In vitro Bioassays to Model Complex Cellular Environments for Better Lead Selection

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Introduction

Bioassays have evolved considerably over the years to aid in the drug discovery process. Many challenges persist in assessing candidate efficacy, when correlated with clinical outcomes. There are numerous benefits of early *in vitro* testing, that can lead to better decision making earlier in the drug discovery process. Early *in vitro* testing can lead to faster timelines, cost-effective solutions and decreases the need for animal studies. The case study presented in this poster, comparing Kadcyla and Enhertu, showcases the breadth of valuable information that can be obtained *in vitro,* and how steadily increasing complexity captures the mode of action of Antibody-drug conjugates (ADCs).

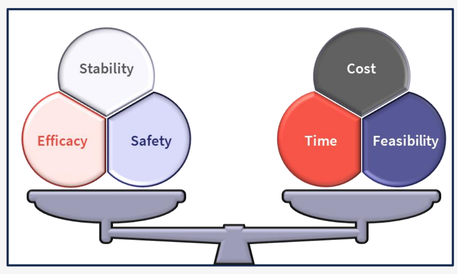


Figure 1. In drug development a balanced approach is essential to maximize the possibility of delivering clinical success.

Methods and Results

Endpoint Cytotoxicity Readouts

Kadcyla (ado-trastuzumab emtansine) 2013		Enhertu (fam-trastuzumab deruxtecan-nxki) 2019
Trastuzumab	**mAb**	Trastuzumab
DM1 (Inhibitor of tubulin polymerization)	**Toxin**	DXd (membrane-permeable topoisomerase I inhibitor)
Non-cleavable	**Release mechanism**	Enzymatic
av. 3.5	**DAR**	8
No	**Clinical Efficacy on heterogenous Tumours**	Yes

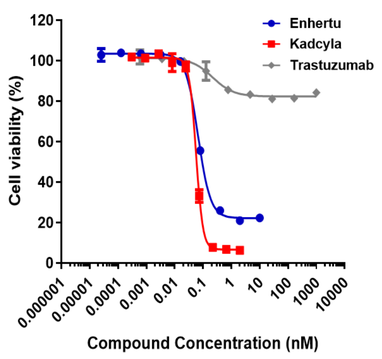


Figure 2. Industry standard *in vitro* endpoint cytotoxicity assessment using the CellTiter-Glo™ luminescence assay. HER2^{POS} cells incubated in the presence of a titration of Enhertu, Kadcyla or Trastuzumab for 96 hours are assessed for viability. Using this readout, potencies are similar for the two ADCs, while slightly better maximum cell killing is observed for Kadcyla.

This assay is key at the early discovery and lead selection phase of the drug discovery process, when screening multiple candidates requires a cost-effective and robust assay. While providing useful initial information on potency, this assay fails to capture complexities such as kinetics of cell killing, effect on solid tumors, heterogenous tumors or presence or absence of immune cells within these environments.

Assessing Kinetics – Real-Time Imaging

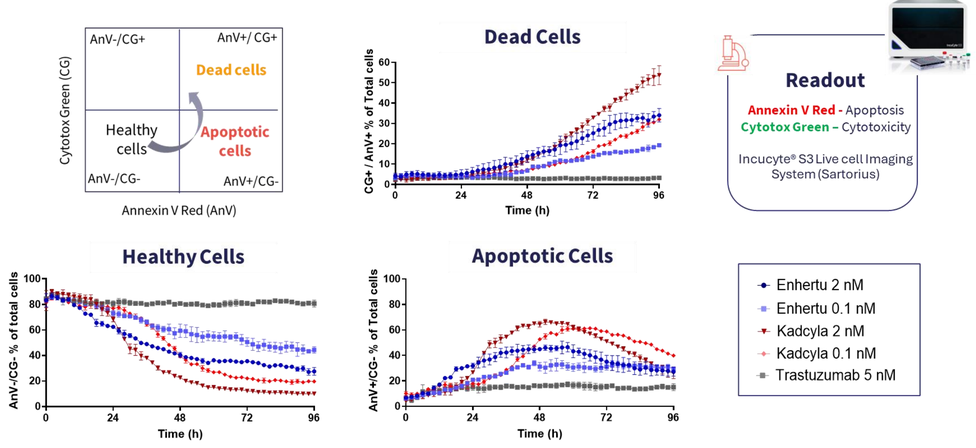


Figure 3. Quantifying cytotoxicity in real time – cell-by-cell analysis on HER2^{POS} cells on the Incucyte® live cell imaging system

The cell-by-cell analysis enables tracking of cells as they enter the apoptotic and cytotoxic phase over time. Here, we show that cells treated with Enhertu enter apoptosis earlier. While the response is delayed for Kadcyla, it displays an increased level of cell killing. These results are consistent with learnings from the endpoint readout but add additional insight into the MoA .

Modelling Solid Tumors – 3D Spheroid Assays

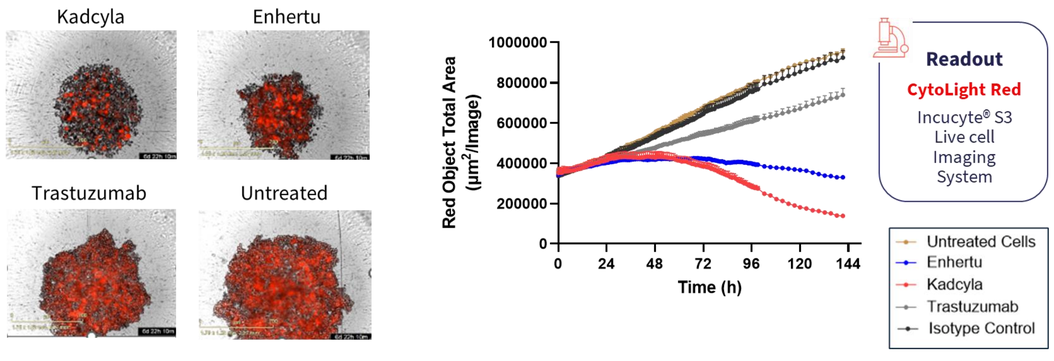
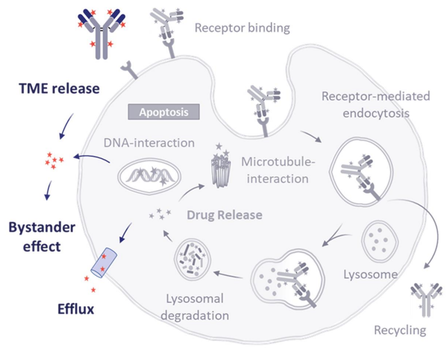


Figure 4. Assessment of 3D spheroid cell viability on the Incucyte® live cell imaging system. HER2^{POS} cells transduced with a fluorescent marker are grown in ultra-low attachment plates, treated with ADCs and controls, and spheroid shrinkage or growth is monitored in real time.

2D monolayer cell-based assays play a pivotal role in *in vitro* testing in the drug discovery process. However, for solid tumor indications, 3D spheroids are more representative of the tumour microenvironment. Due to the compact structure of the 3D spheroids, penetration of test ADCs and their impact on viability is slower compared to monolayers. In this case study, HER2^{POS} spheroids are killed by both Kadcyla and Enhertu after a 48h incubation, noted by decrease in spheroid size and loss of fluorescence intensity. Consistent with the 2D data, Kadcyla demonstrates higher level of killing on HER2^{POS} spheroids at later time points.

Modelling Heterogenous Tumors and the Bystander Effect



The ADC impacting neighboring antigen-negative cells through payload release from the antigen-positive cells

Abzena's bystander assay

- ✓ Real co-culture
- ✓ Real-time
- ✓ Monitors both Target^{POS} and Target^{NEG} cell line

Readout

Cytolight Red (HER2^{POS})
Green Fluorescent Protein (HER2^{NEG})

Incucyte® S3 Live cell Imaging System (Sartorius)

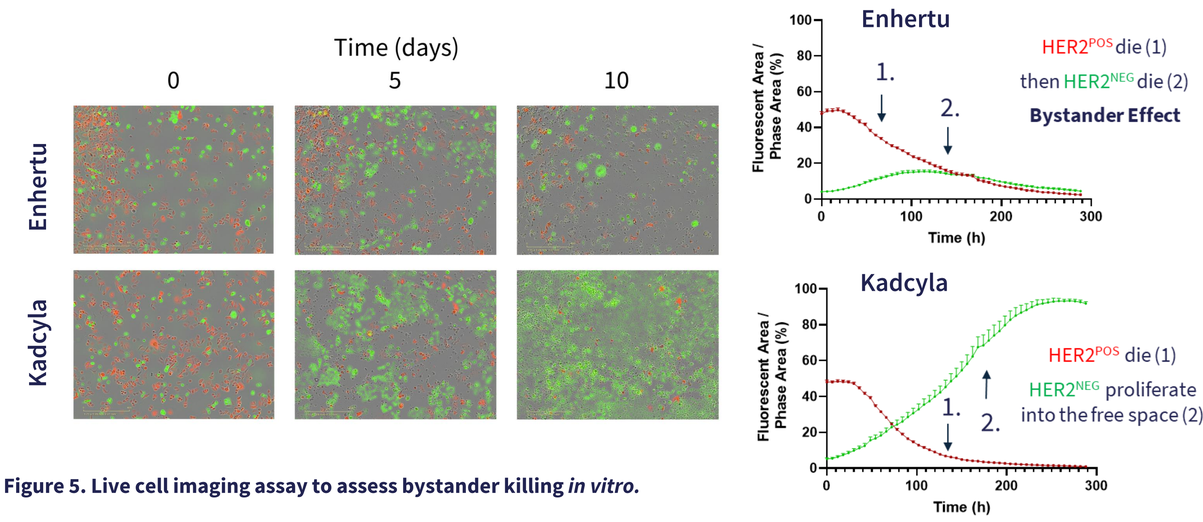


Figure 5. Live cell imaging assay to assess bystander killing *in vitro.*

ADCs with bystander effect, such as Enhertu, are known to be clinically more effective in heterogenous tumors. Using a co-culture method, the bystander effect can be recapitulated, as demonstrated here with Enhertu. In contrast, Kadcyla kills the HER2^{POS} cells (Red), while the HER2^{NEG} cells (Green) remain alive and continue proliferating, suggesting it will perform subpar in such indications.

Adding the Immune Component – Payload and Immune-Mediated Killing

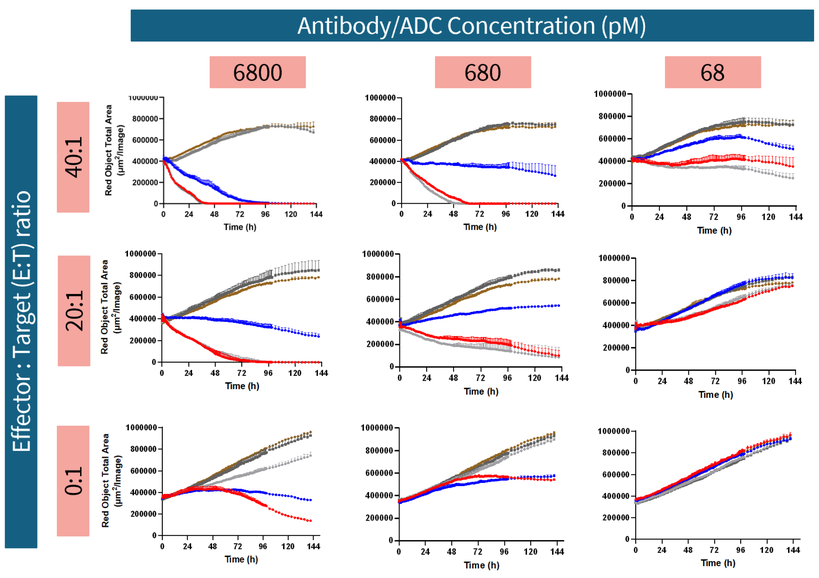


Figure 6. Assessment of Antibody Dependent Cellular Cytotoxicity (ADCC) and payload-mediated killing of 3D spheroids using live cell imaging. The spheroid cytotoxicity assay described in Figure 4 is performed in the presence and absence of peripheral blood mononuclear cells (PBMCs).

Concentration and E:T ratio dependent cell killing of HER2^{POS} spheroids have been demonstrated. ADCC occurs within hours, while payload mediated killing is observed within days. Standard *in vitro* assays will assess these processes individually, whereas here we capture both MoAs in a single assay. Results show that on HER2^{POS} spheroid monocultures, Kadcyla is the more effective ADC regardless of the presence or absence of immune effectors.

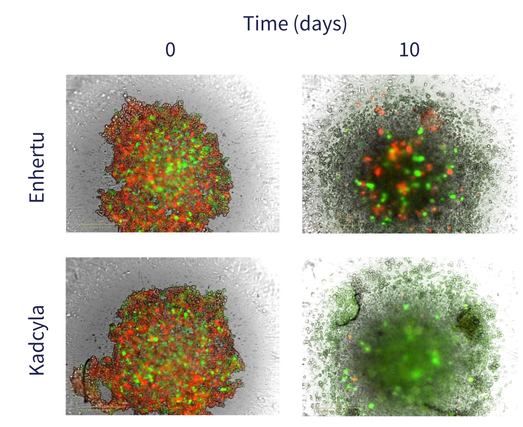


Figure 7. Assessment of ADCC alongside bystander effect in heterogenous 3D spheroid co-cultures. Assay performed as described in Figure 5, starting from spheroid HER2^{POS} (Red) and HER2^{NEG} (Green) co-cultures and adding PBMCs.

This complex experiment, modelling heterogenous solid tumors in the presence of immune effectors, demonstrates ADCC acting in synergy with the cytotoxicity of the ADC, as would happen *in vivo*. In this model, Enhertu eliminates more of the total cell population than Kadcyla.

Note that this assay is performed in 384-well format, assessing multiple culture conditions, providing extensive information on the behavior of these candidates in various conditions.

Summary

By assessing Kadcyla and Enhertu in *in vitro* assays of increasing complexity, we can gain insight into how each candidate is likely to perform in various tumour environments. Kadcyla performs better than Enhertu in monocultures with HER2^{POS} cell lines, and in 2D and 3D cultures, both in the presence and absence of immune effectors. However, in HER2^{POS} and HER2^{NEG} co-cultures, Enhertu shows bystander killing, indicating it is the more effective drug for treating heterogenous tumors, matching the success of Enhertu in the clinic.

In conclusion, the data presented here demonstrates how *in vitro* bioassays can be used to assess the mode of action (MoA) of bioconjugates in-depth, saving both time and cost, as well as ensuring the most successful candidate is taken forward to clinical trials.