Development of three-dimensional cancer cell line spheroid models for the in vitro functional characterization of cytotoxic antibody-drug conjugates

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Introduction

Antibody-Drug Conjugates

Antibody-drug conjugates (ADCs) are a class of cancer therapeutics comprised of a linker-payload conjugated to a monoclonal antibody targeting a tumor-associated antigen (TAA), to enable the delivery of the cytotoxic payload to cancer cells.

In Vitro Models for the Evaluation of Antibody-Based Therapeutics

Current standard *in vitro* monolayer models do not sufficiently represent *in vivo* tumor tissue complexity[1], particularly in consideration of the interaction between protein-based therapeutics, such as antibodies, with its 3-dimensional (3D) environment[2]. We aimed to generate *in vitro* 3D models out of cancer cell lines which could yield spheroids in a rapid, robust and uniform manner. Using these spheroid models, we subsequently developed methods to evaluate the tissue penetration capability and cytotoxic activity of structurally distinct antibodies or ADCs bearing various payload classes targeting multiple TAAs.

Establishment of an automated spheroid generation methodology

A Ultra-low attachment (ULA) surfaces and automated cell seeding enable the fast and reliable scaffold-free self-assembly of cancer cells into a 3D arrangement

Spheroid Generation Workflow

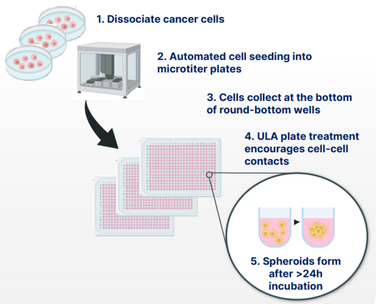
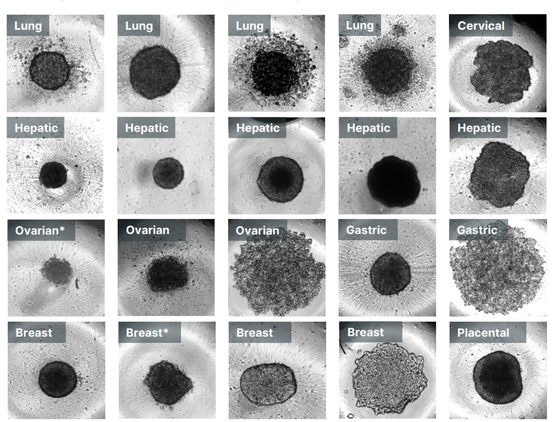


Figure 1. (A) Schematic of high-throughput 3D spheroid generation method using Ultra-Low Attachment (ULA) plates. Cancer cell lines are seeded into microtiter ULA plates using an automated liquid-handling robot, briefly centrifuged, and incubated for 2-3 nights under standard culturing conditions to allow for spheroid formation. **(B)** Representative brightfield images of 3D monoculture spheroids generated from cancer cell lines of varying tissue types or co-cultured with a human fibroblast cell line by ULA method.

B Cancer cell spheroids exhibit a wide range of morphologies not present in traditional monolayer models



* Seeded 1:1 with fibroblast cell line

The spheroid penetration capability of antibodies and ADCs was evaluated by high-content confocal fluorescence imaging

Spheroid Penetration Workflow

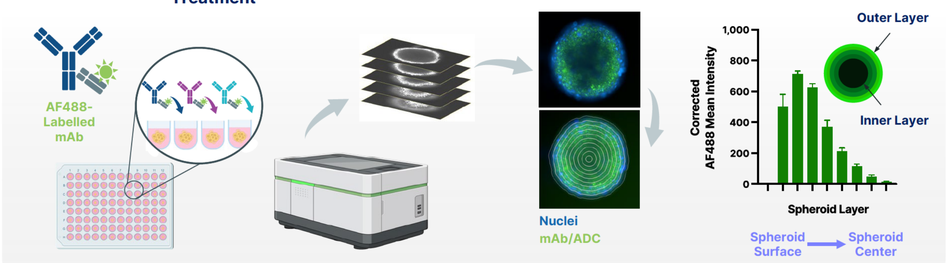
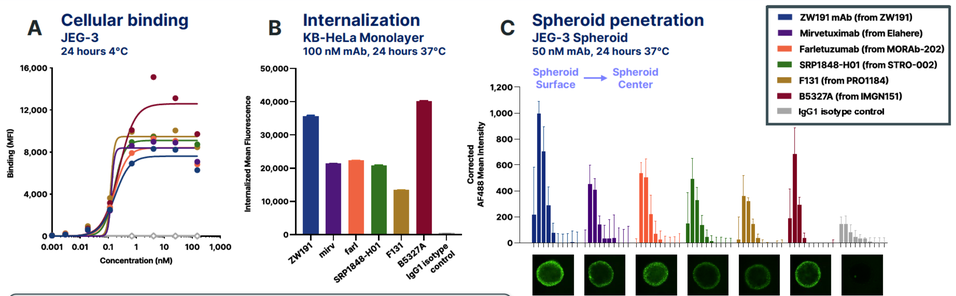


Figure 2. Schematic of high-content confocal fluorescence imaging and analysis methodology for spheroid penetration assay. Briefly, spheroids are treated with antibodies or ADCs, which are indirectly labelled with AlexaFluor® 488 fluorophore. Following treatment, spheroids are stained with a nuclear dye (Hoechst 33342), imaged and analyzed using Harmony® software (Revvity). Mean fluorescence intensity within each layer of the spheroid is quantified and visualized per layer.

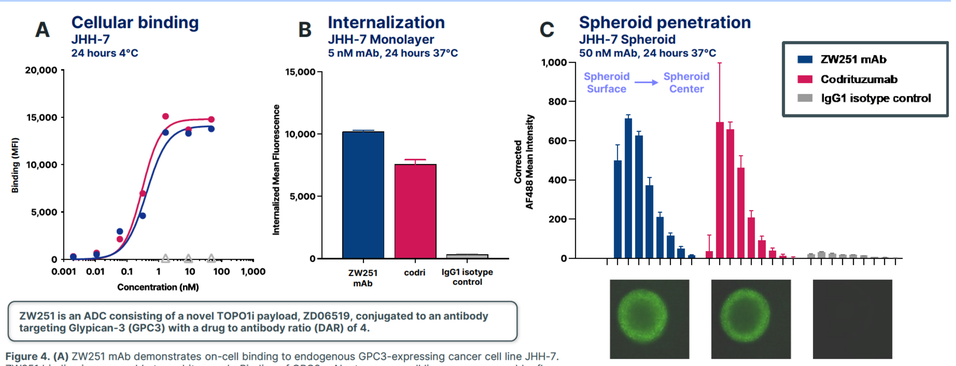
Case study 1: FRα-targeting ADC ZW191 demonstrates superior antigen-mediated binding, internalization and spheroid penetration capabilities compared to other anti-FRα mAbs



ZW191 is an ADC consisting of a novel topoisomerase 1 inhibitor (TOPO1i) payload, ZD06519, conjugated to an antibody targeting Folate Receptor Alpha (FRα) with a drug to antibody ratio (DAR) of 8.

Figure 3. (A) ZW191 mAb demonstrates on-cell binding to endogenous FRα-expressing cancer cell line JEG-3 comparable to other full-sized IgG1 molecules. Binding of FRα mAbs to cancer cell lines was assessed by flow cytometry. **(B)** ZW191 mAb demonstrates FRα-mediated internalization into endogenous FRα-expressing cancer cell line KB-HeLa, superior to other full-sized IgG1 molecules and comparable to the biparatopic antibody, B5327A. Internalized fluorescence of AF488-labelled FRα mAbs into cancer cell lines after 24 hours was determined by flow cytometry (external fluorescence was quenched prior to analysis). **(C)** ZW191 mAb demonstrates greater spheroid penetration capabilities into JEG-3 spheroids, by depth and intensity, compared to other full-sized IgG1 molecules including biparatopic B5327A. Adapted from Lawn et al. 2023[3].

Case study 2: GPC3-targeting ADC ZW251 demonstrates comparable antigen-mediated binding, internalization and spheroid penetration profile to GPC3 mAb, codrituzumab



ZW251 is an ADC consisting of a novel TOPO1i payload, ZD06519, conjugated to an antibody targeting Glypican-3 (GPC3) with a drug to antibody ratio (DAR) of 4.

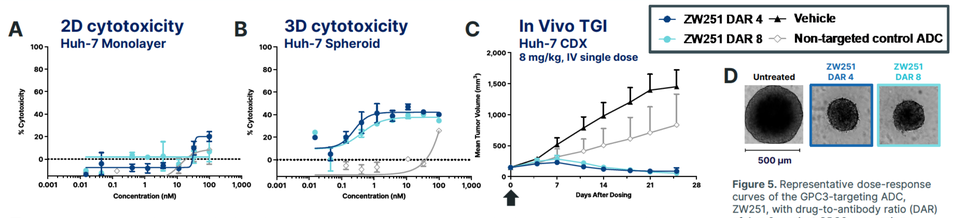
Figure 4. (A) ZW251 mAb demonstrates on-cell binding to endogenous GPC3-expressing cancer cell line JHH-7. ZW251 binding is comparable to codrituzumab. Binding of GPC3 mAbs to cancer cell lines was assessed by flow cytometry. **(B)** ZW251 mAb demonstrates GPC3-mediated internalization into JHH-7 cells. Internalized fluorescence of AF488-labelled GPC3 mAbs into cancer cell lines after 24 hours was determined by flow cytometry (external fluorescence was quenched prior to analysis). **(C)** ZW251 mAb demonstrates good spheroid penetration capabilities into JHH-7 spheroids, comparable to codrituzumab. Adapted from Madera et al. 2023[4].

A novel 3D cytotoxicity assay was developed and optimized to enable high-throughput screening of ADC panels

3D Cytotoxicity Workflow

Spheroids were treated with a serial dilution of test sample and incubated under standard culturing conditions for 6 days. Following treatment, cell viability was quantified using an ATP quantification luminescent reagent, brightfield confocal imaging, and live/dead cell fluorescent stains (data not shown).

Case study 3: A 3D cytotoxicity assay provides differentiated activity from a classic 2D monolayer assay in the context of ZW251 ADC evaluation



E ZW251 DAR differentiation in the 3D assay is more predictive of *in vivo* activity than the 2D assay

Cell Line	Expression • Flow cytometry quant • IHC analysis	2D Activity (Differentiation DAR 8 vs DAR 4)	3D Activity (Differentiation DAR 8 vs DAR 4)	In vivo TGI 8 mg/kg single dose (Differentiation DAR 8 vs DAR 4)
HepG2	• 4,100,000 GPC3/cell • H-score: 278	Active (DAR 8 > DAR 4)	Active (DAR 8 = DAR 4)	Active (DAR 8 = DAR 4)
Hep3B	• 1,300,000 GPC3/cell • H-score: 267	Inactive	Inactive	Active (DAR 8 = DAR 4)
JHH-7	• 1,100,000 GPC3/cell • H-score: 198	Active (DAR 8 > DAR 4)	Active (DAR 8 > DAR 4)	Active (DAR 8 > DAR 4)
JHH-5	• 380,000 GPC3/cell • H-score: 300	Active (DAR 8 > DAR 4)	Active (DAR 8 = DAR 4)	Active (DAR 8 = DAR 4)
Huh-7	• 260,000 GPC3/cell • H-score: 265	Inactive	Active (DAR 8 = DAR 4)	Active (DAR 8 = DAR 4)

Figure 5. Representative dose-response curves of the GPC3-targeting ADC, ZW251, with drug-to-antibody ratio (DAR) of 4 or 8, against GPC3-expressing hepatocellular carcinoma cell line Huh-7 in monolayer **(A)** or spheroid **(B)** formats. In 2D, ZW251 DAR 4 and 8 are inactive at the concentrations tested (EC_{50} >100 nM); in 3D, ZW251 DAR 4 and 8 are comparably active with similar EC_{50} values (0.20 nM and 0.25 nM, respectively). **(C)** In vivo tumor growth inhibition plot of ZW251 in BALB/c nude mice subcutaneously engrafted with Huh-7 cell line derived xenograft (CDX), 5-6 mice per group. **(D)** HepG2 spheroids treated with 150 nM ADC for 6 days exhibit decreased size by brightfield imaging. **(E)** Comparative analysis of in vitro 2D and 3D potency with in vivo cell line derived xenograft (CDX) tumor growth inhibition by ZW251 with DAR 8 or 4 in various cancer cell lines expressing GPC3.

TGI = tumor growth inhibition

Case study 4: The 3D cytotoxicity assay of ADCs bearing different topoisomerase 1 inhibitor (TOPO1i) payloads is strongly predictive of in vivo tumor growth inhibition activity

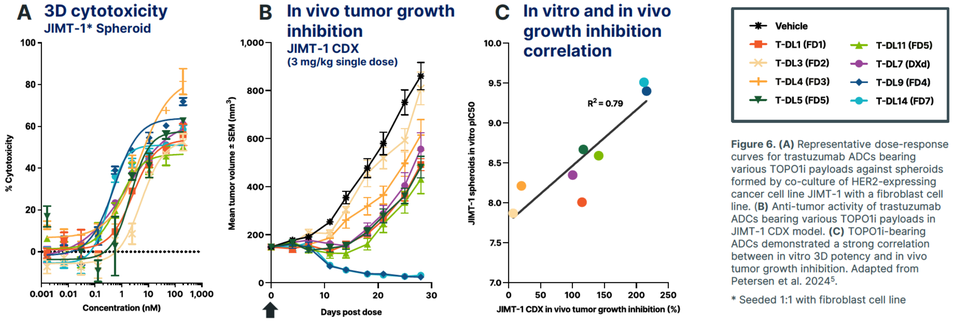


Figure 6. (A) Representative dose-response curves for trastuzumab ADCs bearing various TOPO1i payloads against spheroids formed by co-culture of HER2-expressing cancer cell line JIMT-1 with a fibroblast cell line. **(B)** Anti-tumor activity of trastuzumab ADCs bearing various TOPO1i payloads in JIMT-1 CDX model. **(C)** TOPO1i-bearing ADCs demonstrated a strong correlation between in vitro 3D potency and in vivo tumor growth inhibition. Adapted from Petersen et al. 2024[5].

* Seeded 1:1 with fibroblast cell line

Conclusions

- A readily implementable method for the rapid generation of cancer cell line spheroids was established and applied to over 50 distinct immortalized cancer cell lines derived from over 10 tissue types, demonstrating varying morphological features, with a success rate of >95%.
- In vitro assays were developed to evaluate the spheroid penetration capability and 3D cytotoxic activity of mAbs and ADCs, enabling the interrogation of various antibody formats and payload classes.
- These 3D models and assays serve as valuable functional tools which provide improved translation between *in vitro* and *in vivo* activity, supporting the characterization of therapeutic ADC candidates and their pipeline advancement.

References

1. Voskoglou-Nomikos T, Pater JL, Seymour L. Clinical predictive value of the in vitro cell line, human xenograft, and mouse allograft preclinical cancer models. *Clin Cancer Res.* **2023**, 9(11), 4227-4239.
2. Vasalou C, Helmlinger G, Gomes B. A mechanistic tumor penetration model to guide antibody drug conjugate design. *PLoS One.* **2015**, 10(3), e0118977.
3. Lawn S, Hernández Rojas A, Colombo R, et al. Abstract 2641: ZW191, a novel FRa-targeting antibody drug conjugate bearing a topoisomerase 1 inhibitor payload. *Cancer Res.* **2023**, 83, 2641.
4. Madera L, Hernández Rojas A, Colombo R, et al. Abstract 2658: ZW251, a Novel Glypican 3-Targeting Antibody Drug Conjugate Bearing a Topoisomerase 1 Inhibitor Payload. *Cancer Res.* **2023**, 83, 2658.
5. Petersen ME, Brant MG, Lasalle M, et al. Design and evaluation of ZD06519, a novel camptothecin payload for antibody drug conjugates. *Mol Cancer Ther.* **2024**, https://doi.org/10.1158/1535-7163.mct-23-0822.