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Evaluating Antibody Drug Conjugates (ADCs) *In Vitro* Using 3D Tumor Spheroid Models

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Abstract

Antibody-drug conjugates (ADCs) combine highly targeted delivery of chemotherapeutic drug molecules with an immunotherapeutic intervention for cancer cell killing. A rapid increase in ADC development in recent years has created a need for robust and reliable techniques for assessing novel candidate drugs. The use of 3D advanced cell models can improve the translational ability of these *in vitro* techniques. Here, we present a combined Incucyte® Live-Cell Analysis and iQue® High-Throughput Screening (HTS) by Cytometry workflow for assessing anti-HER2 ADC activity in single and multi-spheroid models. Incucyte® Live-Cell Analysis data demonstrated greater induction of cytotoxicity by anti-HER2 ADCs (Kadcyla® and Enhertu®) compared to the monoclonal antibody (mAb) backbone on which they were based (Trastuzumab). iQue® HTS analysis revealed the unique Enhertu® bystander activity, by allowing the cellular composition of a spheroid co-culture to be measured. These techniques facilitate in-depth analysis of ADC activity and allow mechanistic differences between ADCs to be unpicked.

Introduction

ADCs are a unity between highly specific mAbs and cytotoxic chemotherapeutic drugs. This combination creates a powerful therapeutic for the treatment of several types of cancer: both solid tumors and hematological malignancies. Over the last few years, research and development of ADCs has accelerated, as evidenced by a 35% increase in clinical trials and a 90% increase in phase 1 clinical trials investigating ADCs in 2022 compared to in 2021.[1] The two main targets for ADC development at this time, HER2 and TROP2, are overexpressed on breast cancers and comprise 20% of all ADC studies.[1]

ADCs have three main components: the targeting mAb, the cytotoxic payload, and a stable linker between them. Each element adds an extra layer of tumor targeting specificity to the ADC, on top of the highly specific delivery of the payload to the tumor cell following mAb binding. For example, the linker is highly stable prior to internalization of the antibody into the target cell, which ensures the payload is not released elsewhere in the body. ADC linkers can be categorized into cleavable or non-cleavable types and are either severed by enzymatic activity of proteases or are degraded in the acidic lysosome.[2] Another factor contributing to ADC specificity is the mechanism of action (MoA) of the cytotoxic payload. Since these are typically chemotherapeutic drugs, their MoAs act preferentially towards rapidly proliferating cancer cells, for example by inhibiting microtubule polymerization or inducing DNA damage.[3-5]

Recent years have seen a shift in the choice of *in vitro* models utilized for oncology research. The 2D cell monolayer has long been used due to its simplicity, cost-effectiveness, and scalability, however it can lack some critical features of the 3D tumor microenvironment (TME). For this reason, many researchers now use 3D models, such as spheroids or organoids, to assess drug effects *in vitro*.

These models provide a much closer reflection of the TME, with more complex cell-cell interactions and the option to include extracellular matrix (ECM) proteins. The tumor cells also grow in a more layered structure, with the outer layer undergoing fast proliferation and interaction with the TME, followed by a middle quiescent layer and an inner necrotic core.[6,7] This facilitates a more representative modelling of drug behavior *in vivo*, for example by allowing the comparison of tumor penetration of candidate drugs.

Traditional methods for measuring drug response in 3D models are often limited because they:

1. Require multiple workflows for quantification of different parameters, often using multiple instruments
2. Involve lengthy, time-consuming protocols, which require multiple rounds of optimization, fixation, and repetitive washes
3. Necessitate correlation of data from several different assays for each treatment condition, increasing the risk of data variability
4. Provide bulk measurement of cytotoxicity without a deeper investigation into spheroid cell type composition.

In this application note, we present a combined Incucyte® Live-Cell Analysis and iQue® HTS by Cytometry approach for quantifying the function of two Trastuzumab (anti-HER2) based ADCs: Trastuzumab emtansine (Kadcyla®) and Trastuzumab deruxtecan (Enhertu®), using both single and multi-spheroid 3D models. The Incucyte® Live-Cell Analysis System captures temporal information on mAb internalization and target cell death by quantifying spheroid size and fluorescence intensity. The iQue® 3 Cytometry Instrument measures target cell counts and viability after spheroid dissociation, enabling a closer look at different populations within a co-culture.

Cell Culture and Maintenance

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 from InvivoGen was used as an isotype control.

Cell Lines

BT474 cells (from a breast cancer cell line) or SKOV-3 cells (from an ovarian cancer cell line) were used as high HER2 expressing cell types. MDA-MB-231 cells, from a breast cancer cell line, express low levels of HER2 and were used as a negative control throughout.

Antibody Internalization

Target cells were seeded in an Ultra-Low Attachment (ULA) 96-well microplate (Corning® 7007) for 72 hours to promote

spheroid formation. Antibodies, labeled with Incucyte® Human Fabfluor-pH Orange Antibody Labeling Dye (Sartorius 4812), were then added to spheroids. Phase and fluorescence images (10X) were captured every 15 minutes using the Incucyte® Live-Cell Analysis System. Internalization was quantified as an increase in Total Orange Area (µm² /image).

Single Spheroid ADC Cytotoxicity
Target cells (transduced with Incucyte® Cytolight Green Lentivirus for stable expression of nuclear restricted GFP) were seeded in a ULA plate for 72 hours to promote spheroid formation. Phase and fluorescence images (10X) were captured using the Incucyte® Live-Cell Analysis System on a 3-hour repeating scan schedule for 9 days. Cell death was quantified as a reduction in spheroid area.

Multi-spheroid ADC Cytotoxicity
A flat bottom 96-well plate (Corning® 3595) was coated with a layer of Matrigel® (5 mg/mL) as per a validated Incucyte® protocol (Incucyte® Multi-Spheroid Assay for the Quantification of Multi-Spheroid Growth and Health on a Layer of Matrigel®).[8] Incucyte® Nuclight Green Lentivirus labeled target cells (2 K/well) were seeded on top and incubated for 72 hours to promote multi-spheroid formation. Antibodies were added (2 µg/mL) and cells were monitored using the Incucyte® Live-Cell Analysis System via a repeating scan schedule (every 3 hours) for 10 days. Images were quantified for a Brightfield Green Integrated Intensity (GCU x µm²/image) over time as an indicator of cell death.

Single Spheroid Antibody-Dependent Cellular Cytotoxicity (ADCC)
Target cells labeled with Incucyte® Cytolight or Nuclight Green Lentivirus were seeded in a ULA plate (4 K/well) for 72 hours to promote spheroid formation. As MDA-MB-231 cells require Matrigel® to form tight spheroids, Matrigel® was added to culture media at a final concentration of 2%. Matrigel® was added to both target cell types to ensure differences in diffusion didn't impact ADCC. Test antibodies were added at a range of concentrations. Natural killer (NK) cells were added (16 K/well) alongside IL-12 (10 ng/mL) to improve their longevity. Phase and fluorescence images (4X objective) were captured using the Incucyte® Live-Cell Analysis System on a 3-hour repeating scan schedule for 10 days. Cell death was quantified as a reduction in spheroid green mean intensity.

Single Spheroid Bystander Activity
Single spheroids were formed by mixing high HER2 expressing BT474 cells (labeled with Incucyte® Cytolight Green Lentivirus) and unlabeled, low HER2 expressing MDA-MB-231 cells at a 2:3 ratio. Antibodies were added after 72 hours and images were captured on a repeat scanning schedule (every 3 hours) using the Incucyte® Live-Cell Analysis System. On day 8, the spheroids were dissociated (using a previously validated protocol, T Cell Killing in Single Spheroids) and cells were labeled using the iQue® 3 Cell Membrane Integrity (R/Red) Dye.[9] This enabled live cell counts of each cell type to be quantified using the iQue® HTS by Cytometry Platform.

🌐 Incucyte® Multi-Spheroid Assay for the Quantification of Multi-Spheroid Growth and Health on a Layer of Matrigel®

Results

The described experiments have utilized both single and multi-spheroid tumor models to profile the function of three antibodies: Trastuzumab, Kadcyla®, and Enhertu®. Trastuzumab is an anti-HER2 mAb therapeutic, whilst Kadcyla® and Enhertu® are ADCs which contain a Trastuzumab backbone. Due to this commonality in structure, we expect the antibodies to share many functional capabilities, however, they do also include unique structural features which may distinguish their anti-tumor function (Figure 1). One difference is in the cytotoxic payload included on each ADC, with Kadcyla® including chemotherapy drug emtansine (DM1) whilst Enhertu® is linked to the payload deruxtecan (Dxd). Each ADC also differs in the number and positions of the payload conjugation sites, with DM1 binding to lysine residues in the Trastuzumab backbone in Kadcyla® at an average of 3.5 conjugations per antibody, whilst binding of Dxd in Enhertu® is via thioether bonds with cysteine residues, with 8 conjugations per molecule. [10,11] The cytotoxic payload in the ADCs also has different linker chemistries, with Kadcyla® containing a non-cleavable amine-to-sulfhydryl crosslinker (SMCC) and Enhertu® containing a valine-citrulline cleavable linker.[12]

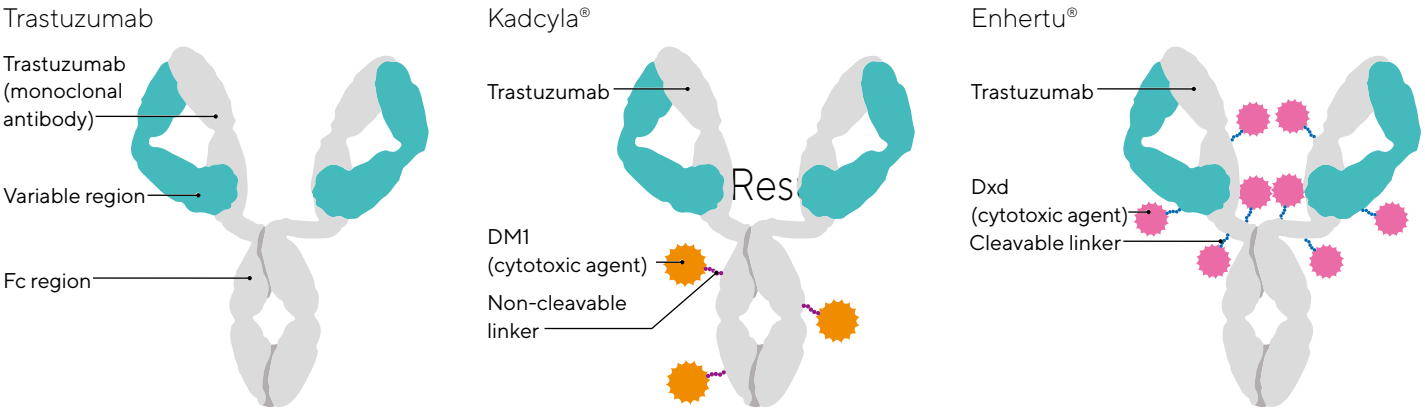

Figure 1. Structures of anti-HER2 antibodies and ADCs
Trastuzumab is an anti-HER2-IgG1 mAb. Kadcyla® is a modified version of Trastuzumab which includes non-cleavable linkers to the chemotherapy drug emtansine (DM1). Enhertu® is also an adapted version of Trastuzumab, with cleavable linkers to attach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

Antibody Internalization

Efficient antibody internalization is critical for the delivery of the ADC cytotoxic payload into target cells. Initial experiments aimed to quantify the relative level of internalization of each of the antibodies into single spheroids formed from high HER2 expressing cells (BT474s). The antibodies were pre-labeled with Incucyte® Human Fabfluor-pH Orange Antibody Labeling Dye, which fluoresces upon internalization into the acidic lysosomal and endosomal pathways. Incucyte® images were captured over time and an increase in internalization was quantified as an increase in spheroid orange mean intensity.

Phase and fluorescence images (Figure 2A) clearly show that fluorescence, and therefore internalization, in the presence of all three anti-HER2 antibodies was much higher than the IgG control. The time course graph (Figure 2B) provides a closer examination of the differences in internalization between the anti-HER2 antibodies and indicates considerably higher internalization of the two ADCs (Kadcyla® and Enhertu®) compared to the backbone antibody (Trastuzumab), with endpoint Orange Calibrated Units (OCU) intensity values of 31.8, 27.0 and 17.7, respectively. These data are in line with results generated previously for internalization of these antibodies in a 2D monolayer assay format (data available in Application Note: Cross-Platform Analysis of the Binding and Function of Anti-HER2 Antibody-Drug Conjugates (ADCs)).[13]

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

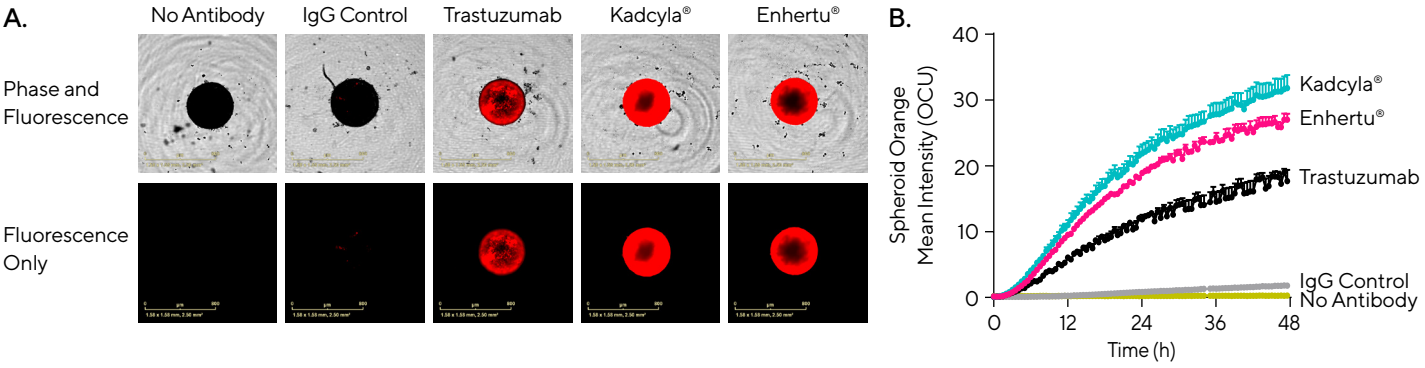

Figure 2. Internalization of ADCs into BT474 spheroids is greater than internalization of Trastuzumab.
BT474 cells (4 K/well) were seeded into ultra-low attachment (ULA) plates for 72 hours to promote spheroid formation. Antibodies were labeled with Incucyte® Human Fabfluor-pH Orange Antibody Labeling Dye then added to spheroids (n=3). Phase and fluorescence images (10X objective) were captured every 15 minutes using the Incucyte® Live-Cell Analysis System. (A) Representative Incucyte® images of single spheroids taken 48 hours after antibody addition. (B) Internalization was quantified as an increase in Spheroid Orange Mean Intensity (OCU) and plotted over time.

Single Spheroid ADC Cytotoxicity

Following internalization of ADCs into target antigen-expressing cells, the chemotherapeutic drug payload is released, either through proteolytic degradation of the linker or by its metabolism in the acidic lysosome. This means the drug molecule can exert a cytotoxic effect, specifically towards the target cell. This reduces off-target cytotoxicity compared to administration of the chemotherapeutic drug alone. The mechanism of this cytotoxic effect can vary depending on the drug used. For example, the DM1 in Kadcyla® acts to inhibit microtubule assembly, whilst the Dxd payload in Enhertu® disrupts DNA replication through inhibition of topoisomerase I.[14,15] To explore this cytotoxicity *in vitro*, a range of concentrations of antibody were tested with a monoculture of BT474 cells in a 3D single spheroid format. Reduction in spheroid size over time was quantified using the Incucyte® Live-Cell Analysis System and was used as a measure of cell death.

Both ADCs induced a concentration dependent reduction in spheroid area over time, indicating induction of cytotoxicity (Figures 3C and 3D). The effect of Kadcyla® varied much more across the concentration range tested, with average spheroid size at endpoint of 0.92×10^5 µm² at the highest concentration and 1.91×10^5 µm² at the lowest concentration. Contrastingly, the sensitivity to varying concentrations of Enhertu® was much lower with the spheroid area ranging only from 1.15×10^5 µm² ¬(25 µg/mL) to 1.68×10^5 µm² (0.39 µg/mL). Only the highest concentration of Trastuzumab (25 µg/mL) had an impact on spheroid size (Figure 3B). Clearly this is not down to the action of chemotherapeutic drugs and instead may be the result of Trastuzumab's other mechanisms of action, including inhibition of cell signaling, for example in the PI3K-AKT pathway.[17] Maintenance of this activity in the Kadcyla® ADC may explain the much larger reduction in spheroid area at the highest Kadcyla® concentration compared to the second highest concentration tested (13 µg/mL). Across the concentration range tested, the IgG control had no effect on spheroid size (Figure 3A).

Comparing the data in Figures 2 and 3, there is a clear difference in the time taken for internalization compared to the cytotoxic response. Internalization begins rapidly within the first 24 hours and starts to plateau between 24-48 hours. In contrast, with the cytotoxic effect, there is minimal difference between the conditions until after the 48 hours when the concentration dependent response starts to materialize. This time course could reflect the MoA of the ADCs in that they need to be internalized into the target cells first before the cytotoxic payload can be cleaved and start to induce cytotoxicity.

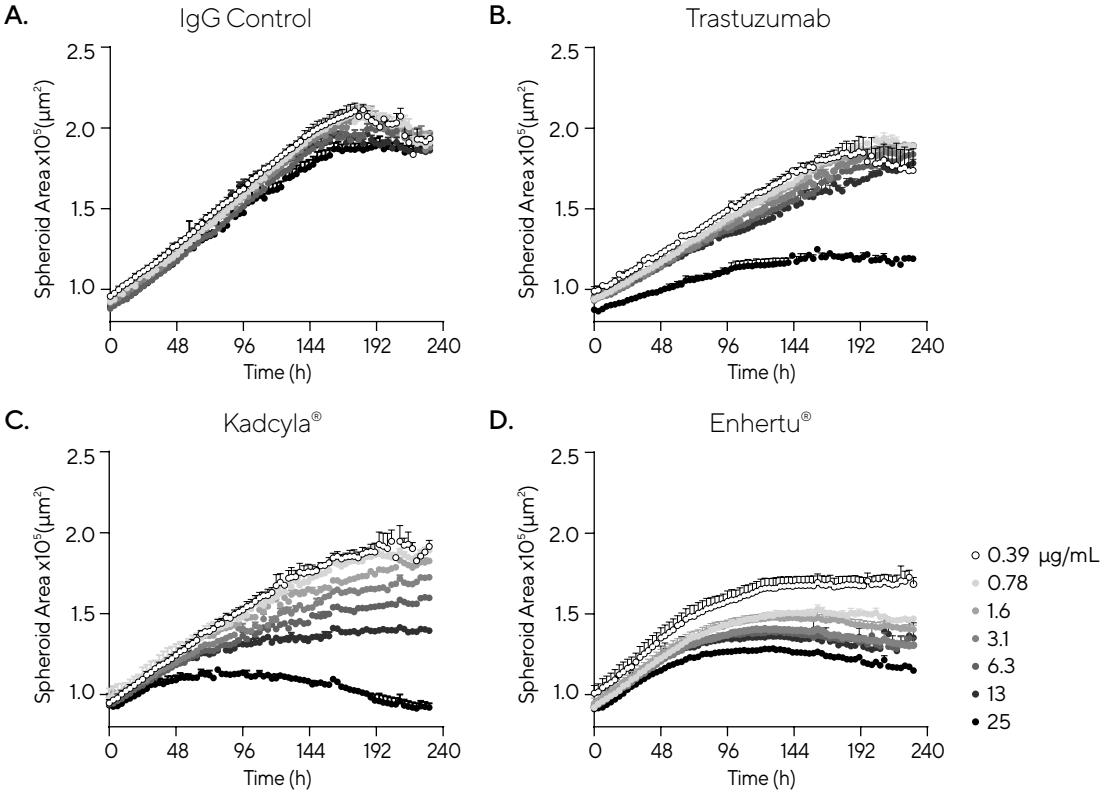

Figure 3. ADCs induced a concentration dependent increase in cytotoxicity of BT474 cells in a 3D single spheroid model.
BT474 cells (1 K/well) were seeded in ULA plates for 72 hours to promote spheroid formation. Antibodies were added to spheroids at a range of concentrations (n=3). Incucyte® images were captured on a 3-hour repeating scan schedule for 9 days. Time-course data for change in Largest Object Area (µm²) for spheroids incubated with (A) IgG control, (B) Trastuzumab, (C) Kadcyla® and (D) Enhertu®.

Multi-spheroid ADC Cytotoxicity

Multi-spheroid tumour models can also be utilized to test the activity of ADCs in conditions more representative of the *in vivo* tumor environment. Adding further 3D complexity with increased cellular and ECM interactions, this model may bring us closer to a translational model for oncology drug research. This involved coating plates with a layer of Matrigel® before seeding the target cells (high HER2 expressing SKOV-3 or low HER2 expressing MDA-MB-231 cells) on top. After 72 hours of multi-spheroid formation, antibodies were added at a single concentration (2 µg/mL). Images were captured using the Incucyte® Live-Cell Analysis System with the multi-spheroid scan type and analysis.

The images in Figure 4A show the difference between SKOV-3 spheroids in the presence of each antibody on day 9. Compared to the IgG control, there was a visible reduction in the number and size of spheroids in the presence of each of the antibody treatments. A mask was applied which allowed quantification of death as a loss of fluorescence intensity within the spheroid brightfield object. Figure 4B shows that the ADCs induced a high level of death in the SKOV-3 spheroids, with a 63.9 and 73.7 % reduction in spheroid intensity at assay endpoint compared to the IgG control for Kadcyla® and Enhertu®, respectively. Trastuzumab also induced considerable cell death, but at a slightly reduced level compared to the ADCs (49.7% reduction from IgG control). Overall, the cell death at this concentration of antibody is considerably higher than was seen in the single spheroid model, which is likely due to differences in cell type and spheroid size. Figure 4C shows that this is an antigen positive cell type specific effect as there was no impact on HER2-low MDA-MB-231 spheroids.

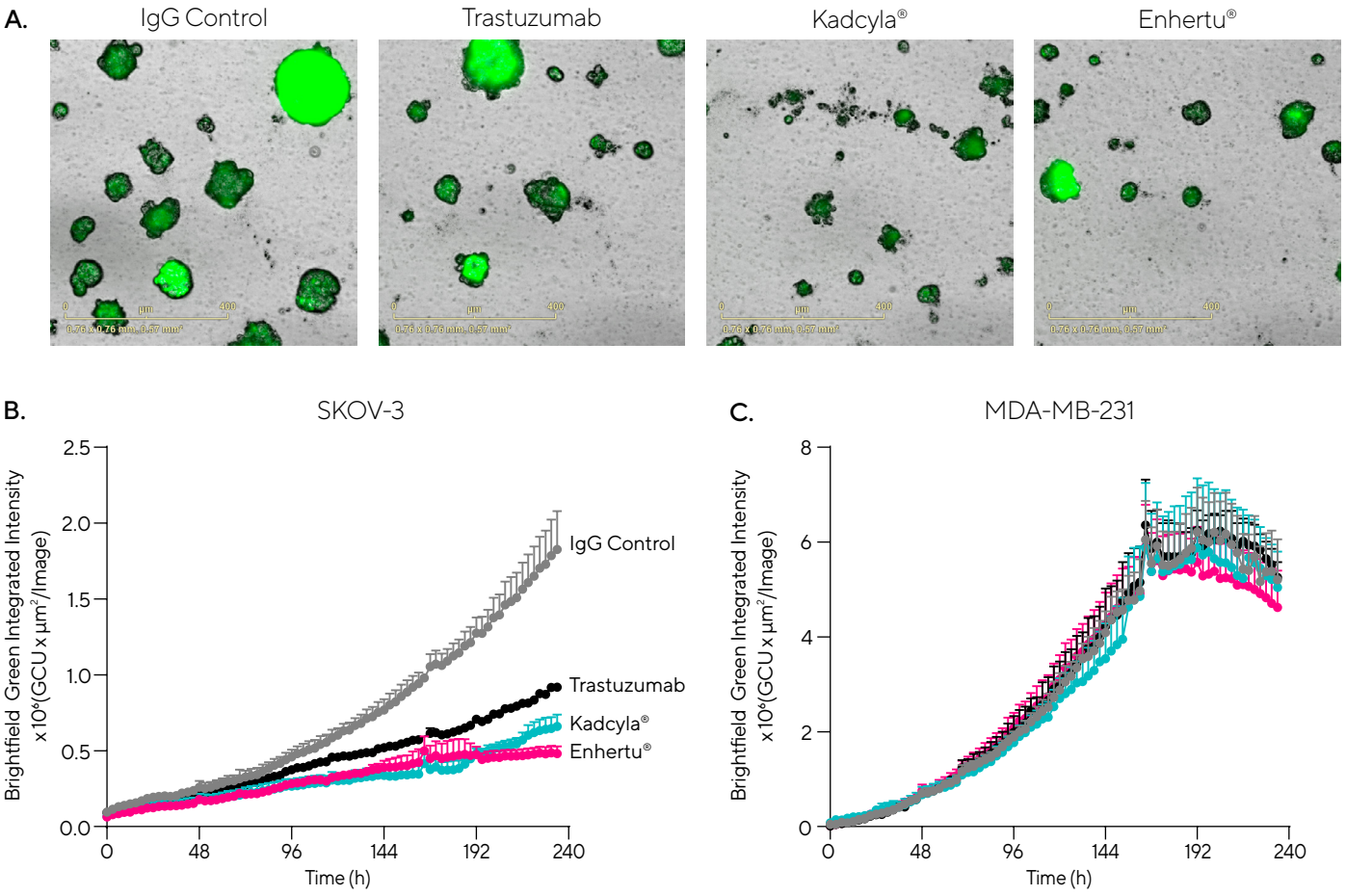

Figure 4. ADCs induce death of HER2 expressing SKOV-3 cells in a multi-spheroid model.
Incucyte® Nuclight Green Lentivirus labeled SKOV-3 and MDA-MB-231 cells (2 K/well) were seeded on a layer of Matrigel® (5 mg/mL) to promote multi-spheroid formation. Antibodies were added (2 µg/mL) and cells were monitored using the Incucyte® Live-Cell Analysis System via a repeating scan schedule (every 3 hours) for 10 days. (A) Representative images of SKOV-3 multi-spheroids from day 9. Cell death in (B) SKOV-3 and (C) MDA-MB-231 spheroids was quantified as a loss of fluorescence intensity of spheroids over time.

Single Spheroid ADCC

A key MoA of Trastuzumab in the killing of HER2 expressing cancers is ADCC.[17] It is expected that ADCs based on Trastuzumab would retain this killing function on top of the payload-induced cytotoxicity. To investigate this, NK cells, which are the main cell type involved in ADCC, were added to the single spheroid model. Incucyte® images were captured on a repeating schedule and cell death was quantified as a reduction in spheroid intensity over time. This metric was chosen as it allowed us to distinguish differences between both antibodies and concentrations in terms of induction of spheroid death.

The temporal microplate graph (Figure 5A) shows the green intensity of spheroids formed from high HER2 expressing, green-labeled BT474 spheroids over time. The data highlights a much larger decrease in spheroid green intensity over time in the presence of the anti-HER2 antibodies, compared with the IgG control. This, combined with the bar chart in Figure 5B, shows that Kadcyla® induced the most cell death, followed by Trastuzumab, then Enhertu®. This contrasts with what was observed in the absence of NK cells (Figure 3), when the Enhertu® induced greater cytotoxicity than Trastuzumab. This may suggest that NK-mediated ADCC has a greater overall cytotoxic effect than ADC-induced cytotoxicity in this model, and that this effect is slightly attenuated in the Enhertu® antibody, perhaps due to the addition of the payload molecules to the Fc region. The antibodies had minimal effect on the intensity of the HER2-low MDA-MB-231 spheroids (Figure 5C).

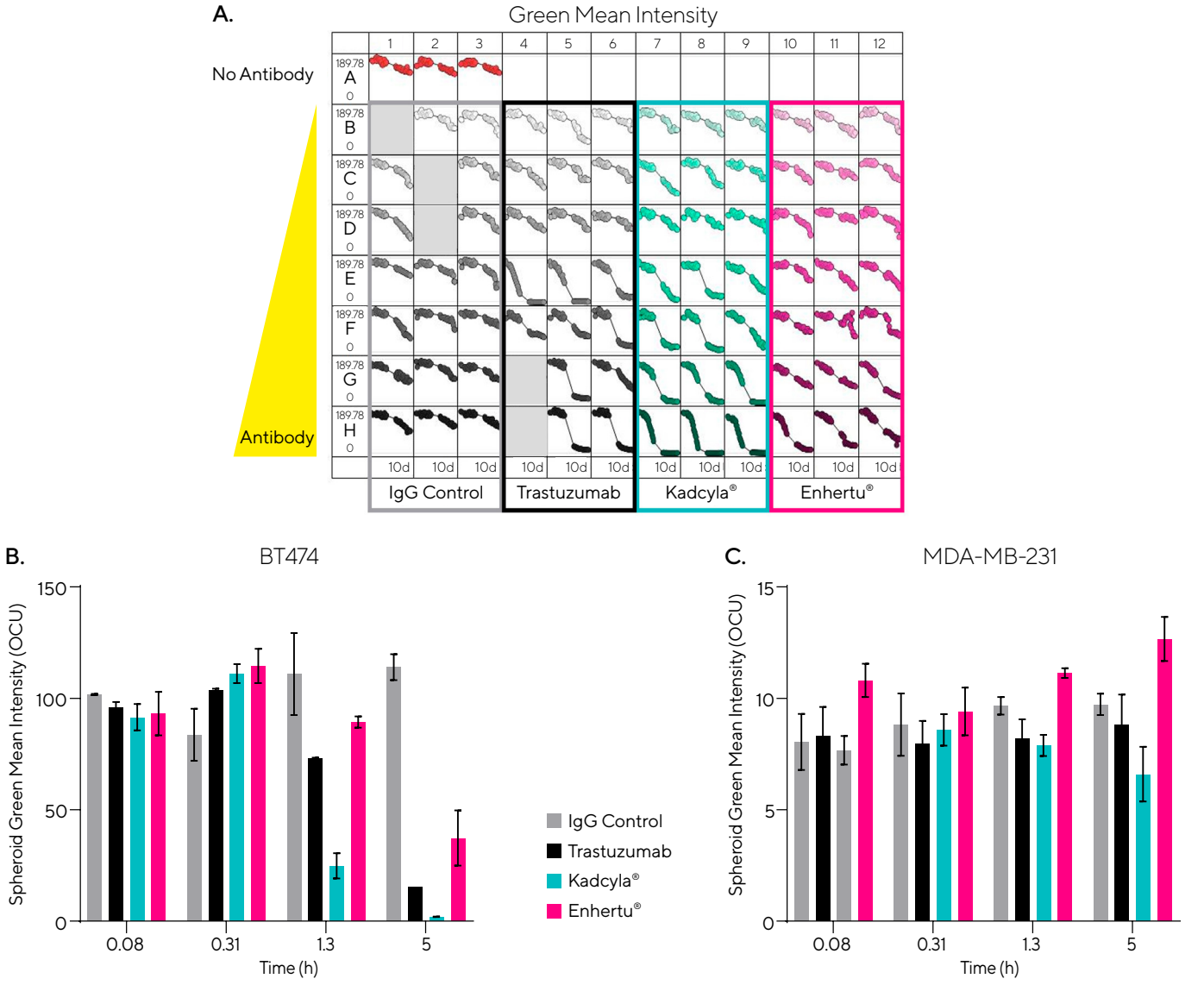

Figure 5. Induction of NK-mediated ADCC activity is much greater with high HER2 expressing targets in the presence of Kadcyla®. Target cells (4 K/well) were MDA-MB-231 (HER2-low) or BT474 (HER2-high) labeled with Incucyte® Nuclight or Cytolight Green Lentivirus, respectively. Matrigel® was added (2%) to aid tight spheroid formation. After 72 hours, cells were mixed with test antibodies and natural killer (NK) cells (20 K/well). Phase and fluorescence images (4X) were captured using the Incucyte® Live-Cell Analysis System on a 3-hour repeating scan schedule for 10 days for spheroid green mean intensity (OCU). (A) Temporal microplate graph of intensity of BT474 spheroids. Outliers are removed in grey. (B) and (C) Bar charts for intensity of spheroids formed from BT474 and MDA-MB-231 targets.

Single Spheroid Bystander Activity

In 2022, Enhertu® was the first HER2-directed therapy to be approved for treatment of patients with low-HER2 expressing breast cancer.[18] The reason it has been given this additional indication (alongside treatment for high HER2-expressing breast cancers) is its potential to exert a unique 'bystander' effect. 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 and result in their death.[19]

To examine this *in vitro* using a 3D single spheroid model, spheroids were first formed from a mixture of of high HER2 expressing BT474 cells (labeled with Incucyte® Cytolight Green Lentivirus) and low HER2 expressing, unlabeled MDA-MB-231 cells. The cells were mixed at a 2:3 BT474 to MDA-MB-231 ratio. Incucyte® images were captured every 3 hours for 8 days and a reduction in spheroid area used to quantify cell death (Figure 6A). This showed that spheroid area was reduced only in the presence of Enhertu® and remained comparable in the presence of the other three antibodies.

However, the Incucyte® Live-Cell Analysis data alone lacked information on the growth of the individual cell types in the co-culture. To determine this, at assay endpoint, the spheroids were dissociated to create a single cell suspension. Cells were then labeled using iQue® 3 Cell Membrane Integrity (R/Red) Dye and analyzed for the green (BT474) and unlabeled (MDA-MB-231) live cell counts using the iQue® HTS by Cytometry Platform. The data in Figure 6B show that, as in each of the previous assays, all three of the anti-HER2 antibodies caused a reduction in the number of high HER2-expressing BT474 cells. The percentage cell death compared to IgG control was again greater for the two ADCs, at 96.4% and 97.2%, respectively, compared to Trastuzumab, which induced 46.4% cell death.

Figure 6B shows that, unlike in the monoculture assays, there has been considerable death of the HER2-low cell type in the presence of Enhertu® when the cells are co-cultured with the high HER2 expressing cells. This provides evidence that the bystander activity of Enhertu® can be observed using this *in vitro* model. These data also show that there has been an increase in the number of MDA-MB-231 cells compared to the IgG control in the wells treated with Kadcyla®. This suggests that as Kadcyla® induces cytotoxicity of the BT474 cells, the MDA-MB-231 cells have more space and/or nutrients to grow. This could explain why the Incucyte® Live-Cell Analysis quantification of spheroid area remains constant unless the antibody has induced death of both cell types in the co-culture (as seen with Enhertu®).

This demonstrates that, although we can measure the bulk reduction in spheroid area using the Incucyte® Live-Cell Analysis System (as in Figure 6A), we get little information on the cellular composition of the spheroid. Using the Incucyte® Live-Cell Analysis System to gather temporal information and then dissociating spheroids for iQue® HTS Cytometry analysis allows quantification of the different cell types comprising the spheroid. This in turn can provide information on differential MoAs of test antibodies.

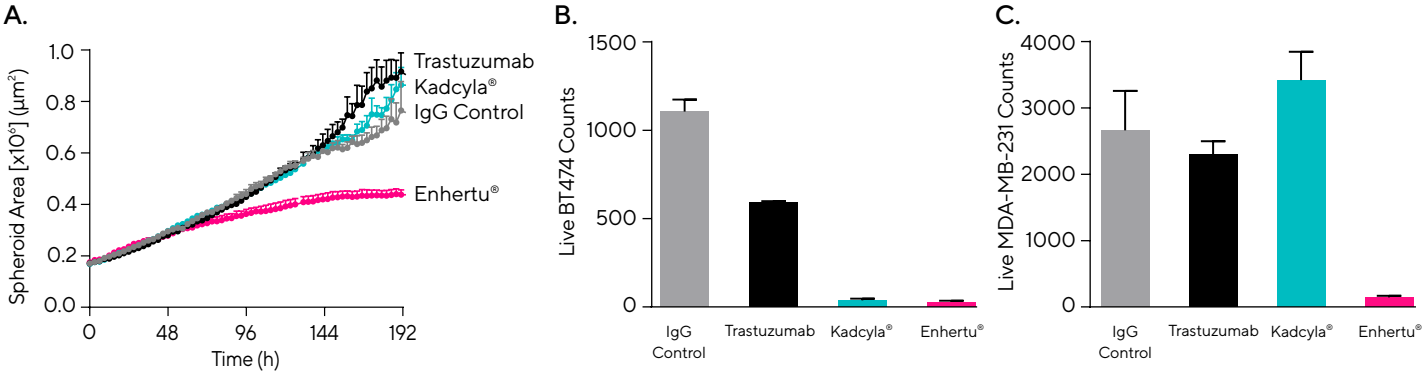

Figure 6. Bystander killing of low HER2 expressing cells is only seen with the Enhertu® ADC.
Single spheroids were formed from a 2:3 ratio of high HER2 expressing BT474 cells (labeled with Incucyte® Cytolight Green Lentivirus) to low HER2 expressing, unlabeled MDA-MB-231 cells. Antibodies were added after 72 hours. Images were captured on a repeat scanning schedule (every 3 hours) using the Incucyte® Live-Cell Analysis System. On day 8, spheroids were dissociated and cells labeled using the iQue® 3 Cell Membrane Integrity (R/Red) Dye. Live cell counts were quantified using the iQue® 3 Platform. (A) Cell death was quantified from Incucyte® images as a reduction in spheroid area over time. Bar graphs show viable cell counts from the iQue® 3 Platform of (B) BT474 cells and (C) MDA-MB-231 cells.

Conclusions

These data describe the use of the Incucyte® Live-Cell Analysis System and iQue® HTS by Cytometry Platform to profile the *in vitro* function of ADCs. Incucyte® assays quantified antibody internalization and cytotoxicity over time, whilst dissociated spheroids could be assessed using the iQue® 3 Cytometry Instrument to reveal differences in spheroid composition. The advantages of this workflow include:

1. 3D single and multi-spheroid models provide a closer reflection of the TME, for example due to more complex cell-cell and cell-ECM interactions. This can mean a more translational model for drug development.
2. The Incucyte® Live-Cell Analysis System provides visual and temporal analysis of spheroids over time, with easy quantification of metrics such as spheroid size and fluorescence intensity allowing antibody internalization and cytotoxicity to be measured over time.
3. The iQue® HTS by Cytometry facilitates high-throughput analysis of dissociated cells, with the ability to distinguish individual cell types within a mixture. This can reveal mechanistic information on a drug's activity.
4. Mix and read reagents combined with validated protocols make experimentation simple and streamlined.
5. This workflow and the described advantages allow a comprehensive assessment of the function of ADCs in 3D models and can enhance drug discovery and biological research applications.

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