Comprehensive *in vitro* Mechanistic Characterization of a Trop2-Directed ADC (Dato-DXd)

Pengfei Gao[1]; Marta Westwood[2]; Xinghan Liu[1]; Yasong Cui[1]; Yuhui Wang[1]; Fang He[1]; Chunyan Han[3]; Cheng Zhang[3];Xiaoxue Wang[3]; Mandy Xu[3]; Tan Pang[4]; Chong Wang[4]; Mengya Tong[4]

[1]*in vitro* Biology, Pharmaron, Beijing, China. [2]*In vitro* Biology, Pharmaron, Hoddesdon, UK. [3]*in vitro* ADMET, Pharmaron, Beijing, China. [4]*in vivo* Pharmacology, Pharmaron, Beijing, China. www.pharmaron.com

Summary

ADC discovery required deconvoluting target engagement, trafficking, stability, and linker/payload behavior to understand efficacy and safety.

Here we present an integrated workflow to mechanistically profile Dato-DXd, showing that the ADC binds and internalizes selectively into Trop2^{high} cells, releases DXd in lysosomes, and drives true donor-dependent bystander killing.

Dato-DXd (datopotamab deruxtecan) was discovered by Daiichi Sankyo and jointly developed by Daiichi Sankyo and AstraZeneca.

It is a Trop2-directed ADC featuring a protease-cleavable tetrapeptide linker, designed for high plasma stability and efficient lysosomal cleavage, making it well-suited for delivering DXd specifically within target-expressing cells. It carries a DXd topoisomerase-I inhibitor payload and received FDA approval in 2025 for HR-positive, HER2-negative breast cancer and EGFR-mutant NSCLC.

Pharmaron's Capabilities Mechanistic *in vitro* Profiling of ADCs

Target Antigen Evaluation
- Profiling on cell lines, organoids, and animal tissues via genomics, transcriptomics, proteomics
- In-house & public database integration
- Target antigen expression quantification, localization, internalization

Functional ADC Testing and Biomarkers
- DNA damage, apoptosis, cell cycle profiling
- Cell death, proliferation, signalling modulation
- Translational organoid models and bystander effects
- Biomarker testing via genomics, proteomics, cellomics

Payload Identification
- High-throughput screening for biochemical and cell-based assays
- Affinity-selection mass spectrometry
- DEL library synthesis and screening

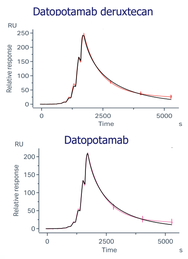
Biophysical and Structural Evaluation
- Epitope accessibility and binding affinities
- Structural integrity, stability or aggregation after bioconjugation
- Payload positioning and linker orientation via CryoEM

Linker Evaluation and *in vitro* ADMET
- Plasma stability and DAR
- Lysosomal and protease-mediated payload release, catabolism
- ADC internalization and payload release after internalization
- Bioanalysis for component tracking

ADC *in vitro* Toxicology
- Fc-mediated immunotoxicity
- Cytokine storm
- Off-target toxicity via normal organoids testing and safety panel screening
- Mitochondrial and liver toxicity

1 Trop2 Binding Affinity is Maintained after Conjugation

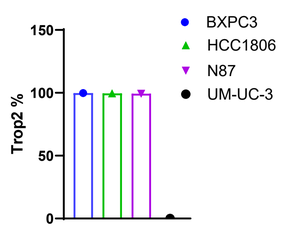
Test article	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Stoichiometry
Datopotamab	2.21E+05	2.13E-03	9.62E-09	221.6	2.0
Dato-Dxd	2.59E+05	2.05E-03	7.91E-09	255.8	2.0



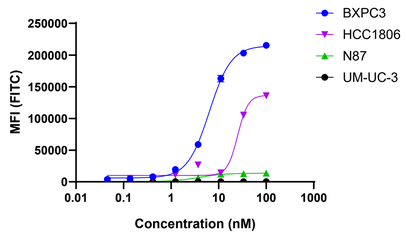
SPR analysis shows that datopotamab and Dato-DXd retain comparable nanomolar Trop2 affinity, indicating that conjugation does not impair Fab–Trop2 binding.

Binding kinetics and stoichiometry values further confirm no loss of epitope accessibility and fully preserved functional binding sites.

2 Selective Binding to Trop2^{high} Cells

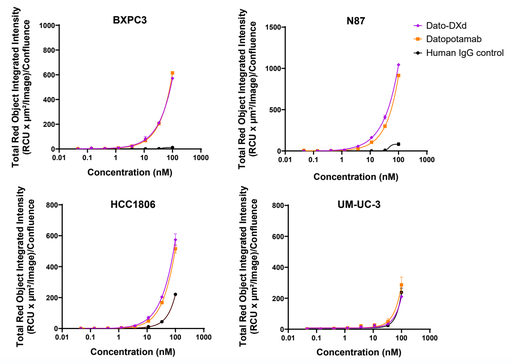


Flow cytometry confirmed high Trop2 surface levels on BxPC-3, HCC1806 and N87, with low expression on UM-UC-3.



Dato-Dxd showed binding only on Trop2^{high} cells, with no binding in the Trop2^{low} line UM-UC-3, demonstrating target-dependent engagement.

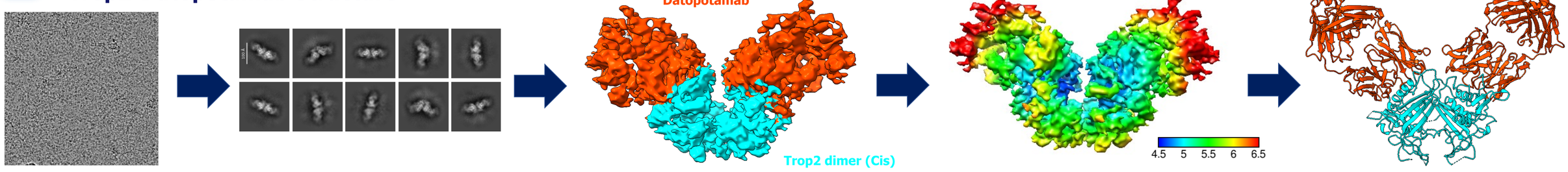
3 Internalization Only in Trop2^{high} Cells



ADC internalization was assessed using a pH-sensitive fluorescence assay (Phrodo-labeled antibodies) and Incucyte imaging.

The results confirmed efficient internalization and lysosomal trafficking in Trop2^{high} cell lines.

4 Trop2-Datopotamab Structure

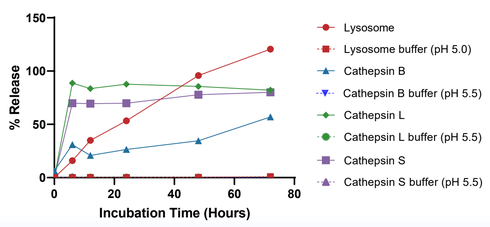


Micrograph of Trop2-Datopotamab | 2D class averages of Trop2-Datopotamab | Cryo-EM map of Trop2-Datopotamab | Local resolution of Trop2-Datopotamab | Model of Trop2-Datopotamab

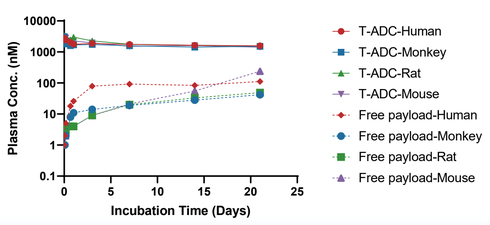
Preliminary structure determination of the Trop2-Datopotamab complex was performed via CryoEM. Trop2 protein was expressed in Hi-5 cells, and the cis-dimeric complex architecture was determined through a complete cryo-EM structure determination workflow from raw micrograph to refined atomic model.

5 Lysosomal Cleavage and Plasma Stability

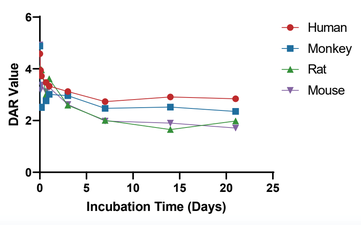
A DXd release is protease dependent



B Dato-DXd is very stable in plasma across species



C Time-dependent DAR changes of Dato-DXd during 21-day plasma incubation

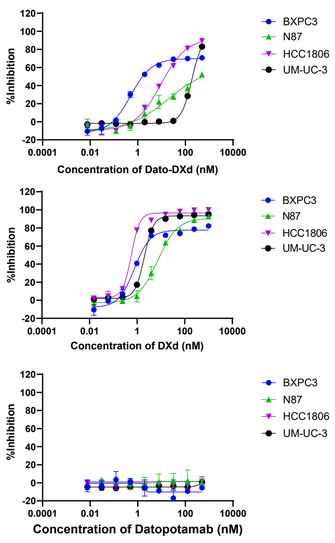


(A) Payload release assay shows that DXd release from Dato-DXd is protease-driven under lysosomal conditions. Cathepsin L and Cathepsin S account for most of the cleavage, while Cathepsin B contributes less and more slowly. These data support Cathepsin L/S as the primary enzymes mediating intracellular linker cleavage and payload release.

(B) *In vitro* plasma stability of Dato-DXd across human, monkey, rat, and mouse plasma over 21 days demonstrates that the intact ADC remains largely stable in all species, while free DXd payload increases gradually over time, with greater release in human and mouse and lower release in monkey and rat, indicating species-dependent linker stability..

(C) DAR values decrease over time in all species, indicating gradual deconjugation of the ADC, with relatively better stability in human and monkey plasma and faster DAR loss in rat and mouse.

6 Trop2-Specific Cytotoxicity



Dato-DXd Cytotoxicity

Dato-Dxd shows higher cytotoxicity in Trop2^{high} cells BXPC3 and N87 compared to Trop2^{low} cell line UM-UC-3, confirming target-dependent payload delivery.

DXd Cytotoxicity

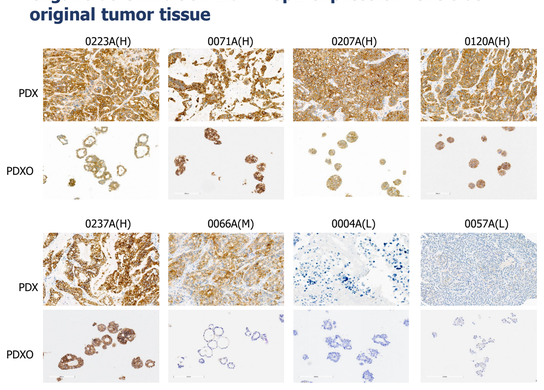
Free Dxd kills both Trop2^{high} and Trop2^{low} cell lines.

Datopotamab Cytotoxicity

The naked antibody has no killing activity.

7 Efficacy in PDX-Derived Organoids Correlates with Trop2 Expression Level

A Organoids exhibit similar Trop2 expression levels as original tumor tissue



B Dato-DXd efficacy in organoids is Trop2-dependent.

Trop2-TPM	Trop2-IHC	PDXO_ID	Datopotamab	Dato-DXd	Deruxtecan	DXd
			IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)
			mAb	ADC	Payload+linker	Payload
1153.77	High	PANC0223A	> 694.4	0.54	189.15	1.3
671.22	High	NSCLC0071A	> 694.4	160.47	1150	3.37
401.43	High	NSCLC0207A	> 694.4	0.54	435.59	1.72
363.69	HIgh	OVC0120A	> 694.4	0.08	182.4	0.47
343.35	HIgh	CRC0038A	> 694.4	0.824	296.2	0.979
250.98	HIgh	GAS0101A	> 694.4	>671.1	854.9	2.67
224.04	HIgh	NSCLC0181A	> 694.4	149.46	595.05	1.64
215	HIgh	NSCLC0238A	> 694.4	10.13	615.57	1.26
170.59	HIgh	NSCLC0196A	> 694.4	86.39	458.4	1.09
140.82	High	NSCLC0237A	> 694.4	440.67	1209	3.759
113.96	High	NSCLC0175A	> 694.4	64	565.2	0.17
58.89	Moderate	NSCLC0066A	> 694.4	581.1	560.28	1.88
11.28	low	CRC0004A	> 694.4	292	183.3	0.42
1	low	GAS0057A	> 694.4	175.97	452.8	1.4

(A) IHC of patient-derived xenograft (PDX) tumors and matched organoids confirm preserved Trop2 expression *ex vivo*.

(B) Dato-DXd induced selective killing of Trop2^{high} organoids, whereas the antibody itself showed no activity. The weaker effect of DXd+linker versus free DXd demonstrates restricted permeability of the conjugated payload.

8 Efficient Bystander Killing *in vitro* Demonstrated by Two Independent Assays

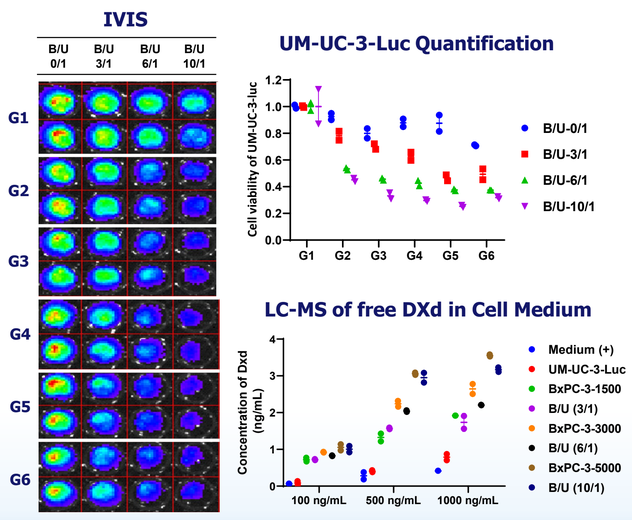
A Luciferase Co-Culture Assay

BxPC-3 (Trop2^{high}) and luciferase-expressing UM-UC-3-Luc (Trop2^{low}) cells were co-cultured at defined B/U ratios (BxPC-3 / UM-UC-3-Luc).

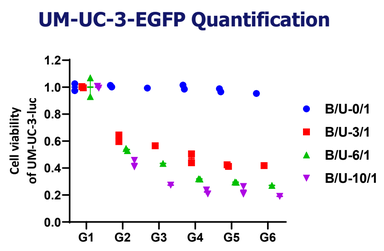
After Dato-DXd treatment, luminescence from UM-UC-3-Luc decreased only when BxPC-3 cells were present, demonstrating donor-dependent DXd-mediated bystander killing.

LC-MS confirmed increasing levels of free DXd in the medium with higher numbers of BxPC-3 cells.

G1. Dato-Dxd-0 ng/mL
G2. Dato-Dxd-100 ng/mL
G3. Dato-Dxd-200 ng/mL
G4. Dato-Dxd-500 ng/mL
G5. Dato-Dxd-1000 ng/mL
G6. Dato-Dxd-2000 ng/mL



B IncuCyte EGFP Co-Culture Assay



A similar experiment was performed via IncuCyte with EGFP-expressing UM-UC-3 cells.

Dato-DXd caused selective loss of EGFP-positive UM-UC-3 cells, again only in the presence of BxPC-3, confirming DXd-driven elimination of neighboring Trop2^{low} bystander cells.

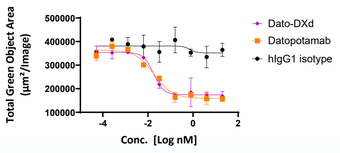
9 Assessment of FcRn Binding, ADCP, and ADCC After Conjugation

FcRn Binding Affinity is Preserved after Bioconjugation

Sample	ka (1/Ms)	kd (1/s)	KD (M)	Affinity KD (M)
Datopotamab	5.36E+05	4.16E-01	7.75E-07	7.93E-07
Dato-DXd	2.92E+05	2.70E-01	9.26E-07	1.03E-06

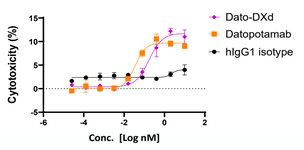
SPR analysis shows Dato-DXd preserves FcRn binding affinity comparable to datopotamab, indicating that conjugation does not disrupt IgG-like recycling, supporting maintained systemic exposure and favorable pharmacokinetics.

ADCP Activity Remains Unchanged after Bioconjugation



Identical ADCP (antibody-dependent cellular phagocytosis) potency using macrophages and N87 cells demonstrates that bioconjugation does not impair macrophage engagement or phagocytic clearance.

ADCC Potency is Moderately Reduced upon Bioconjugation



ADCC (antibody-dependent cellular cytotoxicity) activity with NK cells and N87 cells shows 5-fold reduced Dato-DXd potency, demonstrating partial Fc-effector reduction after conjugation.

PhalVBPos0226v1