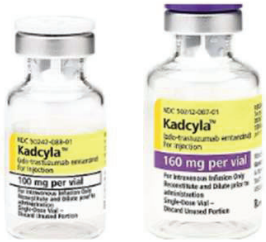
ABZENA

Case Study:
Her2-targeting ADCs

Can *in vitro* bioassays predict clinical success?

Case Study #5 – The story of two Her2-targeting ADCs, Kadcyla and Enhertu

KADCYLA



Approval: 2013

Antibody: trastuzumab

Linker: non-cleavable

Payload: DM1 – inhibitor of tubulin polymerization

DAR: 3.5

Clinical activity: NOT EFFECTIVE in heterogenous tumours

ENHERTU

Approval: 2019

Antibody: trastuzumab

Linker: cleavable

Payload: Dxd - a membrane-permeable topoisomerase I inhibitor

DAR: 8

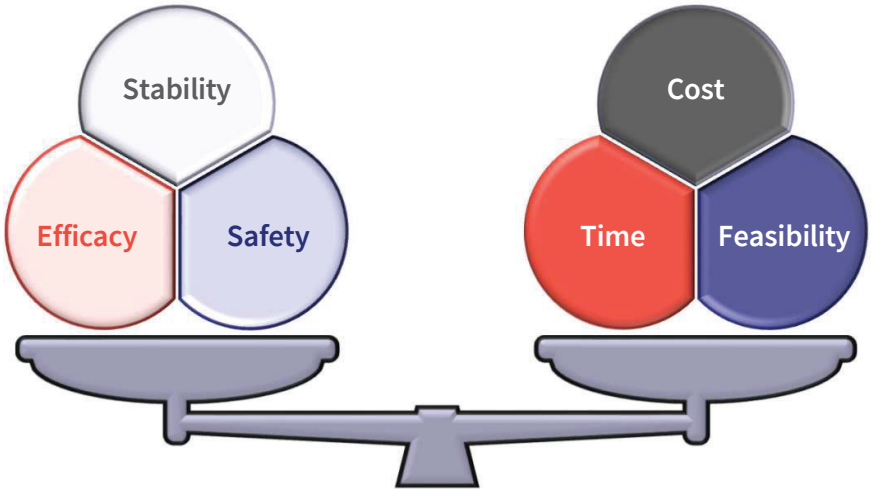
Clinical activity: EFFECTIVE in heterogenous tumours, e.g. gastric cancer and metastatic breast cancer

How can *in vitro* bioassays inform clinical success?
Benefits of early testing

Bioassays have evolved greatly over the years, but there is still some skepticism - do these results correlate with clinical outcomes?

Benefits of early *in vitro* testing are multiple:

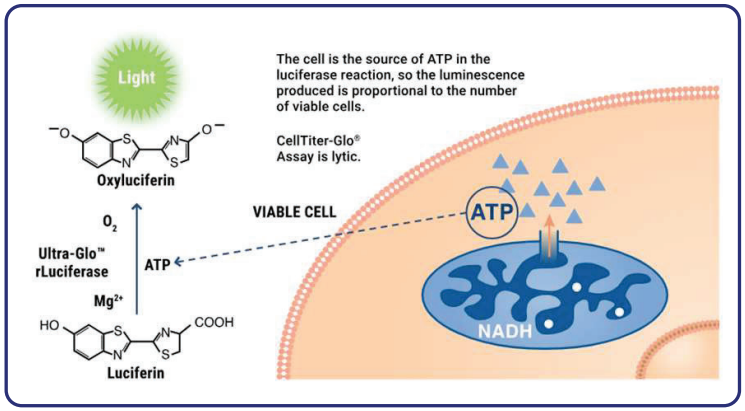
- ✓ Better decision making early on to increase chance of success in the clinic
- ✓ Quicker timelines
- ✓ Cheaper solutions
- ✓ Reducing the need for animal studies

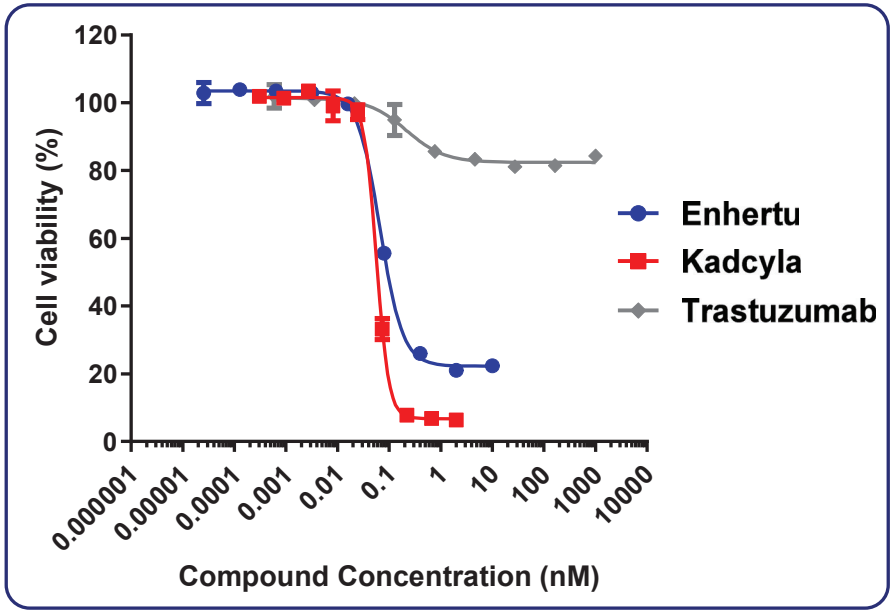


Our case study on Kadcyla and Enhertu demonstrates the amount of valuable information that can be obtained *in vitro*, and how this allows you to start smart and finish fast

How much does a standard end-point anti-proliferative assay reveal?

- CellTiterGlo® assay using a target positive cell line, after 96h co-incubation with a titration of drugs
- Many clients perform only this functional testing as part of their early screening and characterization studies



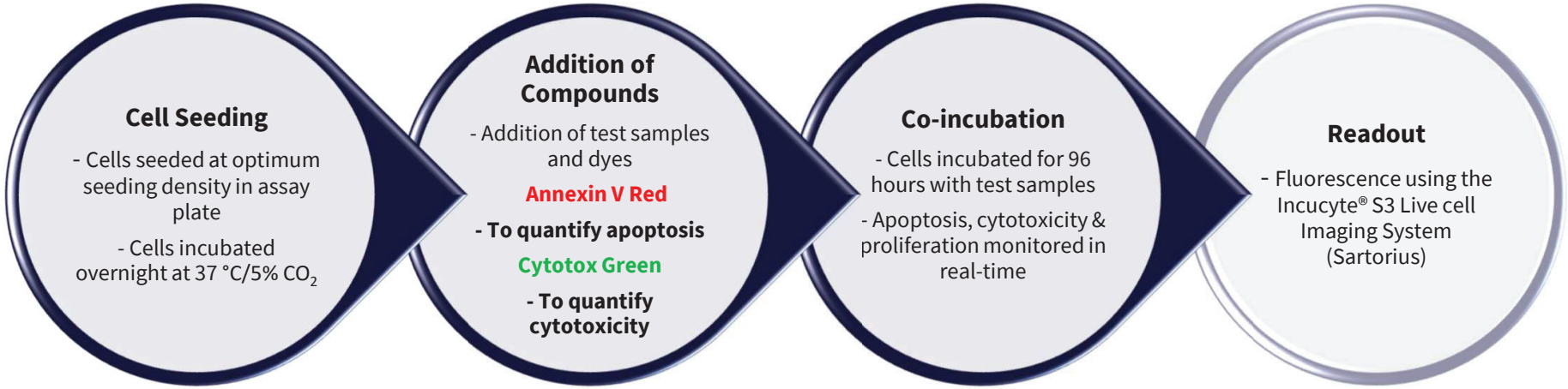


Result: Similar performance with comparable potency, but better max. cell kill for Kadcyla

But if we dig deeper…

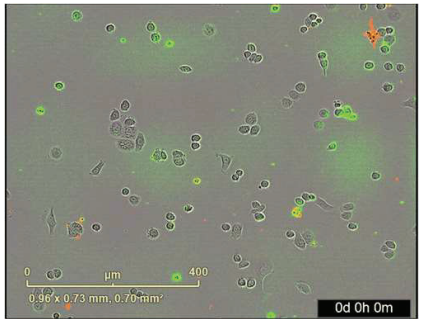
Real-time cytotoxicity assessment via live cell imaging on $Her2^{HIGH}$, $Her2^{LOW}$ and $Her2^{NEG}$ cell lines

Workflow

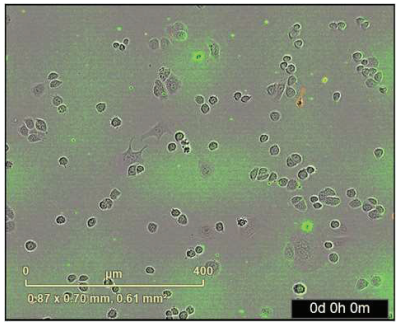


<u>Aim</u>: to assess the cytotoxic and anti-proliferative activity of Kadcyla and Enhertu on cell lines with varying levels of Her-2 expression

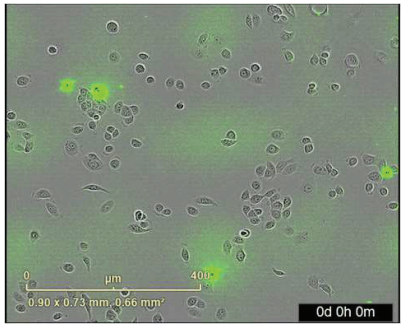
Example Results
Qualitative data



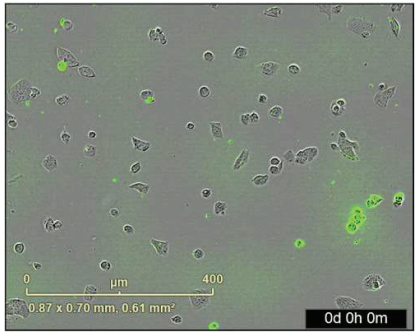
Her2^{HIGH} – Enhertu 2 nM



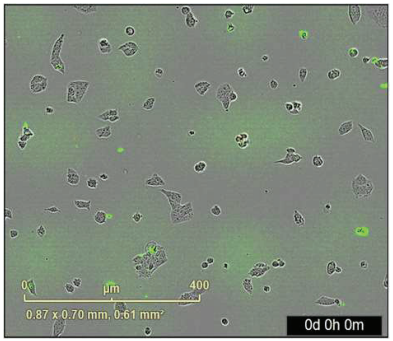
Her2^{HIGH} – Kadcyla 2 nM



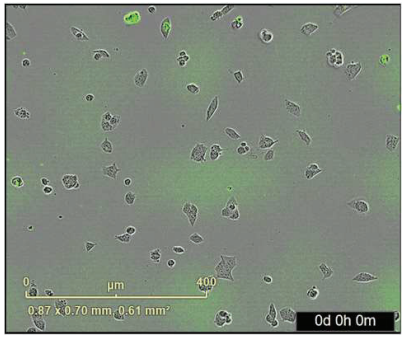
Her2^{HIGH} – Trast 5 nM*



Her2^{NEG} – Enhertu 2 nM



Her2^{NEG} – Kadcyla 2 nM



Her2^{NEG} – Trast 5 nM*

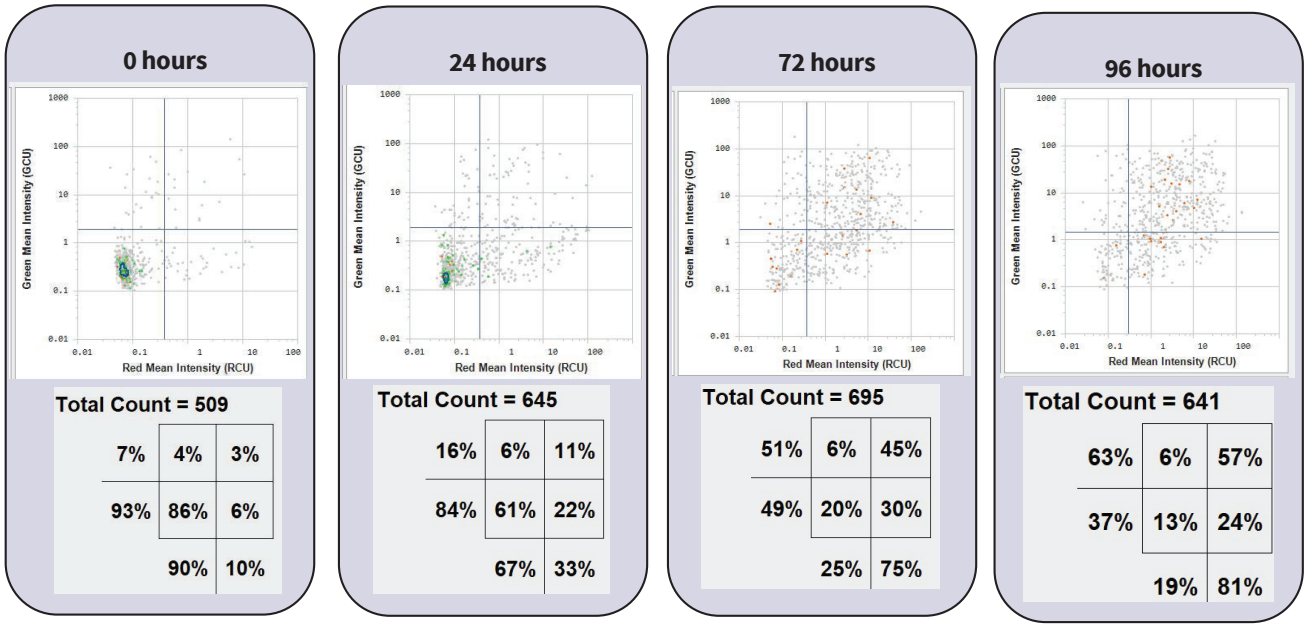
In the Her2^{HIGH} cell line, anti-proliferative and cytotoxic activity is observed with Enhertu and Kadcyla treatment

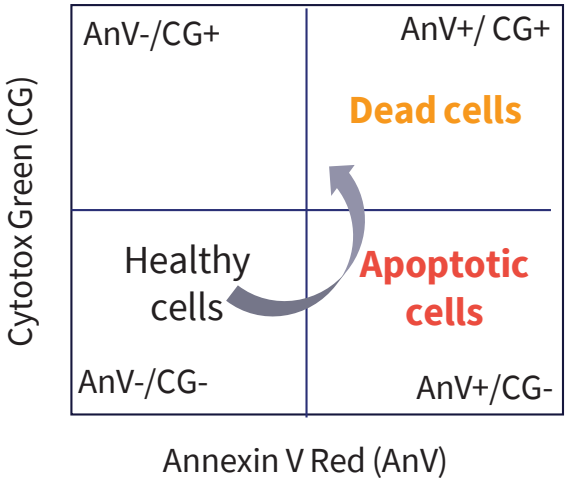
In the Her2^{NEG} cell line, cells are not impacted by either treatment

* Concentrations differ due to different serial dilutions used

Quantifying cytotoxicity - cell-by-cell analysis

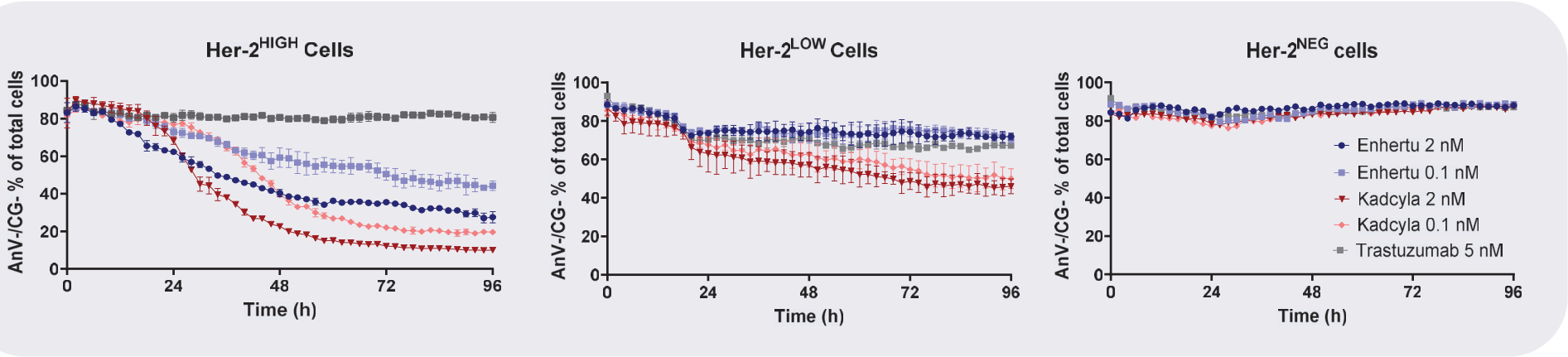
Monitoring as cells enter early and late apoptosis

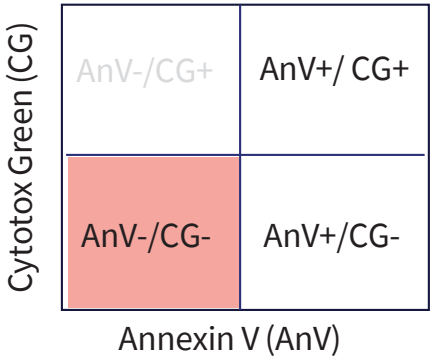




Assessment of Healthy Cells

Monitoring the AnV-/CG- population

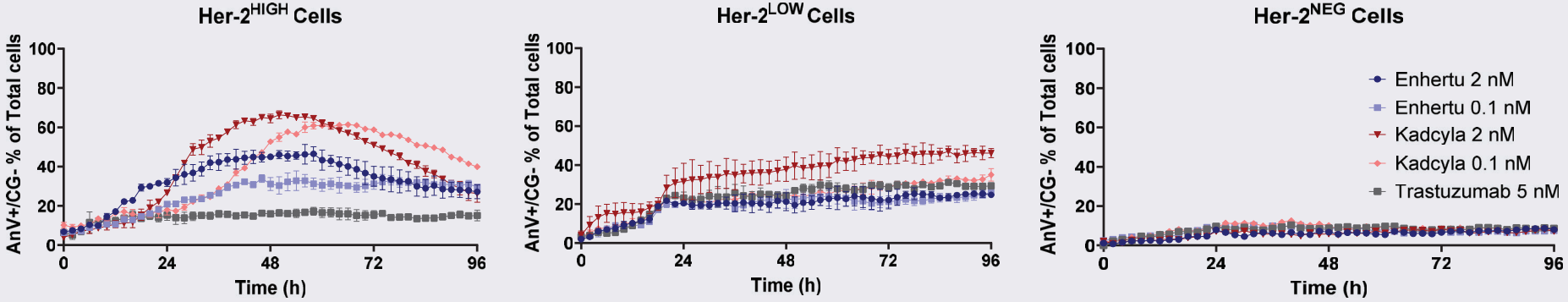


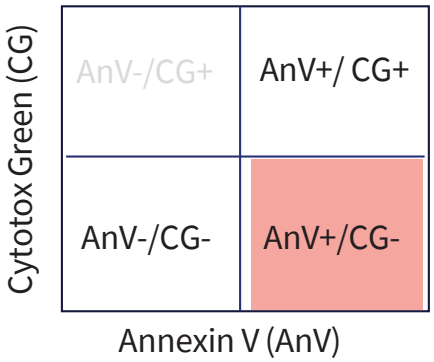


- In the Her2^{HIGH} cells, a reduction in the percentage of healthy cells over time is apparent.
  - The Kadcyla response is a bit delayed compared to Enhertu, but results in a more efficient elimination of the healthy population
- In the Her2^{LOW} and Her2^{NEG} cell line, Enhertu does not have an impact, whereas Kadcyla is reducing healthy cell numbers in the Her2^{LOW} cells.
  - Albeit to a lesser extent than in the Her2^{HIGH} cell line.

Assessment of Apoptosis

Monitoring the AnV+/CG- population

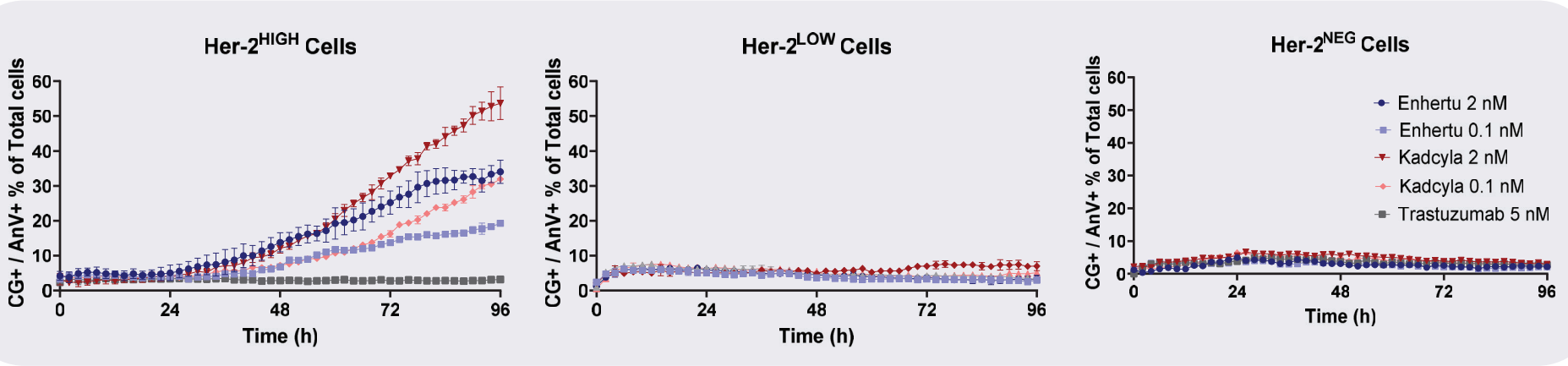


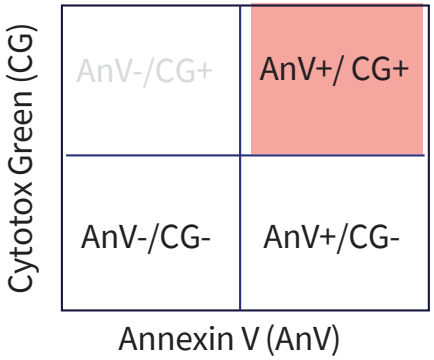


- In the Her2^{HIGH} cells, the early apoptosis signal is transient, peaking earlier and higher for higher concentrations
  - The Kadcyla response is again a bit delayed compared to Enhertu, but then peaks higher, consistent with the previous results
- In the Her2^{LOW} and Her2^{NEG} cell line, Enhertu shows no significant impact, whereas Kadcyla is resulting in a plateaued apoptotic response of the Her2^{LOW} cells.
  - Again, to a lesser extent than in the Her2^{HIGH} cell line.

Assessment of Cell Death

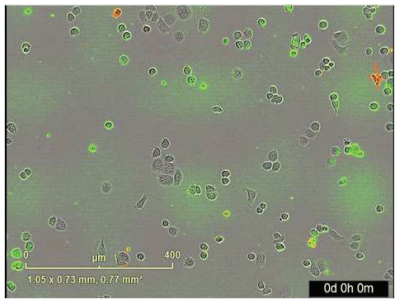
Monitoring the AnV+/CG+ population



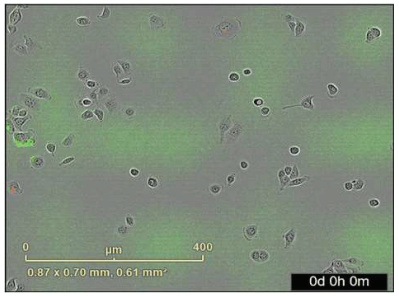


- In the Her2^{HIGH} cells, dead cells with compromised membranes appear after around 40h of treatment
  - The Kadcyla response is steeper, resulting in more cell kill than Enhertu at the same concentrations
- In the Her2^{LOW} and Her2^{NEG} cell line, treatment with neither ADCs lead to significant cell death

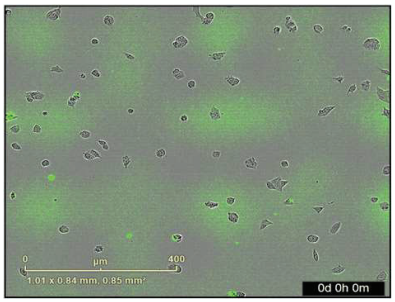
Further Investigating Cell Line Selectivity at High Concentrations



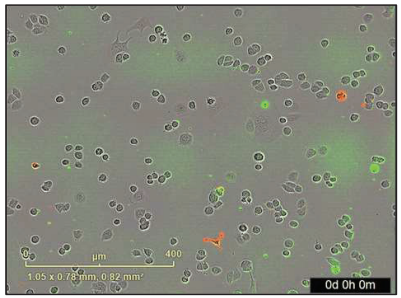
Her2^{HIGH} – Enhertu 2 nM*



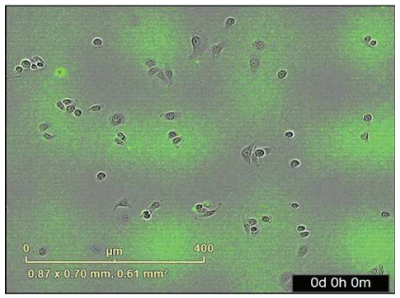
Her2^{LOW} – Enhertu 222 nM&



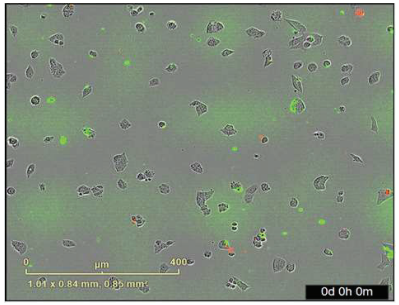
Her2^{NEG} – Enhertu 222 nM&



Her2^{HIGH} – Kadcyla 2 nM*



Her2^{LOW} – Kadcyla 102 nM&



Her2^{NEG} – Kadcyla 102 nM&

Enhertu does not impact the Her2^{LOW} and Her2^{NEG} cell line even at high concentrations.

Kadcyla at high concentrations shows and anti-proliferative and cytotoxic effect even on the Her2^{LOW} and Her2^{NEG} cell lines.

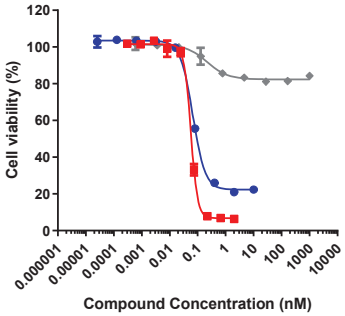
* Top concentration on Her2^{HIGH} cell line

& Concentrations differ due to different serial dilutions used

What have we learnt so far?
Which ADC is the more promising lead?

What happens in a model mimicking the solid tumour environment?

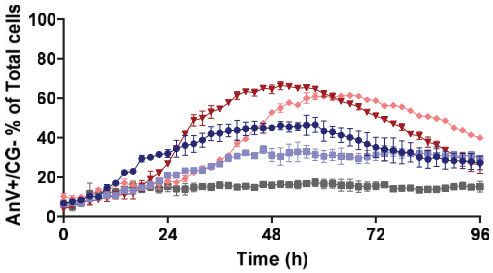
Endpoint single cultures



Comparable potency, but better max. cell kill for Kadcyla

Kadcyla kills Her2^{HIGH} cells more efficiently, also active on Her2^{LOW} cells, and even on Her2^{NEG} cells at high concentrations

Real-time single cultures



Assessment of 3D Spheroid Viability via Live Cell Imaging

Workflow

Cell Seeding

- **CytoLight Red** transduced cells seeded in 384w ultra-low attachment spheroid plates

Spheroid Formation

- Cells incubated for 72-h at 37 °C/5% CO_2 to allow spheroid formation

Addition of Compounds

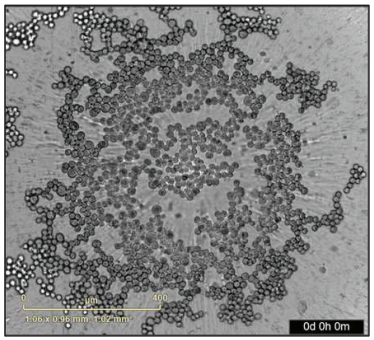
- Addition of titration of test samples

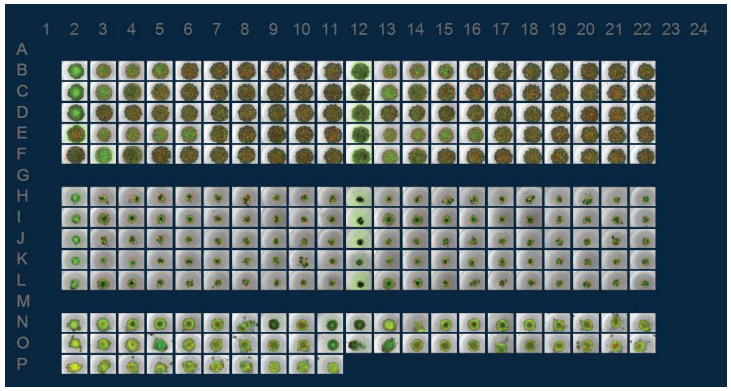
Co-incubation

- Cells incubated for 6 days with test samples
- Spheroid size and health monitored in real-time

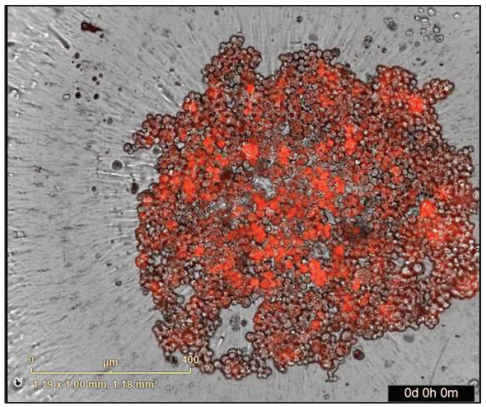
Readout

- Fluorescence using the Incucyte® S3 Live cell Imaging System (Sartorius)

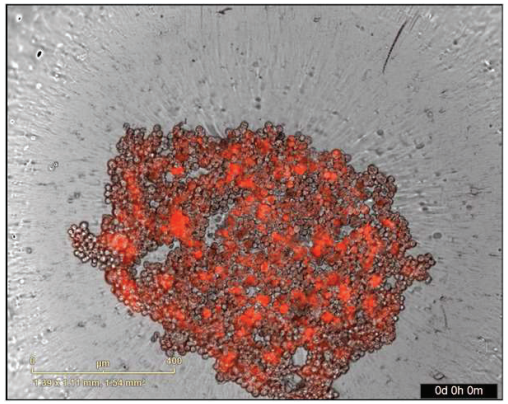




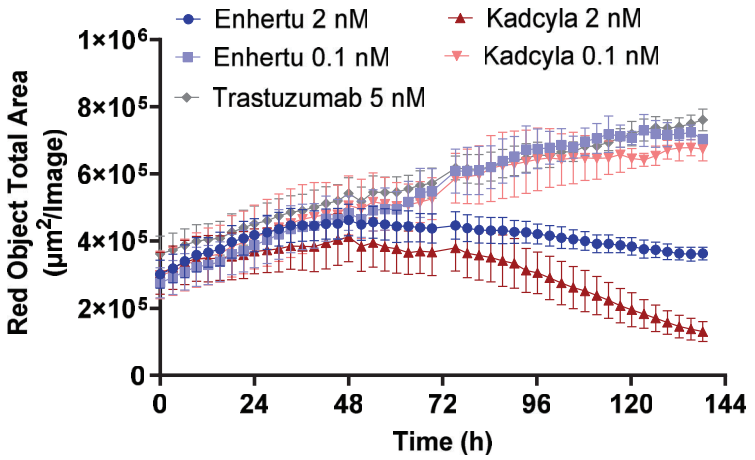
Assessment of 3D Spheroid Viability
Results



Her2^{HIGH} cells – Kadcyla 2 nM



Her2^{HIGH} cells – Trastuzumab 5 nM

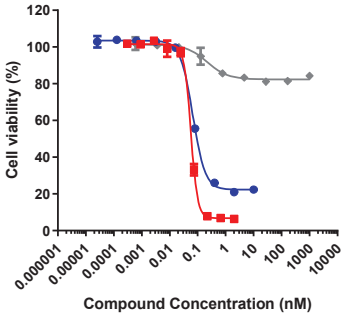


- Spheroids established from Her2^{HIGH} cells are destroyed by both Kadcyla and Enhertu after 48 h of treatment at higher concentrations.
  - IC_{50} shifted compared to 2D cultures, spheroids are 'harder' to kill.
- As with the 2D cell cultures, the Kadcyla response is more pronounced, resulting in increased shrinking of the spheroid.

What have we learnt so far?
Which ADC is the more promising lead?

What happens in a model mimicking a heterogenous tumour environment?

Endpoint single cultures

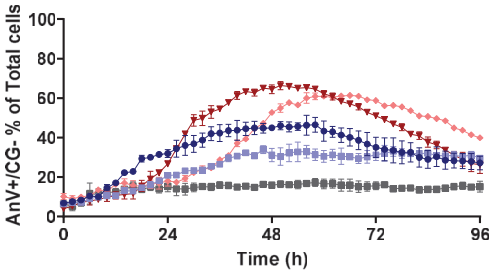


Comparable potency, but better max. cell kill for Kadcyla

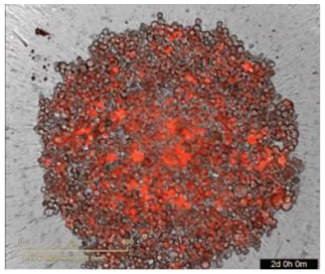
Kadcyla kills Her2^{HIGH} cells more efficiently, also active on Her2^{LOW} cells, and even on Her2^{NEG} cells at high concentrations

Kadcyla shrinks spheroids more efficiently

Real-time single cultures



3D spheroid cultures



Assessment of Cell Killing in Co-Cultures – Bystander Assay

Workflow

Preparation of fluorescently labelled cells

- Transduction by Cytolight Red ($Her2^{POS}$) or Green ($Her2^{NEG}$) lentivirus reagent

Cell seeding

- Cells plated at 4 to 1 ratio $Her2^{POS}$ to $Her2^{NEG}$

Addition of Compounds

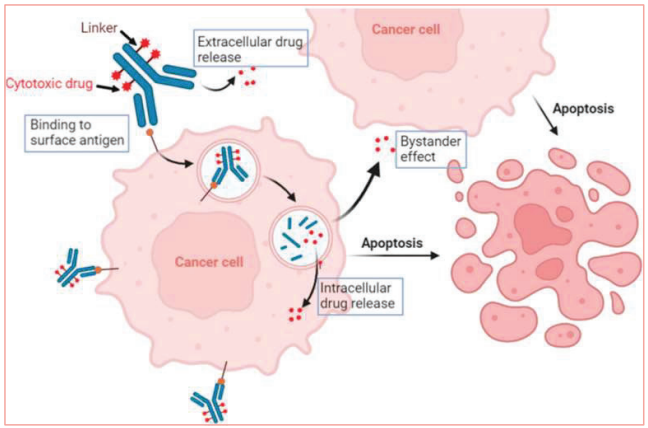
- Addition of titration of test samples

Co-incubation

- Cells incubated for 12 days with test samples

Readout

- Fluorescence using the Incucyte® S3 Live cell Imaging System (Sartorius)
- Reduced fluorescence upon death



TRADITIONAL BYSTANDER ASSAYS

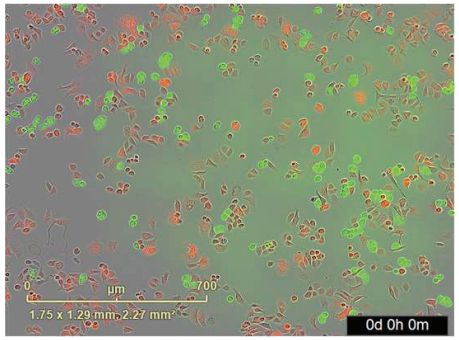
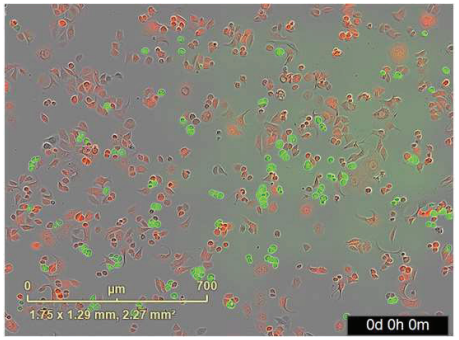
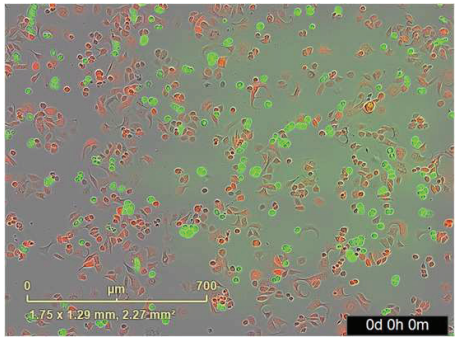
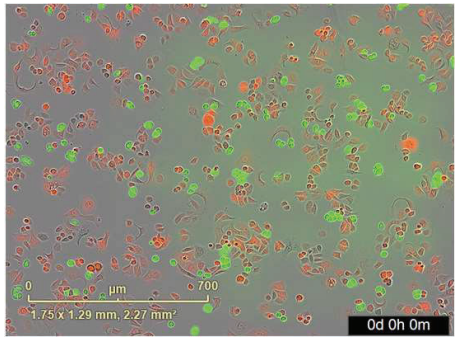
- Not real co-culture (e.g. supernatant transfer)
- Endpoint (e.g. flow cytometry assays)
- Focus only on $Target^{NEG}$ cell line

ABZENA'S BYSTANDER ASSAY

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

Bystander Assay
Qualitative results

Red cells = Her2^{POS} Green cells = Her2^{NEG}

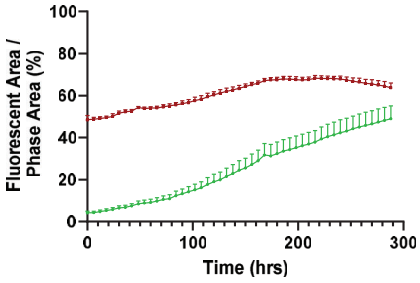
No treatment	Trastuzumab (1 µM)*	Kadcyla (0.5 nM)*	Enhertu (5 nM)*
			
Both cell lines proliferate	Red cells proliferate at a slower rate Green cells proliferate as normal	Red cells die Green cells proliferate into the free space NO BYSTANDER EFFECT	Red cells die then Green cells die BYSTANDER EFFECT

* Cells dosed to maximize killing effect on Her2^{POS} cells, while not impacting Her2^{NEG} cells in single cultures

Bystander Assay
Quantitative results

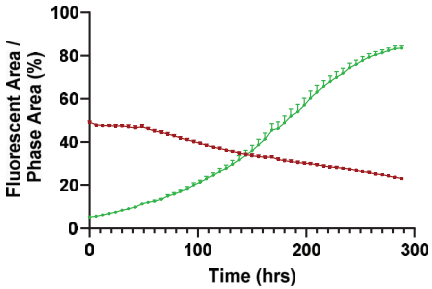
Red cells = Her2^{POS} Green cells = Her2^{NEG}

No treatment



Both cell lines proliferate

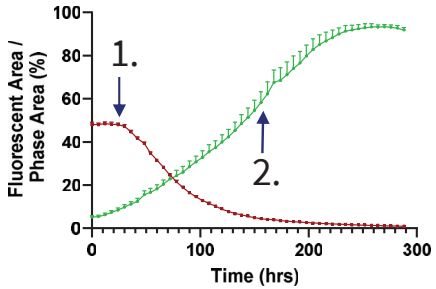
Trastuzumab (1 µM)*



Red cells proliferate at a slower rate

Green cells proliferate as normal

Kadcyla (0.5 nM)*

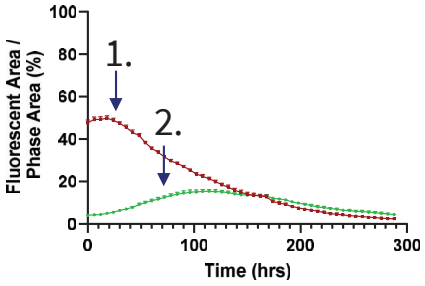


Red cells die (1)

Green cells proliferate into the free space (2)

NO BYSTANDER EFFECT

Enhertu (5 nM)*



Red cells die (1)

then Green cells die (2)

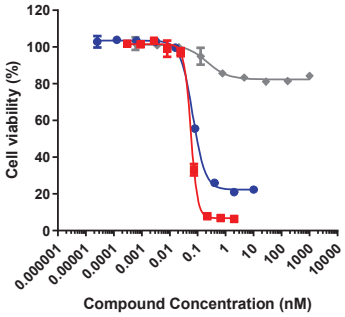
BYSTANDER EFFECT

* Cells dosed to maximize killing effect on Her2^{POS} cells, while not impacting Her2^{NEG} cells in single cultures

What have we learnt so far?
Which ADC is the more promising lead?

In vitro assays help to understand the complex MoAs involved in clinical success, and allow for better lead selection early on

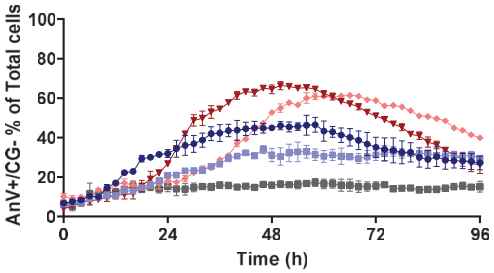
Endpoint single cultures



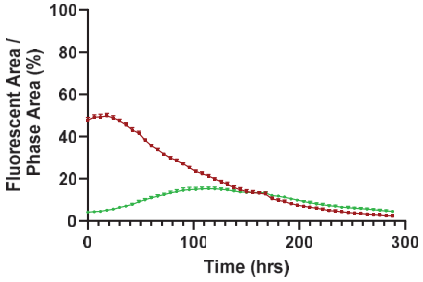
Comparable potency, but better max. cell kill for Kadcyla

Kadcyla kills Her2^{HIGH} cells more efficiently, also active on Her2^{LOW} cells, and even on Her2^{NEG} cells at high concentrations

Real-time single cultures



Co-cultures



Enhertu has a clear bystander effect, and is more likely to be effective on heterogenous tumours

Kadcyla shrinks spheroids more efficiently

3D spheroid cultures