM1 on cell cycle can be further investigated using cells expressing the Incucyte® Cell Cycle indicator, in combination with the Incucyte® Cell-by-Cell Analysis Software Module. This software enables segmentation of individual cells and quantification of their fluorescence so sub-populations can be identified and tracked.

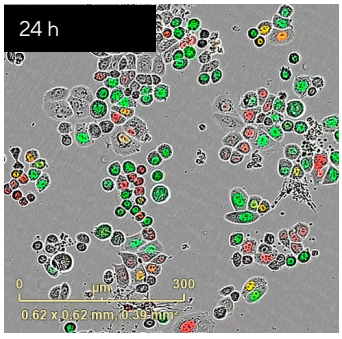
Under normal conditions, AU565 cells expressing the Incucyte® Green | Orange Cell Cycle Lentivirus display a mix of cell cycle phases shown as a mix of green (S | G2 | M, 40 ± 3%), orange (G1, 24 ± 2%), yellow (G1 | S, 15 ± 2%) or colorless (M | G1, 20 ± 4%) cells. Images (Figure 5A) and quantification (Figure 5C) of AU565 cells treated with Kadcyla® (3 µg/mL) show a shift over the first 24 h, with a loss of orange cells (down to 13%) and an increase in colorless cells (up to 27%), indicating a stalling of the cycle in the M | G1 phase. At 36 h, the image shows mainly green (24%) and non-fluorescent cells (52%) which, coupled with their increasingly rounded morphology, indicates that the stall is around the mitosis stage of the cycle. By 48 h, the cells look unhealthy and are dying. There is little effect on the populations with the isotype control IgG (Figure 5B and 5D) or with trastuzumab (data not shown).

Figure 5: *Effect of Kadcyla® on Cell Cycle*

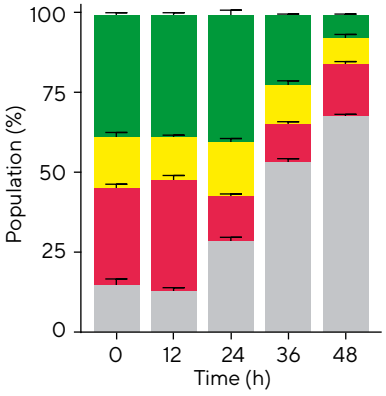
A. Kadcyla® (3 µg/mL)



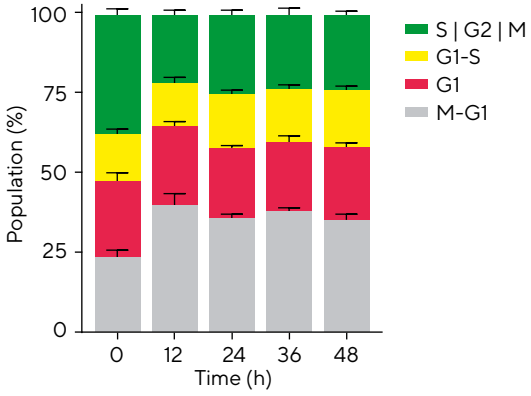
B. IgG (3 µg/mL)



C. Kadcyla® Populations



D. IgG Populations



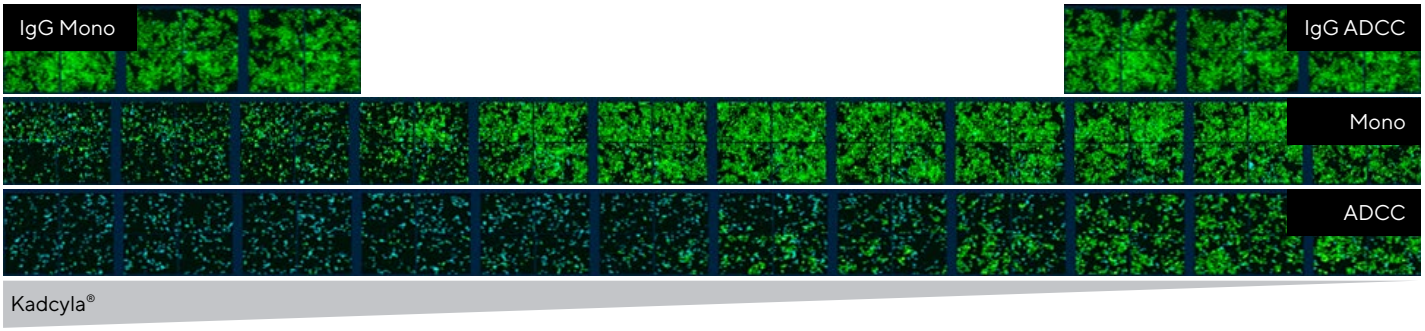
Note. AU565 cells expressing the Incucyte® Cell Cycle indicator were treated with Kadcyla® or isotype IgG. Series of images (A) depicting the changes in cell cycle phases after treatment with Kadcyla® or IgG (3 µg/mL, B). The time course graphs (C and D) display the change in populations post-treatment. All data shown as the mean ± SEM for at least 3 wells.

Effect of Kadcyla® in an ADCC Co-Culture Model

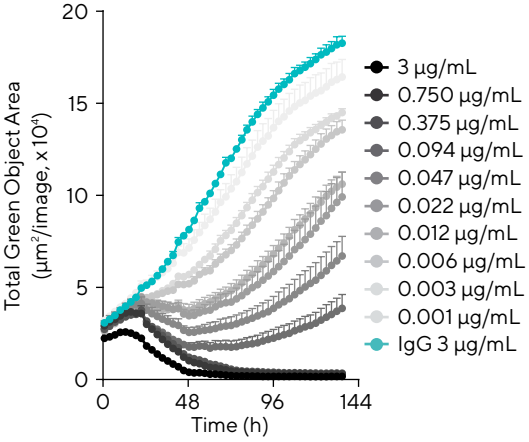
Kadcyla® has been designed to combine the targeting ability of trastuzumab with the delivery of a cytotoxic payload to specific cells. To demonstrate the combined power of Kadcyla®, its ability to kill target cells through conventional ADCC (in combination with direct killing) was compared to its direct cytotoxic effects in monoculture. To quantify this, AU565 cells expressing a nuclear restricted green fluorescent protein (Incucyte® Nuclight Green Lentivirus), were either treated with Kadcyla® in monoculture or as a co-culture with pre-isolated natural killer cells (1:5, target:effector ratio). Incucyte® Annexin V NIR was added to define target cell apoptosis. The images (Figure 6A) display a concentration-related decrease in green fluorescence in both the monoculture and co-culture wells, indicating loss of target cells with Kadcyla® treatment. It is clear from the images that the additional ADCC effect in the presence of NKs is more potent than the direct cytotoxic effect alone. The pseudo-colored blue fluorescence in the images indicates the Annexin V response and shows a concentration-related increase in the presence of Kadcyla®. There is no effect seen with the isotype control IgG in either assay setup. Quantification of the green cell fluorescence in the images confirms the rapid, concentration-related killing of target cells (Figure 6B). When the effect in co-culture is directly compared to the effect in monoculture a clear difference can be seen (Figure 6C). The killing of target cells occurs faster through ADCC and is some 25-fold more potent (EC_{50} direct cytotoxic 0.27 µg/mL compared to 0.011 µg/mL for ADCC). Similar data was reported following quantification of the Annexin V signal (EC_{50} direct cytotoxic 0.38 µg/mL compared to 0.012 µg/mL for ADCC, graphs not shown). The data at 96 h suggests an improved clearance of target cells in the ADCC co-culture model, which is in line with Kadcyla's® high clinical efficacy for tumor cell clearance.

Figure 6: *Enhanced effect of Kadcyla® with ADCC*

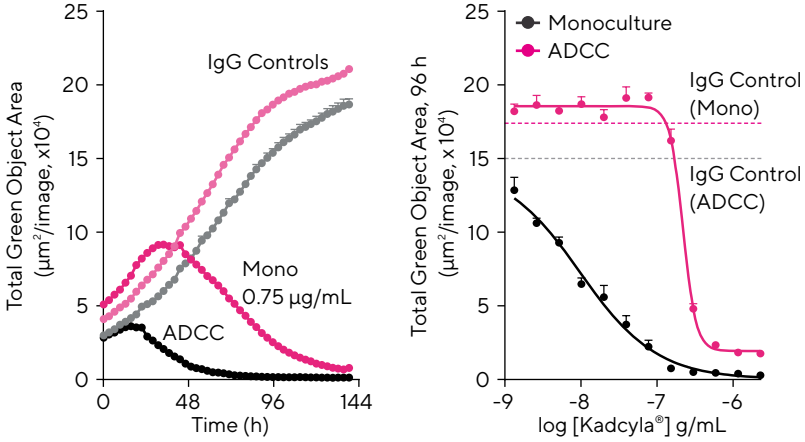
A. Incucyte Images (72 h)



B. ADCC



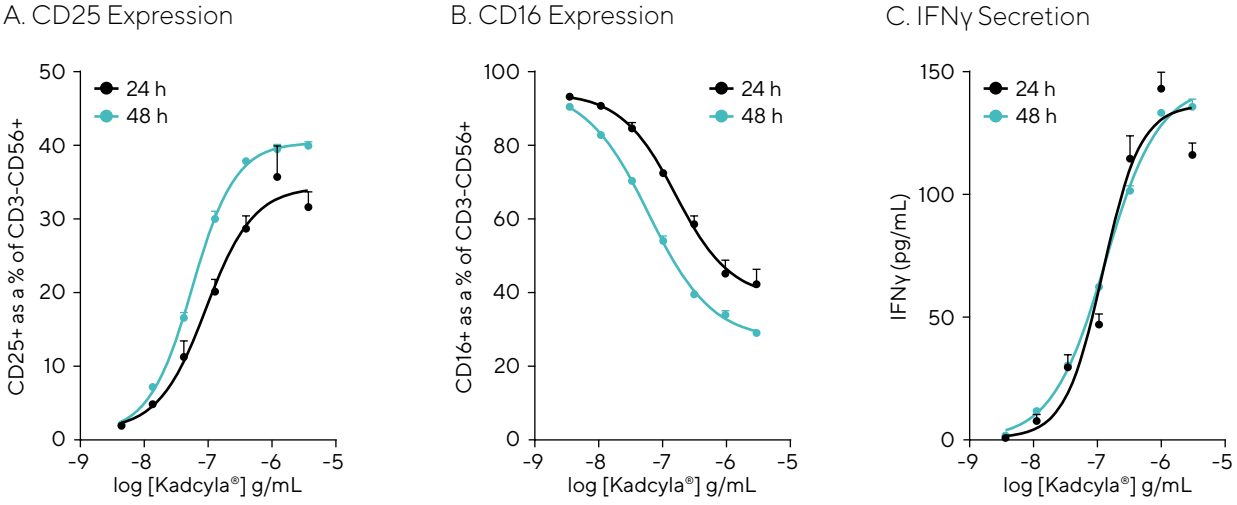
C. ADCC vs. Monoculture



Note. Nuclight Green-expressing AU565 cells were tested as a monoculture or as a co-culture with NK cells in a 1:5 target:effector cell ratio. Cells were treated with either Kadcyla® or isotype IgG in the presence of Incucyte® Annexin V NIR Dye. Images (A) depict the concentration-related decrease in green target cells and increase in Annexin V signal (blue, 72 h) in both assay formats. The effect is enhanced in the presence of NK cells (ADCC). The time course (B) demonstrates the quantification of this ADCC response. When directly compared, there is an enhanced target cell killing effect due to ADCC in the co-culture compared to monoculture (C). The isotype control has no effect on cell death (A, B and C). All data shown as the mean ± SEM for at least 3 wells.

To gain further insight on the biological action of Kadcyla® in the ADCC assay, replicate plates were set up with wild type AU565 cells and NK cells. These plates were analyzed using the iQue® Human NK Cell Killing Kit in combination with the iQue® Advanced Flow Cytometry Platform to enable analysis of the activation state of the NK cells. The co-culture plates were treated with Kadcyla® for either 24 h or 48 h before the contents of the well were lifted and stained with the antibody cocktail provided in the kit. The data shows a clear concentration-dependent increase in CD25 expression on the CD3-CD56+ NK cells induced by Kadcyla® at both 24 h and 48 h (EC_{50} 0.08 µg/mL, 0.05 µg/mL) indicating activation of the NK cells (Figure 7A). Kadcyla® also induced a reduction in the levels of CD16 on the NK cells (Figure 7B). This shedding event was enhanced after 48 h compared to 24 h with a slight shift in mid-point determination (EC_{50} 24 h 0.17 µg/mL, 48 h 0.07 µg/mL). CD16 shedding has been linked to activation of NK cells where it is involved in the detachment of NK and target cells during ADCC and can lead to an increase in serial target engagement.[6] There was also a concentration-dependent increase in IFNγ secretion in response to Kadcyla® treatment (EC_{50} 0.13 µg/mL, Figure 7C) only measured in the co-culture wells indicating a link to NK activation. Taken together the data supports that Kadcyla® treatment of HER2 expressing target cells in the presence of NK cells results in the activation of NK cells to induce the ADCC event.

Figure 7: *Quantification of NK Activation During ADCC*



Note. Co-cultures of AU565 and NK cells in a 1:5 target:effector cell ratio were set up and treated with Kadcyla® for 24 h or 48 h. The iQue® Human NK Cell Killing Kit was used to assess activation of the NK cells. The graphs show the concentration dependent effect of Kadcyla® on CD25 expression on NK cells (A), shedding of CD16 (B) and secreted IFNγ levels (C). All data shown as the mean ± SEM for at least 3 wells.

Conclusion and Summary

The datasets displayed in this document clearly exemplify the detailed characterization of Kadcyla® as an example of an ADC complex using live-cell analysis on the Incucyte® Live-Cell Analysis System. The data demonstrates how the various analysis readouts can be applied in the characterization of this class of biotherapeutics.

The assays enable simple, clear visualization and quantification of various effects of Kadcyla®.

- Kadcyla® binds specifically to HER2 expressing cells and is internalized with similar efficiency and potency as trastuzumab.
- Unlike trastuzumab, once internalized Kadcyla® results in directed cell death through the release of the cytotoxic payload DM1, a potent tubulin inhibitor.
- Kadcyla's® combined effect of ADCC activity and direct killing results in improved efficacy for tumor cell clearance.

The biological analysis of these assays is further enhanced by the combination with flow cytometric analysis to quantify immune effector activation states and cytokine secretion profiles. Utilizing both technologies together they offer great potential for use in the development and research for future ADC therapeutics.

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