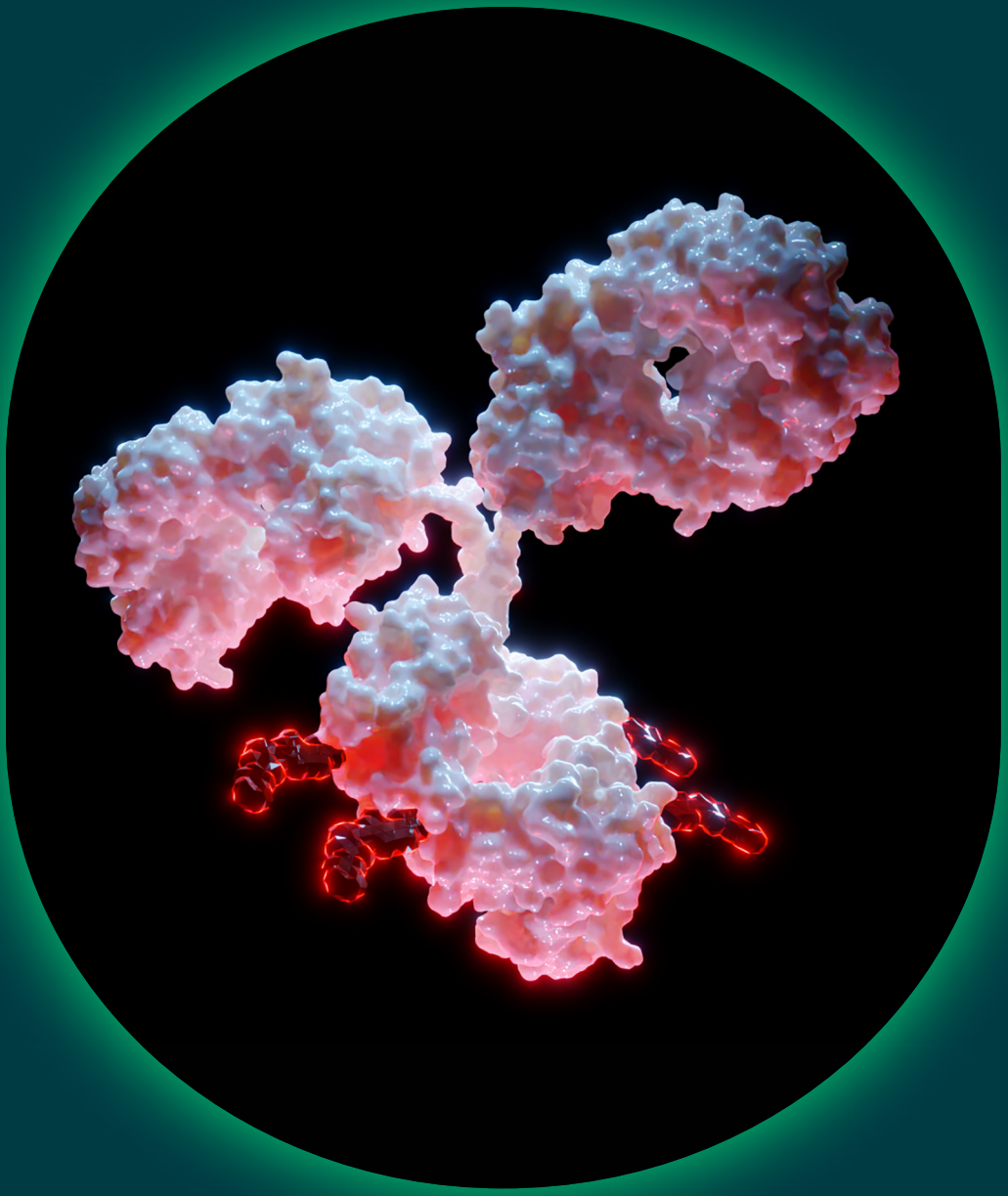
EXPERT INSIGHTS

Comprehensive Insights into Antibody–Drug Conjugates (ADC) for Precision Cancer Therapy



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Contents

Introduction

Despite decades of progress in oncology, the fundamental challenge of delivering potent therapeutics specifically to tumor cells while minimizing harm to healthy tissue remains a central goal of cancer treatment. Traditional chemotherapy, while effective against rapidly dividing cells, cannot distinguish between malignant and normal tissues, resulting in severe systemic toxicity and dose-limiting side effects. Antibody-drug conjugates (ADCs) represent a paradigm shift in this pursuit, offering a precision medicine approach that combines the targeting specificity of monoclonal antibodies with the cell-killing potency of cytotoxic drugs.

Each ADC consists of three essential components: a monoclonal antibody that recognizes a tumor-associated antigen expressed on cancer cells, a linker molecule that connects the antibody to the payload and releases the drug at tumor sites, and a cytotoxic payload that induces cell death upon internalization by tumor cells. This elegant architecture enables ADCs to deliver chemotherapeutic agents directly to malignant cells, achieving dramatically higher efficacy and reduced systemic toxicity compared to conventional chemotherapy.

The clinical impact of ADCs has been substantial. Since the first FDA approval in 2000, the field has expanded to over 15 approved ADCs for treating hematologic malignancies and solid tumors, including breakthrough therapies such as trastuzumab deruxtecan (Enhertu®) for HER2-positive breast cancer and brentuximab vedotin (Adcetris®) for Hodgkin lymphoma. Hundreds of ADC candidates are currently in clinical development, and the global ADC market continues to grow rapidly, reflecting both commercial success and genuine therapeutic advancement.

However, despite these clinical successes, two critical limitations continue to constrain the full potential of ADC therapy and represent major frontiers for innovation. At the biological level, tumor heterogeneity poses a fundamental challenge. ADCs act primarily on antigen-positive cell populations, leaving cancer cells with insufficient or absent antigen expression untouched. This heterogeneity within tumors—where antigen expression varies widely across individual cells—reduces overall treatment efficacy and creates reservoirs of resistant cells that can drive disease relapse. The ability to eliminate not only antigen-positive cells but also neighboring antigen-negative cells through “bystander killing” has emerged as a crucial design goal for next-generation ADCs.

At the manufacturing level, traditional ADC synthesis remains complex, costly, and time-intensive. Conventional production requires multiple purification steps before conjugation, followed by additional modifications and quality control procedures. This multistep process increases production costs, extends development timelines, introduces batch-to-batch variability, and ultimately limits the scalability and accessibility of these potentially life-saving therapies. Streamlining ADC synthesis without compromising product quality represents a critical technical bottleneck for the field.

This collection presents two complementary innovations that directly address these biological and manufacturing challenges, demonstrating how rational design and process innovation can advance ADC therapeutics toward broader impact.

Overcoming Tumor Heterogeneity through AI-Guided Payload Design

The first study, by Guo *et al*. [1], presents a machine learning-guided framework for rational payload optimization. Using computational modeling to predict structure-activity relationships, the authors designed Ed9, an optimized exatecan derivative engineered for enhanced bystander killing. The resulting ADC, T-VEd9, demonstrated superior tumor regression compared to the FDA-approved reference ADC trastuzumab deruxtecan (DS-8201/Enhertu®) in preclinical models of antigen-heterogeneous tumors. Notably, T-VEd9 achieved effective killing of both antigen-positive and antigen-negative cancer cells through payload diffusion, directly addressing the clinical challenge of tumor heterogeneity. This work exemplifies how artificial intelligence can accelerate the discovery of next-generation payloads with tailored pharmacological properties, moving beyond trial-and-error approaches toward rational, prediction-driven design.

Simplifying ADC Manufacturing through One-Pot Synthesis

The second study, by Lu *et al*. [2], tackles the manufacturing bottleneck with an innovative "one-pot" strategy that enables direct preparation of site-specific ADCs from unpurified antibodies in cell culture medium. This approach eliminates conventional antibody purification steps entirely, reducing production time from days to approximately two hours while maintaining mild physiological conditions throughout. Remarkably, the ADCs generated through this streamlined process exhibited physicochemical stability and cytotoxic activity comparable to traditionally prepared conjugates, with the added advantage of site-specific conjugation that ensures product homogeneity. By dramatically simplifying the production workflow, this method offers a cost-effective platform for rapid, large-scale ADC manufacturing that could significantly improve accessibility to these therapies.

Ensuring Quality through Advanced Analytical Characterization

Comprehensive characterization of ADC conjugation sites remains essential for quality control and regulatory compliance. Peptide mapping, a pivotal analytical technique, provides site-specific information about where cytotoxic payloads attach to antibody scaffolds—critical data for understanding product heterogeneity in lysine-conjugated ADCs. The third contribution to this Expert Insights eBook demonstrates this characterization approach using ado-trastuzumab emtansine (T-DM1) as a model lysine-linked ADC. Through an integrated workflow combining automated sample preparation, high-resolution liquid chromatography, and advanced mass spectrometry, twenty-six drug conjugation sites were confidently identified with exceptional mass accuracy (within 3 ppm) and sequence coverage (94%). The analysis achieved outstanding reproducibility across replicate samples, with retention time variations below 0.27%. This work illustrates how modern analytical platforms—exemplified by Agilent's peptide mapping solutions, including the AssayMAP Bravo, 1290 Infinity II bio LC, and 6545XT AdvanceBio LC/Q-TOF systems—enable the precise site-specific characterization necessary for developing and manufacturing these heterogeneous biotherapeutics.

Looking Forward

Together, these studies represent a pivotal transition in ADC development—from increasingly complex molecules requiring elaborate production methods toward more rationally designed, efficiently manufactured therapeutics with enhanced clinical potential. The integration of AI-guided payload design, streamlined synthesis, and advanced analytical characterization creates a comprehensive framework for accelerating ADC development and improving patient outcomes in precision cancer therapy. While this collection focuses specifically on payload optimization, synthesis innovation, and quality characterization, the ADC field continues to evolve across multiple dimensions, including novel linker chemistries, alternative antibody formats, and strategies for combination therapy. The innovations presented here demonstrate that overcoming current limitations in cancer treatment requires both biological insight and engineering creativity.

This collection is designed for researchers, clinicians, and industry professionals seeking to understand emerging strategies in ADC development for oncology applications. Through the methods and applications presented in these studies, we aim to provide practical insights into how computational design, process engineering, and analytical precision can advance the next generation of targeted cancer therapeutics. The findings underscore that enhanced accessibility, improved scalability, and greater clinical impact are not merely aspirational goals but achievable outcomes through thoughtful innovation in molecular design, manufacturing strategy, and quality control.

For researchers looking to implement these approaches or explore complementary technologies in ADC development, additional resources and technical guidance are available on the **Agilent** website.

Róisín Murtagh
Senior Content Strategist, Wiley

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[1] Guo, Y. *et al*. (2024). Rational Identification of Novel Antibody-Drug Conjugate with High Bystander Killing Effect against Heterogeneous Tumors. *Advanced Science*. **https://doi.org/10.1002/advs.202306309**.

[2] Lu, M. *et al*. (2025). Direct Preparation of Site-Specific Antibody-Drug Conjugates with Unpurified Antibodies in Culture Medium. *Chembiochem*. **https://doi.org/10.1002/cbic.202401082**.

Rational Identification of Novel Antibody-Drug Conjugate with High Bystander Killing Effect against Heterogeneous Tumors

Adapted from Guo *et al.* [1]

In modern antibody–drug conjugate (ADC) design, "bystander-killing" payloads have emerged as a critical issue. In a heterogeneous tumor microenvironment, cells express the target antigen unevenly. As a result, traditional ADCs that depend on antigen binding for uptake may miss the cells that do not express the 'target antigens', leading to incomplete elimination of tumor cells during targeted chemotherapy and allowing chemotherapy-resistant clones to survive and propagate. On the other hand, ADCs with bystander killing payloads release membrane-permeable cytotoxins that diffuse out of the targeted, antigen-positive cell and then kill neighboring cells with low or even negative antigen expression. However, rational designing of payloads, or systematically selecting and optimizing payloads based on quantifiable biological, chemical, and pharmacological features, is challenging. The lack of a comprehensive rational design, instead of a trial-and-error approach in payload designing, limits the clinical potential of ADCs in killing heterogeneous tumors.

To address this gap, the authors of this study focused on tumors heterogeneously expressing human epidermal receptor 2 (HER2) antigen, a target for ADC therapy, and adopted the strategy to rationally design the ADC-payload using artificial intelligence, followed by developing the payloads optimized for bystander activity. They employed a graph-attention-network (GAT) computational model, a neural network was designed to work on graph-structured data in a context-specific way—paying closer attention to defined criteria of different hyperparameters of the model while mapping molecular structures of different candidate payload molecules for predicting permeability and activity. Using the GAT model, they rationally designed potent exatecan-derived bystander payloads for use in ADC.

Once several efficient bystander-killing exatecan/camptothecin derivative payloads were designed by the GAT model, the authors identified the most potent variant among the payloads, identified as ED9, with satisfactory permeability and bioactivity. Through linker optimization and conjugation, they designed a novel ADC (T-VEd9) with excellent anti-tumor efficacy and bystander-killing effect when evaluated in several cancer cell lines expressing varying levels of HER2 and in HER2+ cancer-bearing mice. The novel ADC showed superior anti-tumor efficacy when compared to DS-8201, the conventional trastuzumab-deruxtecan ADC targeting HER2, in heterogeneous tumor models.

Methods

Mass spectrometry

- Mass spectra were collected using Agilent 1260/G6125B liquid chromatography single quadrupole mass spectrometer.
- Agilent Technologies 6224 quadrupole/time of flight LC/MS spectrometer and Agilent 6540 quadrupole/time of flight LC/MS were used for High-resolution mass spectrum (HRMS).

Chromatographic Characterization

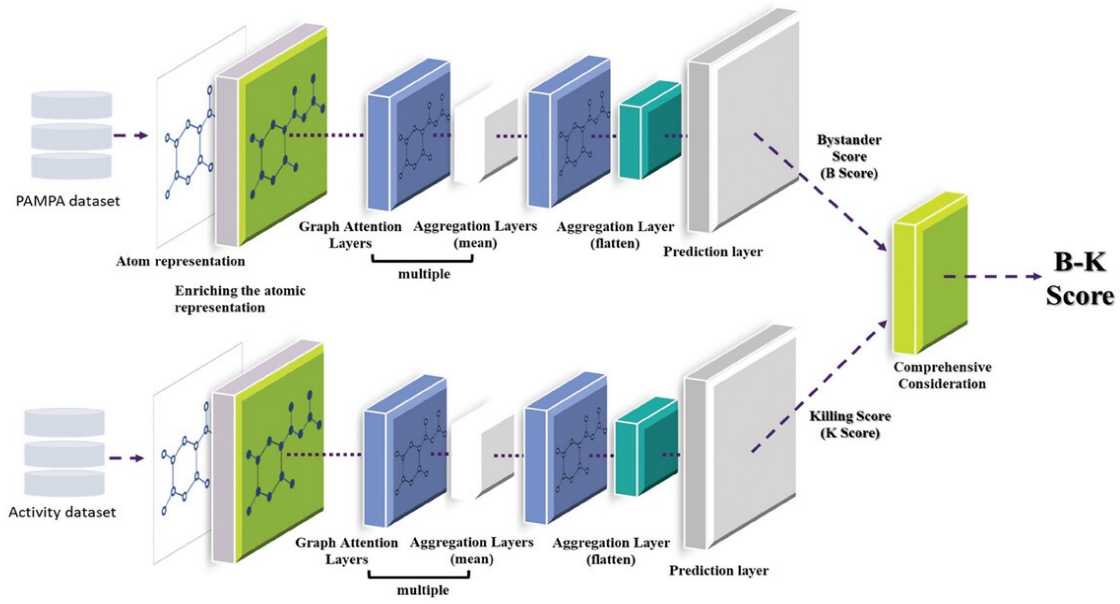
- High-performance liquid chromatography (HPLC) for small molecules was performed on an Agilent 1260 Infinity II (LC03) instrument equipped with a C18 reversed-phase column (Agilent Eclipse XDB-C18, 4.6×250 mm, 5 μm).
- Reversed-phase high-performance liquid chromatography (RP-HPLC) for antibodies and conjugates was performed on an Agilent 1260 Infinity II (LC03) instrument equipped with a reversed-phase column (Agilent PLRP-S 1000 Å, 2.1×150 mm, 8 μm).
- HPLC for antibodies and conjugates was performed on an Agilent 1260 Infinity II (LC03) instrument equipped with columns.
- Hydrophobic interaction chromatography (HIC) for antibodies and conjugates was performed on an Agilent 1260 Infinity II (LC03) instrument equipped with a HIC column.

Results:

The authors argued that a single permeable descriptor, such as cLogD, defining a single physicochemical property derived from calculations, is oversimplistic for designing complex scaffolds. Therefore, they first developed a GAT-driven "bystander score" (B-score) involving comprehensive molecular characterizations to capture molecular features tied to both potency and membrane permeability. This approach facilitated the selective aggregation of information from the most relevant neighboring nodes, offering deeper insights into the underlying molecular properties. Then they trained and validated the GAT model on curated payload data (Fig. 1A).

FIGURE 1

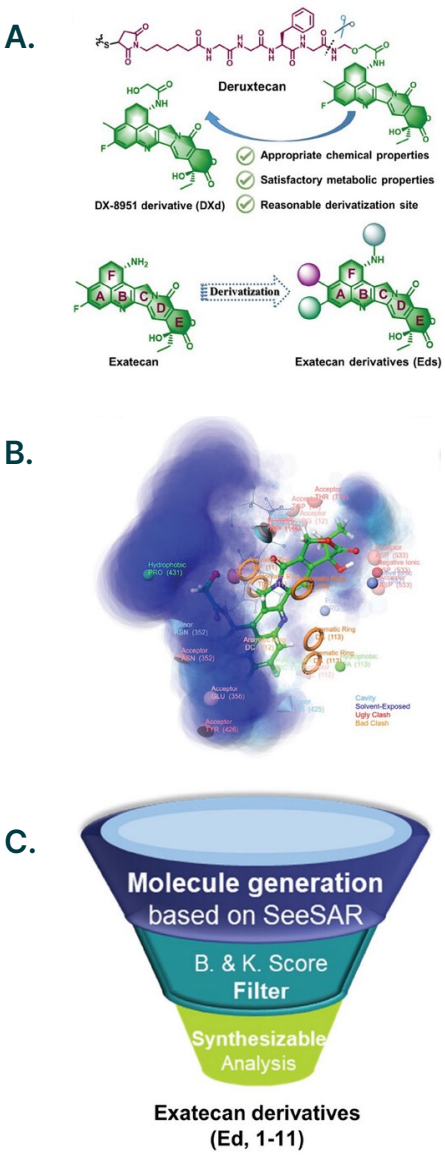
A.



The architecture of the model is divided into two sections, each consisting of a molecular characterization layer, multiple GAT layers, aggregation layers, and a prediction layer. These sections are identical in structure but process different input data. Finally, the scores calculated by these two sections are comprehensively considered and serve as the final B-K score.

Then they used the model alongside multi-parameter molecular characterization to rank molecules with bystander potential. As the therapeutic molecule, they selected exatecan, a structural analog of camptothecin, a class of chemotherapeutic drugs with moderate lipophilicity and unique metabolic properties acting on topoisomerase I. A similar GAT model was used to predict the killing scores (K score) for predicting the Topo I inhibitory effect of the derivative molecules. The *in silico* B-K Score screening led to a shortlisted set of candidates for synthesis (Fig. 2A-C).

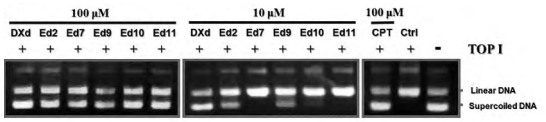
FIGURE 2



A) Schematic illustration of the structure and generation strategy of exatecan derivatives (Eds). **B)** Modification site analysis by determining solvent exposue of DXd in DXd/Topo I/DNA complex. **C)** Generation and screening process of Eds.

Next, the authors synthesized multiple bystander-killing exatecan derivatives (Eds) and profiled their biochemical properties using the DXd molecule as a reference. Among these, Ed9 emerged as the top candidate, combining high permeability and satisfactory cytotoxicity in multiple tumor cell lines with different expression levels of HER2 (NCI-N87, SKBR-3, MDA-MB-453, MDA-MB-361, JIMT-1, and MDA-MB-231) (Fig. 2A-B).

FIGURE 2



IC_{50} (nM)	DXd	Ed9
NCI-N87 (HER2++)	9.03	1.17
SKBR-3 (HER2++)	73.93	13.14
MDA-MB-453 (HER2+)	261.5	120.3
MDA-MB-361 (HER2+)	16.42	1.69
JIMT-1 (HER2+)	576.6	193.3
MDA-MB-231 (HER2-)	25.77	2.69

A) Topo I inhibitory activity of Eds and DXd at 10 μM and 100 μM, with CPT (100 μM) as positive control. **B)** Proliferation inhibitory activity of Ed9 and DXd against human cancer cell lines with different HER2 expression.

TABLE 1

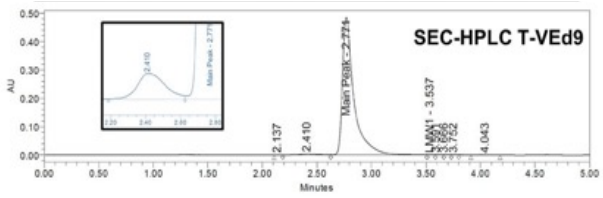
IC_{50} (nM)	NCI-N87 (HER2+)	MDA-MB-453 (HER2+)
T-DXd	0.089	0.029
T-Ed9	0.331	0.091
T-VDXd	0.052	0.025
T-VEd9	0.091	0.071

The half-maximal inhibitory concentration (IC_{50}) of ADCs with different L-Ps.

The authors then optimized linker chemistry for Ed9 payloads. ADCs were constructed on trastuzumab with a controlled drug-antibody ratio (DAR) of 8 and characterized by reversed-phase high-performance liquid chromatography (RP-HPLC), hydrophobic interaction chromatography (HIC), and size-exclusion chromatography (SEC) to confirm homogeneity and binding. They found the canonical tetrapeptide linker used in some DXd ADCs was incompatible with Ed9 in T-Ed9 ADC, due to unsatisfactory linker cleavage, monitored by HPLC and LC/ESI-MS, and payload release. Substituting the tetrapeptide linker with a Val-Ala-based dipeptide linker (T-Ved9 ADC) improved payload release, reflected in superior proliferation inhibitory activity (Table 1), and the ADC mainly existed in monomer (>98.5% monomer) (Fig. 3), guiding the final ADC development.

In *in vitro* bystander assays, T-VEd9 ADC showed HER2-specific cytotoxicity in single-cell lines with different HER2 expressions (MDA-MB-231/GFP, HER2-; SKBR-3, HER2++; and NCI-N87, HER2++) and, importantly, killed neighboring HER2-negative cells in co-culture systems more effectively than comparator ADCs (T-DXd and T-DM1) (Fig. 4A-C). Mixed-cell proliferation assays and assays using conditioned supernatants demonstrated that released Ed9 was membrane-permeable and capable of transferring cytotoxicity to antigen-negative cells, producing strong bystander killing activity (Fig. 4D-F).

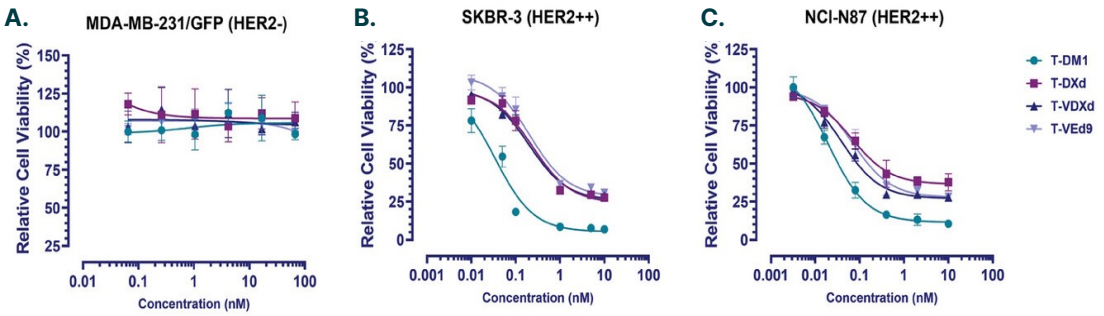
FIGURE 3



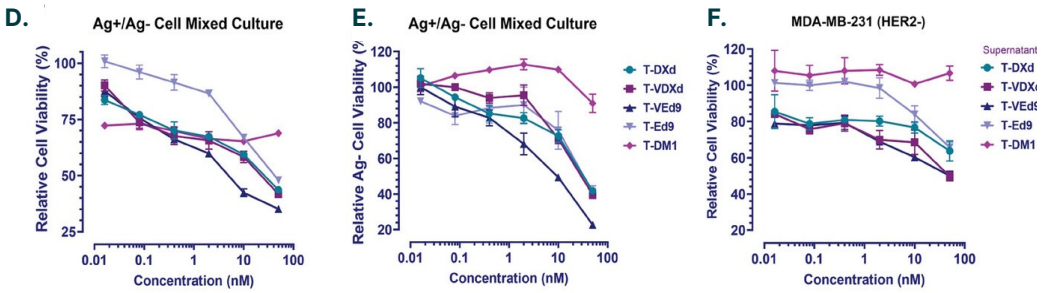
Area	Tras	T-DXd	T-VEd9
HMW	1.3	1.4	1.1
Monomer	98.6	98.6	98.7

Size-exclusion chromatography (SEC) of DAR8 ADCs. Absorbance wavelength was 280 nm. Isomer ratio was calculated by absorbance area. DAR, drug-to-antibody ratio.

FIGURE 4



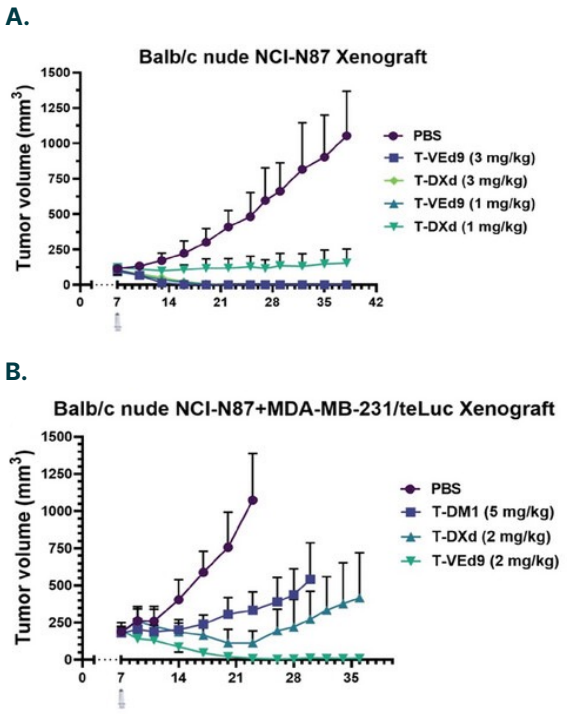
In vitro evaluation of ADCs. Proliferation inhibitory activity of ADCs against **A)** MDA-MB-231/GFP (HER2-) cells, **B)** SKBR-3 (HER2+) cells, and **C)** NCI-N87 (HER2+) cells. Tumor cells were treated with ADCs for 5 days, and relative cell viability (%) was calculated.



D) Proliferation inhibitory activity of ADCs against both HER2+ and HER2- cells in the co-culture system, error bars represent S.E.M. **E)** Proliferation inhibitory activity of ADCs against HER2- cells in the co-culture system, error bars represent S.E.M. **F)** Proliferation inhibitory activity against HER2- cells of HER2+ cells supernatant cultured with ADCs, error bars represent S.E.M. All data shown are representative of more than two independent duplicates.

In HER2+ breast cancer (SKBR-3)-bearing mice, a single dose of T-VEd9 (10 mg·kg^{-1}) produced curative responses in SKBR-3 tumors without observed weight loss or systemic toxicity, and no signs of tumor recurrence (Fig. 5A). In HER2+ gastric cancer (NCI-N87) bearing mice, T-VEd9 showed significant potency even at lower doses (1 mg·kg^{-1}) where T-DXd produced only partial inhibition. In a xenograft model of heterogeneous tumor having both HER2+ NCI-N87 cells and HER2- MDA-MB-231/teLuc cells, T-VEd9 substantially suppressed luciferase signal and tumor growth in comparison to controls, indicating a pronounced in vivo bystander killing effect (Fig. 5B).

FIGURE 5



***In vivo* evaluation of ADCs. A) Anti-tumor activity in HER2+ NCI-N87 gastric cancer model following a single intravenous ADC dose of 1 or 3 mg kg^{-1}. B) Tumor volume change. Bystander killing in a co-inoculation xenograft model following a single intravenous ADC dose of 2 or 5 mg kg^{-1}. Luciferase activity was detected by *in vivo* imager after intraperitoneal injection of substrate.**

Discussion

The heterogeneity of the "target" is a critical factor affecting the efficacy of ADCs. The findings showed that the effective scoring approach facilitates the discovery of novel ADCs with appropriate payloads having promising efficiency to eliminate bystanders in heterogeneous tumors. The key findings included the ability of the GAT model to successfully rank exatecan derivatives for potency and permeability, strong cytotoxicity of Ed9 on antigen-positive cells and measurable bystander killing, superior tumor control in heterogeneous xenograft models, and acceptable stability and pharmacokinetics for the conjugates.

The study not only performed novel integration of machine learning (GAT) with payload chemistry and ADC construction, resulting in a clear rational design pipeline, but also carried out a preclinical comparison with a clinically relevant ADC (DS-8201) in tumor heterogeneity-focused animal models.

Conclusion

In summary, the study showed that GAT-driven in silico scoring can rationally identify efficient bystander-capable payloads (like Ed9) for targeting heterogeneous tumors. The corresponding ADC (T-VEd9) showed improved anti-tumor activity in heterogeneous tumors relative to a clinically used exatecan-based ADC (DS-8201). In the future, the GAT scoring approach could be applied to diversify payload chemistry beyond exatecan scaffolds for discovering next-generation ADC payloads.

REFERENCE

[1] Guo, Y. *et al*. (2024). Rational Identification of Novel Antibody-Drug Conjugate with High Bystander Killing Effect against Heterogeneous Tumors. *Advanced Science*. https://doi.org/10.1002/advs.202306309.

Direct Preparation of Site-Specific Antibody–Drug Conjugates with Unpurified Antibodies in Culture Medium

Antibody–drug conjugates (ADCs) are an important class of targeted cancer therapeutics that offer improved selectivity and reduced systemic toxicity. ADCs combine the specificity of monoclonal antibodies with the potent cytotoxic effects of small-molecule traditional chemotherapeutic drugs, linked to the antibody through a chemical linker. Although more than a dozen ADCs have been approved for clinical use, owing to the structural complexity of ADCs, their production remains challenging.

Adapted from Lu *et al.* [1]

The main challenge arises from the complex, multi-step manufacturing process entailing expression of recombinant antibodies, purification of those antibodies, synthesis of the linker-payload, drug-antibody conjugation, and a final purification step to isolate the ADC. While each step requires sophisticated equipment and quality control, antibody purification in particular adds substantial cost and time, hindering rapid scale-up. Thus, a long-standing bottleneck in ADC-mediated cancer therapy is the absence of a strategy for direct synthesis from unpurified antibody culture media.

In this study, the authors adopted a new strategy that enables direct synthesis of site-specific ADCs from unpurified antibodies present in cell culture medium, thereby eliminating one major purification step. The study is built on a previously reported one-step synthesis of site-specific ADCs, where precise ligand-directed conjugation was achieved by a peptide-directed acyl-transfer mechanism to couple drug payloads onto antibody molecules at a defined lysine residue (K248) in the Fc domain of native IgG antibodies [2].

The authors applied their Fc-binding peptide (FcBP)-directed thioester (TE)-based conjugation strategy directly to antibody-containing culture supernatants. The method included transient expression of recombinant antibody in HEK293F cells, followed by direct reaction with a culture supernatant containing the unpurified antibody in a ‘one-pot’ conjugation step, and a final single purification step to isolate the ADC. This ‘one-pot’ approach aims to match the quality of ADCs obtained by the conventional multi-step process, in which the antibody is purified first and then conjugated. The strategy applies to a broad range of antibodies and diverse substrates. Its robustness and substrate tolerance suggested that it might work even in a varied span of complex cell-culture media.

Methods

Liquid Chromatography tandem time of flight mass spectrometer

- Agilent 6545 Liquid Chromatography tandem time of flight mass spectrometer (LC-TOF-MS) with an Agilent column (AdvanceBio RP-mAb C4 2.5 × 50 mm, 3.5 µm) was the key analytical instrument that was essential for characterizing the antibodies and ADCs, specifically for LC–MS mass confirmation of the site-specific conjugation of the molecules, determining the precise DAR, and checking product homogeneity and purity.

Hydrophobic interaction chromatography

- Analytical hydrophobic interaction chromatography (HIC) was performed using a HIC column (Agilent, 4.6 x 100 mm, 3.5 µm) at 25°C while profiling the conjugation homogeneity and hydrophobicity of ADCs.

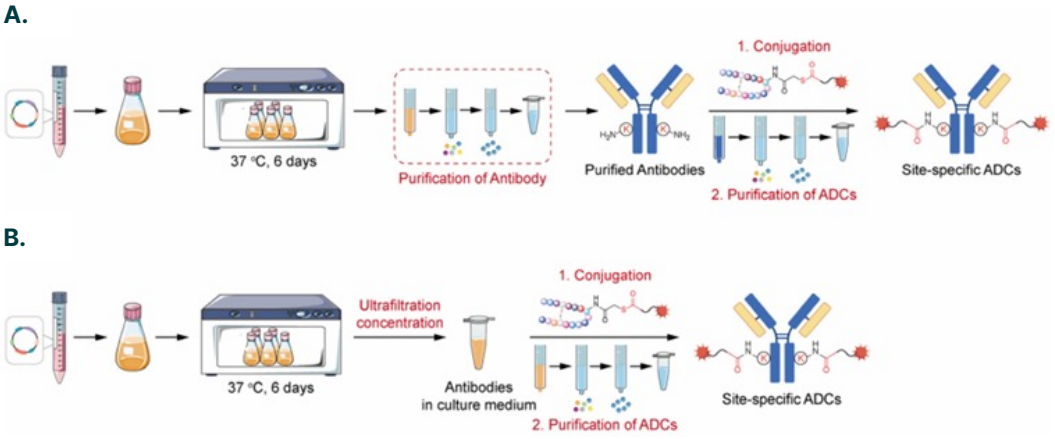
Results

Experimental Verification and Optimization

The authors first tested the system using Zilovertamab, a well-characterized monoclonal antibody targeting cancer-associated ROR1 protein, which conjugates the microtubule inhibitor Monomethyl auristatin E (MMAE), forming the approved ADC Zilovertamab Vedotin.

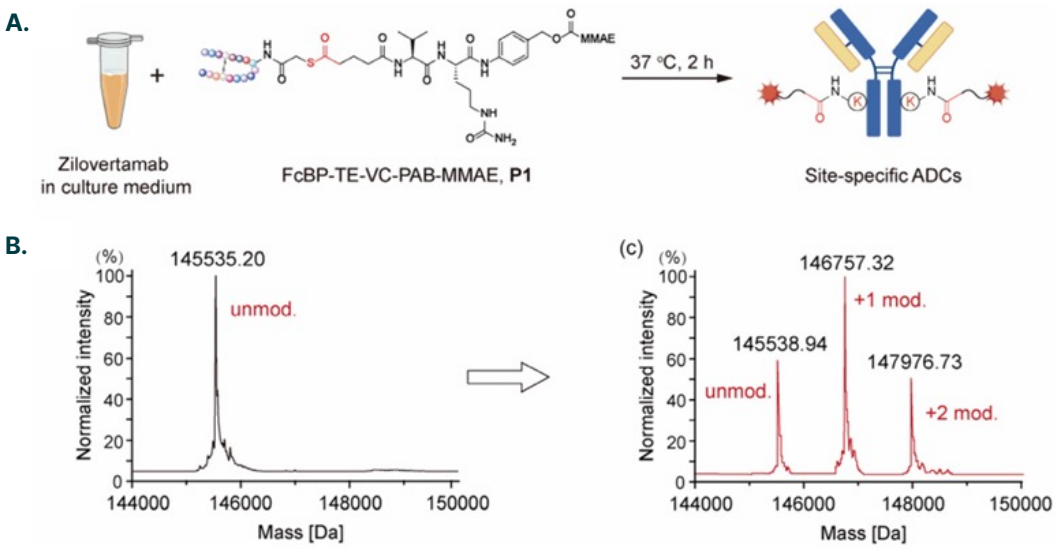
First, the antibody plasmid was transfected into HEK293F cells for 7 days. Then, the culture medium containing Zilovertamab from HEK293F cells was brought to a requisite concentration (2 mg mL^{-1}). The unpurified culture medium containing Zilovertamab was reacted directly with a thioester-based drug-linker FcBP-TE-valine–citrulline (VC)-p-Aminobenzyl (PAB)-MMAE (FcBP-TE-VC-PAB-MMAE; denoted as P1) at 37°C for two hours. Liquid chromatography-mass spectrometry (LC-MS) confirmed successful conjugation, with drug-to-antibody ratios (DARs) that increased with longer reaction times and/or higher drug concentrations (Fig. 1).

FIGURE 1



The synthesis of site-specific ADCs in the antibody expression system. **A)** Previous two-step preparation of site-specific ADCs with purified antibodies. **B)** Direct one-pot preparation of site-specific ADCs in culture medium within cellular expression systems without antibody purification.

FIGURE 2



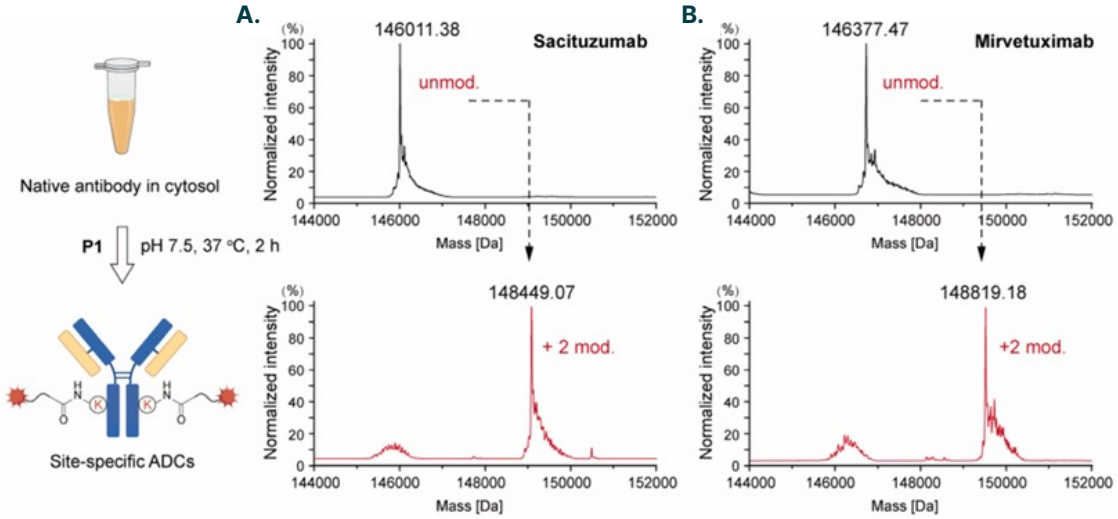
Synthesis of site-specific ADCs with unpurified antibodies in culture medium. **A)** Schematic illustration of synthesizing site-specific ADCs with P1; LC-MS analysis of **B)** Zilovertamab and c) after incubation with P1 at 37°C for 2h.

To improve the efficiency of the reaction, several reaction parameters such as time (extending reaction time from 2 h to 19 h increased DAR from 0.85 to 1.86), reagent equivalents (increasing linker-drug concentration modestly improved DAR to about 2.0 up to a plateau gained at 20 eq), and pH (adjusting reaction pH from 6.2 to 7.5 accelerated conjugation) were optimized. The optimized conditions were thus 20 equivalents of P1, pH 7.5, 37 °C, reaction time 2 h, producing homogeneous site-specific ADCs with a DAR of about 2 (Fig. 2).

Antibody and Linker Versatility

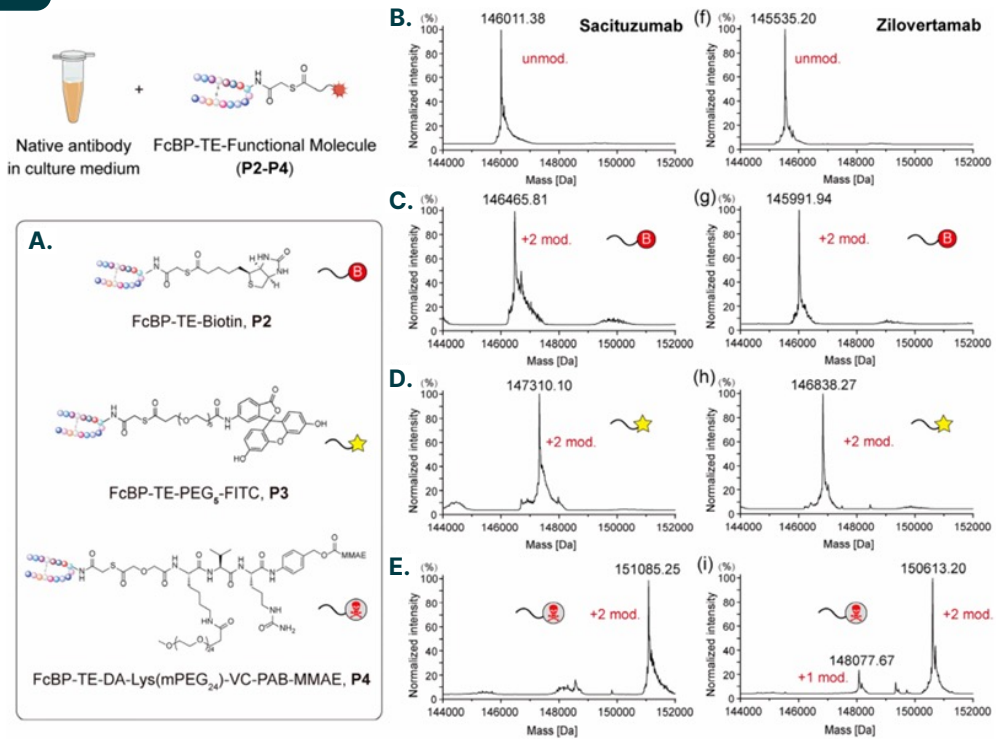
To test the universality of the approach, the authors applied the method to Sacituzumab (anti-Trop2) and Mirvetuximab (anti-FRα) antibodies to prepare site-specific antibodies. Under the optimized reaction condition, for both the drugs, homogeneous site-specific ADCs with P1 were prepared successfully in a single culture medium (Fig. 3).

FIGURE 3



Application to different antibodies. LC-MS analysis of site-specific ADCs made from Sacituzumab **A)** and Mirvetuximab **B)** in culture medium.

FIGURE 4



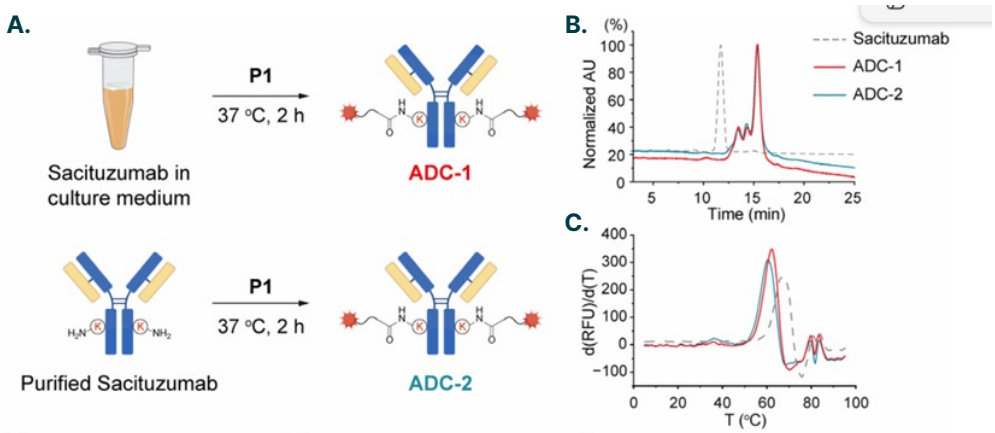
Synthesis of site-specific functional antibodies in culture medium. **A)** Structure of FcBP-TE-functional molecule, P2–P4; b) LC-MS analysis of Sacituzumab, and site-specific functional antibody made from Sacituzumab expression system with **C)** P2, d) P3, e) P4; f) LC-MS analysis of Zilovertamab and **G)** site-specific functional antibody made from Zilovertamab expression system with P2, h) P3, i) P4

The compatibility of this conjugation chemistry of the antibody with a variety of linker payloads containing additional functional groups, including FcBP-TE-biotin (P2), FcBP-TE-FITC (P3), and FcBP-TE-branched PEG-linked (P4), was assessed (Fig. 4). LC-MS confirmed successful conjugation in every case.

Analytical Characterization and Biological Activity

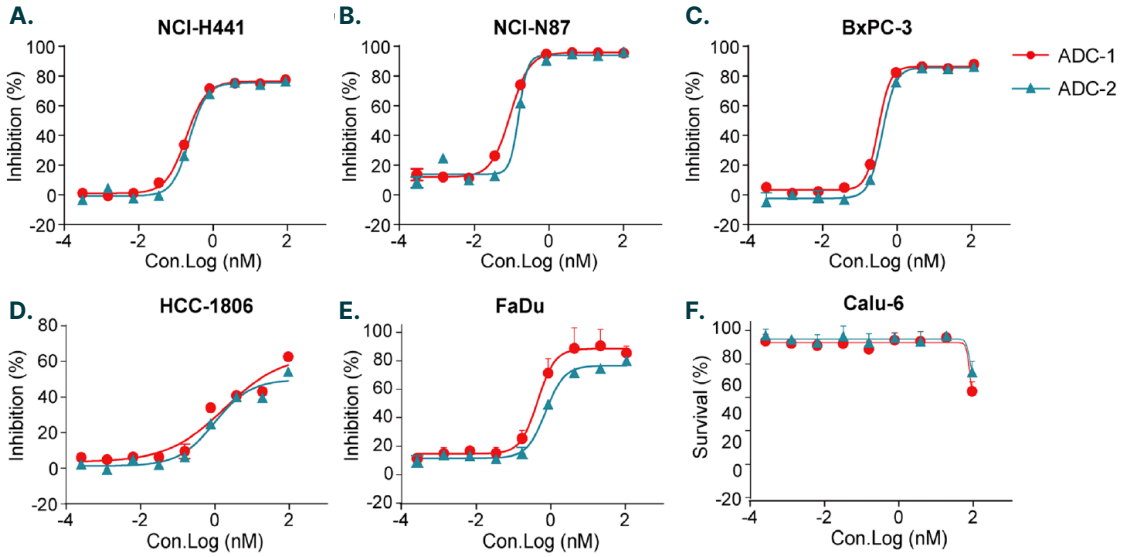
Physicochemical properties of ADCs prepared from unpurified antibody in the direct method were compared to those from antibody purified by the conventional method (Fig. 5a). Hydrophobic interaction chromatography confirmed homogeneous product formation in both approaches, with similar retention times, indicative of comparable hydrophilicity (Fig. 5b) and thermal stabilities above 60°C for both types of ADCs (Fig. 5c).

FIGURE 5



Physicochemical properties of site-specific ADCs. **A)** Schematic preparation of ADC-1 and ADC-2; **B)** hydrophilicity and homogeneity of ADCs; **C)** thermal stabilities of both types of ADCs.

FIGURE 6



Physicochemical properties of site-specific ADCs. **A)** Schematic preparation of ADC-1 and ADC-2; **B)** hydrophilicity and homogeneity of ADCs; **C)** thermal stabilities of both types of ADCs.

Finally, anti-tumor efficacy was characterized in vitro using several Trop2-positive cell lines (NCI-H441, NCI-N87, BxPC-3, HCC-1806, FaDu) and a negative control line (Calu-6). The ADCs generated by both the direct and classical methods showed similar IC50 values, demonstrating similar potency and consistency in cell-killing abilities in antigen-positive cells (Fig. 6).

Discussion

The authors applied their Fc-binding peptide (FcBP)-directed thioester (TE)-based conjugation strategy directly to antibody-containing culture supernatants. The method employs a multifunctional acyl transfer reagent, FcBP-TE-VC-PAB-MMAE, which binds the antibody Fc domain, transfers the payload to the K248 residue, and then self-cleaves to leave a stable amide linkage between the antibody and the drug MMAE. The modification process did not need antibody engineering or complex bioorthogonal reactions. The method produced homogeneous ADCs (DAR ≈ 2) for three therapeutic antibodies—Zilovertamab, Sacituzumab, and Mirvetuximab—within two hours at mild conditions, requiring only one final purification step.

The critical advance in this work is the demonstration that such conjugation can occur directly within the antibody expression system, meaning the culture medium containing secreted antibody can undergo ADC preparation with only a single purification step afterward. This represents a major simplification compared to the classical two-step protocol that requires prior antibody purification. The direct conjugation approach led to the generation of ADCs with comparable biochemical and cytotoxic characteristics, affirming that the necessary qualities for pharmaceutical applications were retained. The advantages of the technique included reduced process complexity and cost, mild and efficient reaction conditions, site-specific and homogeneous products, broad compatibility, comparable quality, and bioactivity.

Conclusion

Overall, this study introduces a practical and scalable method for rapid, site-specific ADC preparation without extensive antibody pre-purification that can accelerate preclinical research. The findings suggested broad application potential not only in therapeutic ADCs but also in fluorescent labeling, biochemical, and diagnostic applications.

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Application Note

Biopharma

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Identification of Conjugation Sites in an Antibody Drug Conjugate

Using the Agilent 6545XT AdvanceBio LC/Q-TOF system

Suitable for Agilent
1290 Infinity III LC

Author

Yulan Bian
Agilent Technologies, Inc.

Abstract

This application note highlights the workflow of drug conjugation site identification in an antibody drug conjugate (ADC). The workflow uses an Agilent AssayMAP Bravo protein sample prep platform, an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF system, and Agilent MassHunter BioConfirm 12.1 software. Twenty-six conjugation sites were confidently identified in a lysine-linked ado-trastuzumab emtansine (T-DM1) sample. The results also showed exceptional mass accuracy and consistent reproducibility.

Introduction

ADCs represent a cutting-edge class of biopharmaceuticals. ADCs are designed to deliver a cytotoxic payload specifically to a targeted site while minimizing off-target effects and enhancing therapeutic efficacy. ADCs are composed of a monoclonal antibody (mAb) linked to a potent cytotoxic agent using a chemical linker through a conjugation process. Since conjugation can occur at several available sites on lysine-based ADCs, multiple conjugation molecules can be present. Therefore, a lysine-conjugated ADC is a heterogenous mixture of conjugated biomolecules.[1]

Peptide mapping is a pivotal tool for the in-depth characterization of ADCs. Peptide mapping provides site-specific information about ADC conjugation sites. In this application note, the drug conjugation sites of a lysine-linked ADC, T-DM1, were characterized following the Agilent peptide mapping workflow. A 1290 Infinity II bio LC system and a 6545XT AdvanceBio LC/Q-TOF system were used in conjunction with an automated AssayMAP Bravo protein sample prep platform. Data analysis and mapping of conjugation sites were performed with MassHunter BioConfirm 12.1 software. The integrated workflow is illustrated in Figure 1.

Experimental

Materials

Urea, Trizma base, dithiothreitol (DTT), 2-iodoacetamide (IAA), trypsin, trifluoroacetic acid (TFA), and acetonitrile (LC/MS grade) were purchased from MilliporeSigma (Burlington, MA, USA). Formic acid (LC/MS grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). The T-DM1 sample was purchased from Alliance Pharm (Singapore, Singapore). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system (Burlington, MA, USA).

Sample preparation

During sample preparation, 6.5 mg of TDM-1 were dissolved into 1.3 mL of denaturation buffer comprised of 8 M urea, 50 mM Tris (pH 8.0). Aliquots of 100 µL of the dissolved sample were transferred into the AssayMap sample plate wells. The digestion protocol was selected using the In-Solution Digestion: Single-Plate application. In this protocol, 10 µL of 100 mM DTT were added for sample reduction. The plate was incubated at 37 °C for two hours. For alkylation, 12 µL of 200 mM IAA were added, followed by incubation at room temperature in the dark for one hour. The sample was then diluted with 400 µL of water, and 20 µL of 0.5 µg/µL trypsin was added. After incubation at 37 °C overnight, the reaction was quenched by the addition of 60 µL of 10% TFA. The digested samples were subjected to LC/MS analysis.

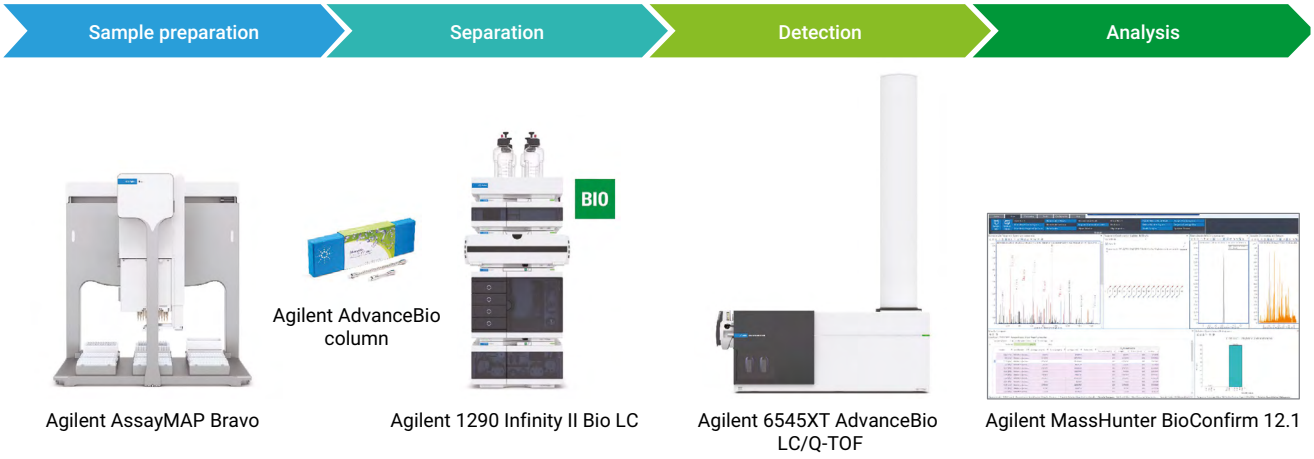

Figure 1. Agilent peptide mapping workflow.

Instrumentation

- Agilent AssayMAP Bravo protein sample prep platform (G5571AA)
- Agilent 1290 Infinity II bio LC system including:
  - Agilent 1290 Infinity II bio high-speed pumps (G7132A)
  - Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent Infinity II sample cooler (option #101)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B) equipped with Agilent bioinert QuickConnect heat exchanger, standard flow (option #065)
  - Agilent 1290 Infinity II diode array detector (G7117B) with Agilent Max-Light cartridge cell, 10 mm
- Agilent 6545XT AdvanceBio LC/Q-TOF system with Agilent Dual Jet Stream ESI source

Software

- Agilent VWorks automation control software 14.1
- Agilent MassHunter data acquisition software 11.0
- Agilent MassHunter BioConfirm software 12.1

LC/MS analysis

Tables 1 and 2 list the parameters for LC and MS data acquisition used in the workflow.

Table 1. Liquid chromatography (LC) parameters.

LC Parameters	
Column	Agilent AdvanceBio peptide mapping, 2.1 × 150 mm, 2.7 µm, 120 Å (p/n 653750-902)
Thermostat	4 °C
Solvent A	0.1% Formic acid in H_2O
Solvent B	90% Acetonitrile and 0.1% formic acid in H_2O
Flow Rate	0.4 mL/min
Gradient	Time (min) %B 0.0 3 1.0 3 70.0 45 71.0 90 73.0 90
Post Time	5 min
Injection Volume	20 µL
Column Temperature	60 °C

Table 2. Mass spectrometry (MS) data acquisition parameters.

Parameter	Value
Source	Agilent Dual Jet Stream ESI
Polarity	Positive
Drying Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Acquisition Mode	Extended dynamic range (2 GHz)
Mass Range	*m/z* 300 to 1,700
Acquisition Rate	8 Spectra/sec
Auto MS/MS range	*m/z* 50 to 1,700
Minimum MS/MS Acquisition Rate	3 Spectra/sec
Isolation Width	Narrow (~*m/z* 1.3)
Max Precursor/Cycle	Top 10
Collision Energy	3.1 × (*m/z*)/100 + 1 for charge 2; 3.6 × (*m/z*)/100 – 4.8 for charge 3 or greater than charge 3
Threshold for MS/MS	1,000 Counts and 0.001%
Dynamic Exclusion On	1 Repeat, then exclude for 0.2 min
Precursor Abundance-Based Scan Speed	Yes
Target	25,000 Counts/spectrum
Use MS/MS Accumulation Time Limit	Yes
Purity	100% Sstringency, 30% cutoff
Isotope Model	Peptides
Sort Precursors	By charge state, then abundance; +2, +3, > +3

Data analysis

Data were processed following the Protein Digest workflow in MassHunter BioConfirm 12.1 software. The conjugation was defined as a new type of modification, MCC-DM1, in the Chemical Data Dictionary Tool. The modification adds 956.3644 Da in mass specifically to the lysine residue. The processing method parameters are listed in Table 3.

Table 3. Protein digest processing method parameters.

Parameter	Value
Condition	Reduced
Mods and Profiles	Alkylation (iodoacetamide), MCC-DM1
Enzyme	Trypsin
Find Peptides	Display biomolecules containing MS/MS scans
Match Tolerances	MS match tolerance: ± 20 ppm MS/MS match tolerance: ± 50 ppm Warn if score is < 3.00 Do not match if score is < 3.00 Allow missed cleavages up to 2 Peptide length range: 4 to 70 Allow terminal truncation Max number of modifications: 4

Results and discussion

The antibody backbone of T-DM1 is trastuzumab. The lysine amines of trastuzumab and the cytotoxic agent DM1 (emtansine) are conjugated by a nonreducible thioether linker, N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC).[2] The structure of T-DM1 is shown in Figure 2.

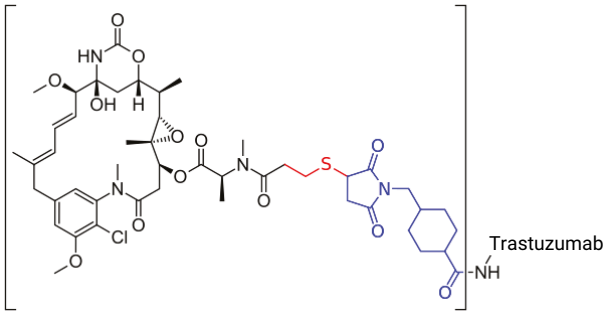

Figure 2. Molecular structure of T-DM1.

To locate the drug conjugation sites of T-DM1, the ADC was reduced, alkylated, and trypsin-digested using the In-Solution Digestion workflow on the AssayMAP Bravo platform. The digestion workflow was followed by LC/MS/MS analysis using the 6545XT LC/Q-TOF system coupled to the 1290 Infinity II bio LC system.

The DM1 payload has strong ultraviolet (UV) absorbance at 252 nm due to the presence of an aromatic ring and other chromophores in the structure. A comparison between UV chromatograms at 214 and 252 nm indicates that the main elution region of drug-conjugated peptides is from 36 minutes onwards (Figures 3A and 3B). This is further confirmed by the extracted ion chromatogram (EIC) of product ion *m/z* 547.22 (Figure 3C). This signature ion was produced from DM1 fragmentation during MS/MS. The conjugation of DM1 increases the overall hydrophobicity of the peptides, causing them to elute later in reversed-phase LC conditions.

An average sequence coverage of 94% was achieved for T-DM1. Figure 4 displays the overlaid biomolecule MS chromatograms of the peptides included in the coverage map from a single sample.

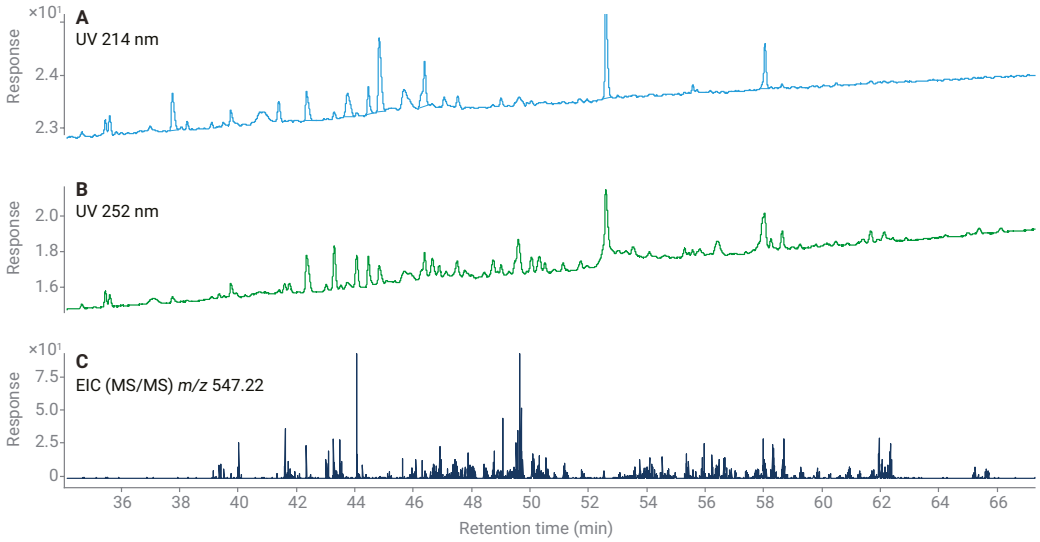

Figure 3. UV chromatograms of T-DM1 at (A) 214 nm and (B) 252 nm, and (C) the extracted ion chromatogram of T-DM1 product ion *m/z* 547.22.

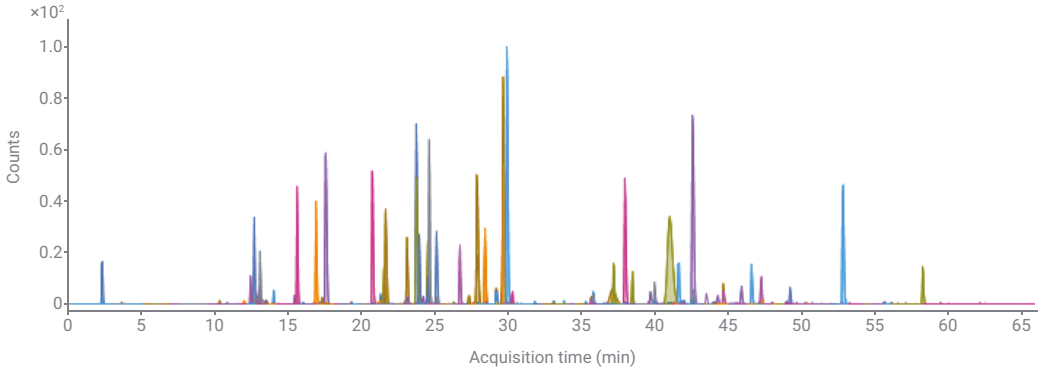

Figure 4. Overlaid biomolecule MS chromatograms of trypsin-digested T-DM1.

Among these peptides, BioConfirm software automatically identified 26 out of 44 lysine sites with conjugated MCC-DM1. The mass accuracy of all identified drug-conjugated peptides was within 3 ppm. The site locations and peptide sequences are listed in Table 4.

Table 4. MCC-DM1 conjugated peptides identified in T-DM1. The conjugation sites are marked with an asterisk (*).

No.	Chain	Site	Sequence	RT (min)	Delta Mass (ppm)
1	Light	K42	YQQKPGK*APK	41.73	0.70
2	Light	K107	VEIK*R	50.37	1.03
3	Light	K145	EAK*VQWK	51.27	1.11
4	Light	K188	ADYEK*HK	43.47	1.74
5	Light	K190	HK*VYACEVTHQGLSSPVTK	43.30	2.15
6	Light	K207	VYACEVTHQGLSSPVTK*SFNR	50.23	0.58
7	Heavy	K30	LSCAASGFNIK*DTYIHWVR	58.42	1.05
8	Heavy	K43	QAPGK*GLEWVAR	55.43	0.28
9	Heavy	K65	YADSVK*GR	49.53	1.32
10	Heavy	K76	FTISADTSK*NTAYLQMNSLR	55.9	1.96
11	Heavy	K136	GPSVFPLAPSSK*STSGGTAALGCLVK	59.34	0.63
12	Heavy	K208	ICNVNHK*PSNTK	41.86	0.76
13	Heavy	K213	ICNVNHKPSNTK*VDK	39.39	1.77
14	Heavy	K216	VDK*K	48.28	0.86
15	Heavy	K217	K*VEPK	47.03	0.93
16	Heavy	K225	SCDK*THTCPPCPAPELLGGPSVFLFPPKPK	56.47	1.05
17	Heavy	K249	THTCPPCPAPELLGGPSVFLFPPK*PK	62.15	0.16
18	Heavy	K251	LFPPKPK*DTLMISR	54.10	0.69
19	Heavy	K291	FNWYVDGVEVHNAK*TKPR	49.09	0.18
20	Heavy	K293	TK*PR	46.72	0.26
21	Heavy	K323	EYK*CK	46.75	0.93
22	Heavy	K325	CK*VSNK	46.08	0.84
23	Heavy	K329	VSNK*ALPAPIEK	52.66	0.31
24	Heavy	K337	ALPAPIEK*TISK	55.56	0.26
25	Heavy	K343	AK*GQPR	46.47	0.00
26	Heavy	K417	LTVDK*SR	50.48	0.81

Each identified sequence was verified through the following three criteria. LTVDKSR peptide with MCC-DM1 conjugated on lysine was used as an example in Figure 5 to illustrate the verification process.

- First, high quality MS/MS spectra with credible b and y ions were required to cover the peptide sequence. The fragment confirmation ladder feature in BioConfirm 12.1 software marks b and y ions based on their occurrence in the MS/MS spectra. This feature offers a rapid assessment of the quality of the MS/MS spectra for the biomolecule (Figure 5A). The enhanced peptide mapping algorithm in BioConfirm software also largely reduces artifacts for improved variable modification assignment, in this case MCC-DM1 modification.
- Second, the stereocenter in the DM1 molecule causes the drug-conjugated peptide to elute chromatographically as duplet peaks. The EIC of the peptide precursor ion confirms the presence of the stereoisomers (Figure 5B).
- Third, during peptide fragmentation, DM1 was also partially fragmented, generating DM1-associated ions. As shown in Figure 3C, *m/z* 547.22 is the most common and abundant fragment ion of DM1. Additionally, ions such as *m/z* 140.07, 453.19, and 485.22 are also present in the MS/MS spectra of the drug-conjugated peptides (Figure 5C). Furthermore, the loss of *m/z* 546.21 on y ions is commonly observed as partially fragmented DM1-linker-peptides.

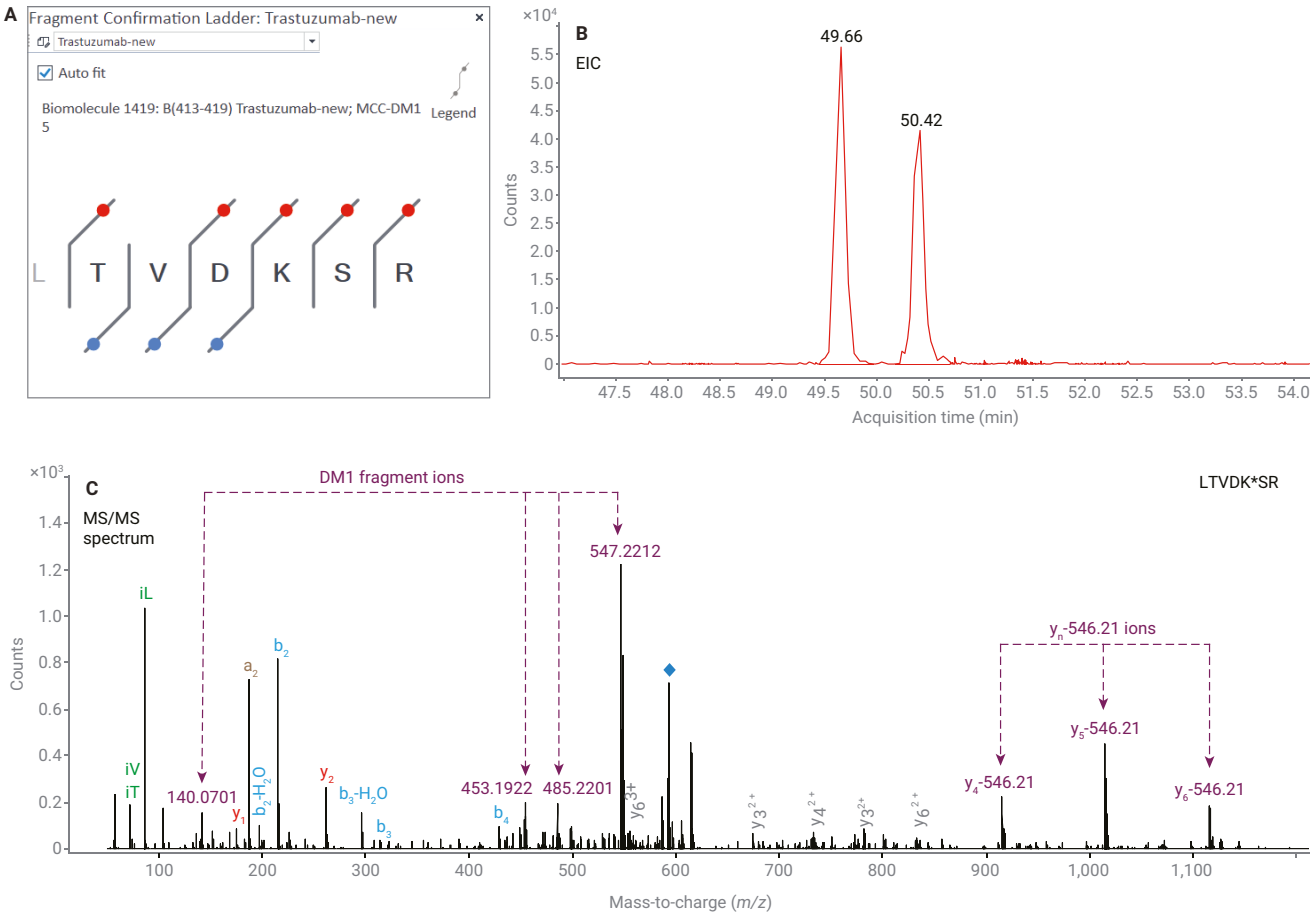

Figure 5. LTVDKSR peptide with MCC-DM1 modification on lysine. (A) The fragment confirmation ladder view in BioConfirm 12.1, (B) the extracted ion chromatogram (EIC) of precursor ion *m/z* 592.29^{3+}, and (C) the MS/MS spectra of LTVDKSR with MCC-DM1 modification.

The results also showed excellent reproducibility between seven replicated samples. Figure 6 shows overlaid biomolecule MS chromatograms of drug-conjugated peptides, including YQQKPGKAPK from the light chain and ALPAPIEKTISK from the heavy chain. Retention time (RT) relative standard deviations (RSDs) for both peptides were 0.27 and 0.03%, respectively. RSDs of abundance, in terms of signal volume, were 4.80 and 5.01%, respectively. This superior reproducibility is attributed to the automated sample preparation, reliable acquisition engine, and advanced software algorithm.

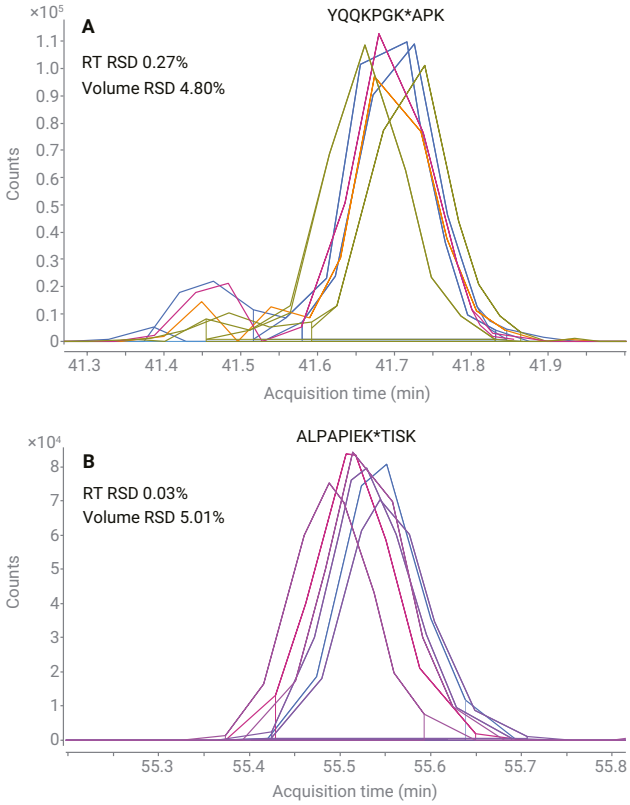

Figure 6. Overlaid biomolecule MS chromatograms of drug-conjugated peptides (A) YQQKPGK*APK and (B) ALPAPIEK*TISK.

Conclusion

In this application note, a lysine-linked ADC, T-DM1, was analyzed using the Agilent peptide mapping workflow to identify its drug conjugation sites. This workflow included an Agilent AssayMAP Bravo protein sample prep platform for automated sample preparation, an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF system, and Agilent MassHunter BioConfirm software. Using this workflow, 26 lysines were confirmed to be drug-conjugated. These results demonstrate that the Agilent peptide mapping workflow enables accurate and reproducible identification of drug conjugation sites in ADCs.

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DE-002358

Printed in the USA, November 6, 2024
5994-7922EN

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Advancing Antibody-Drug Conjugates

Interview with Prof. Kyoji Tsuchikama

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Website: Tsuchikama Laboratory

Kyoji Tsuchikama, PhD, is Associate Professor and Barbara and Peer Boedeker Professor in Cancer Research at the University of Texas Health Science Center at Houston (UTHealth Houston). He leads an interdisciplinary program developing next-generation ADCs for cancer therapy. Prof. Tsuchikama earned his PhD in organic chemistry from Waseda University, Japan, and completed postdoctoral training in chemical biology at Scripps Research.

You made a significant transition from transition metal-catalyzed reactions at Waseda University to medicinal chemistry and ADCs at Scripps Research. What sparked that pivot, and was there a particular moment or project that made you realize therapeutic chemistry was your calling?

During my time at Waseda University, I was completely absorbed in pure organic chemistry research, particularly in developing novel transition metal-catalyzed reactions. I enjoyed the intellectual challenge of building complex molecules from simple starting materials. The pivot really began when I started to read emerging chemical biology papers, such as click-chemistry-based protein and cell labeling. I could not resist the desire to step into that new field, so I moved to the **Scripps Research** Institute (currently Scripps Research) for my postdoctoral work. I developed chemical tools to study the bacterial cell-to-cell communication system known as quorum sensing. Seeing a compound that I had drawn in a notebook change gene expression patterns or bacterial behavior was a very different kind of satisfaction from developing a new chemical reaction. That experience convinced me that precise molecular design could directly influence disease-relevant biology. Eventually, I moved further into the biomedical realm and began medicinal chemistry projects to develop novel antibody-drug conjugate platforms when I started my own lab at UTHealth Houston.

Starting an independent lab in 2014 as a young investigator in the competitive ADC field must have been daunting. What were those early years like, and how did you build your interdisciplinary team combining organic chemistry, peptide synthesis, chemical biology, and pharmacology?

It was definitely not an easy path. In the first few years after starting my lab in 2014, several of my early federal grant applications were not funded, and I faced a very real financial crisis. I seriously had to rethink how to position the lab and where we could make a distinctive contribution in a very competitive field.

One turning point was a suggestion from Dr. Zhiqiang An, the chair of my department, that I write a review article on ADCs. It is somewhat peculiar to write a comprehensive review before you are recognized as an expert, but I decided to spend several months on this mission, and received a huge reward; through the process of reading and critiquing the literature and going through rigorous peer review, I was able to build a solid knowledge base and identify many critical gaps in the field that chemists can fill, such as linker instability and the lack of a practical dual-conjugation strategy. That exercise essentially gave me a roadmap for what my lab should work on.

I was also very fortunate with the people around me. Dr. An provided access to engineered antibodies that allowed us to move quickly from concepts to real ADC constructs. Dr. Yasuaki Anami (currently a senior scientist at **CrossBridge Bio**) joined my lab as a postdoc with a rare combination of organic synthesis and pharmacology expertise. My wife, Dr. Chisato Tsuchikama, also joined my lab as a postdoc and contributed her deep experience in peptide synthesis and animal studies. My lab would not have been successful if either of these pieces had been missing.

Your dual-drug ADC platform has shown remarkable success in addressing tumor heterogeneity and drug resistance in breast cancer. What inspired you to pursue the concept of loading two different payloads onto a single antibody, and what were the biggest technical challenges in making this work?

The idea of dual-drug ADCs was not brand new when we started this project. There were already a few important precedents, including work by Levengood and colleagues [1] where two similar but different microtubule inhibitors, MMAE and MMAF, were co-loaded onto the same antibody. What had not really been addressed, however, was the central question of how a dual-drug format could better address tumor heterogeneity and acquired resistance compared to single-drug constructs, and how to choose payload combinations that would accomplish that goal. Another gap at that time was that there was no flexible way to customize the drug-to-antibody ratio for each payload without going through long and complicated syntheses of linker–payload constructs.

Our approach was to adapt the branched linker-based conjugation platform we had already developed to dual conjugation. We did this by incorporating two orthogonal click reaction pairs, namely azide with cycloalkyne and tetrazine with trans-cyclooctene, so that each linker-payload could be attached in a controlled and modular way. Fortunately, we were able to find workable conjugation conditions relatively quickly. The bigger challenge turned out to be biological rather than chemical. It took almost three years to establish an appropriate heterogeneous and refractory breast tumor model and to generate in vivo antitumor and mechanistic data that convincingly showed dual-drug ADCs outperforming the co-administration of two single-drug ADCs. That was the point where we felt confident that dual conjugation was not just a clever chemical concept, but a real solution to clinically relevant problems.

You've developed novel glutamic acid-valine-citrulline linkers and branched linker systems for ADCs. How do these linker technologies improve upon existing approaches, and what makes them particularly well-suited for creating multi-drug conjugates?

The glutamic acid–valine–citrulline (EGCit, GluGlyCit) linker provides several mechanistic and practical advantages over traditional dipeptide linkers. First, it preserves efficient protease-mediated traceless cleavage inside cancer cells while markedly improving stability against a broad spectrum of proteases, including serum proteases and neutrophil-derived enzymes such as elastase and proteinase 3. Many newer linkers are optimized for serum stability, but resistance to neutrophil proteases is often overlooked, even though neutropenia is one of the most common and dose-limiting toxicities observed with clinical ADCs. So our EGCit linker can improve the therapeutic index and potentially reduce myelosuppression.

Second, the EGCit motif is less hydrophobic than conventional valine-containing linkers. The negatively charged glutamic acid and the absence of a bulky hydrophobic side chain significantly decrease overall hydrophobicity, which reduces the tendency to aggregate. This property becomes particularly important in dual-payload or higher-DAR settings, where hydrophobic interactions often drive aggregation, fast clearance, and off-target toxicity. Historically, many early dual-drug ADC efforts struggled with poor developability, and I am convinced that linker- and payload-driven aggregation was a major reason for those failures. Using our minimal footprint platform with well-defined conjugation chemistry, we can access dual-payload formats that have been previously very difficult, if not practically impossible, to advance toward translational studies.

Your research has demonstrated that ADC homogeneity significantly impacts blood-brain barrier penetration in glioblastoma models. Could you explain why homogeneous ADCs outperform heterogeneous ones in brain tumor targeting, and what implications this has for treating CNS malignancies?

In that study [2], we asked whether conjugation homogeneity alone, while keeping target, payload, and average DAR constant, affects brain delivery. The answer was clearly yes. Most ADCs in the clinic are heterogeneous conjugates, which contain high-DAR, very hydrophobic species. By constructing and evaluating high-DAR conjugates using our site-specific conjugation, we found that they were very inefficient at crossing the blood–brain barrier, even though those species looked fine in vitro and showed similar plasma PK to the homogeneous ADCs. So High-DAR components contributed little to actual payload delivery in orthotopic GBM tumors. In other words, the "effective DAR" of heterogeneous ADCs in the brain was much lower than the nominal average DAR. With homogeneous ADCs, every molecule has the same, optimized DAR and more controlled hydrophobicity, so essentially the entire dose can efficiently participate in brain tumor targeting. We saw higher intratumoral drug levels and better antitumor efficacy in GBM models without differences in systemic exposure, which points directly to BBB penetration as the key factor. The broader implication is that, for CNS malignancies, ADC homogeneity is not just a chemistry preference but a functional requirement for maximal therapeutic efficacy.

In your analytical workflows, you extensively use Agilent instrumentation, including LC/ESI-MS systems and HPLC platforms for ADC characterization. What specific capabilities of these Agilent instruments are most critical for your ADC research, and how have they enabled discoveries that might not have been possible with other analytical platforms?

When we started our ADC program, we did not have the sensitive and high-resolution mass spectrometers we use now. Our budget only allowed a used Agilent 1100 HPLC with a 1946 single-quad LC/ESI-MS, an earlier generation of the 6100 series. Even so, two capabilities turned out to be critical. First, the system was sensitive and robust enough not only to characterize linker-payloads and monitor catabolite formation, but, with optimized methods, to give rough heavy-chain and light-chain mass profiles after reduction [3]. That allowed us to quickly assess DAR distribution and conjugation efficiency in-house and to iterate chemistry on a day-to-day basis instead of sending everything to a core.

Second, the Agilent LC platform was highly flexible and reproducible. We could use the same system for small-molecule purification one day and denaturing ADC analysis the next, and still compare retention shifts to track hydrophobicity and aggregation. That tight analytical feedback loop was essential for refining our transglutaminase-based site-specific conjugation and for developing our dual-drug formats. Now we also run newer high-resolution systems, but the key point is that even that "old" 1100/1946 setup was powerful enough for a small academic lab to carry out serious ADC chemistry and discovery. I am sure the current generation of Agilent instruments is more than sufficient for this purpose.

You co-founded CrossBridge Bio to commercialize your ADC technologies. How do you balance the demands of running an academic research lab with entrepreneurial responsibilities, and has this dual role changed how you approach research questions?

When I co-founded CrossBridge Bio, it actually started from an educational experience. I joined the **Texas Medical Center Innovation** (TMCi) accelerator program for cancer therapeutics (ACT), supported by the **Cancer Prevention Research Institute of Texas** (CPRIT), mainly to learn basic entrepreneurship skills and knowledge needed to build a university spinoff. Through that program, I met Dr. Michael Torres, an entrepreneur in residence at that time and now the CEO of CrossBridge Bio. After completing the program, he led the company formation, built the team, shaped the early R&D strategy, and raised funds. What makes our situation unique is that my lab and CrossBridge are in the same **TMC3 building**, a new translational campus that co-locates academic labs and startups to help move ideas toward patients. The physical proximity makes our collaboration really easy.

I have a strong belief that academic labs should focus on the discovery of seeds of innovation, and that business should be run by people trained for it, so I did not join in an operational role. Instead, I serve as a board director and a member of the scientific advisory board, while keeping my primary effort in my lab at UTHealth Houston. This way, I can be fully committed to addressing scientific questions about ADC chemistry and cancer biology, and that has not changed after the company was formed. At the same time, the dual role has made me more conscious of translational potential when I frame research questions, while still starting from fundamental scientific curiosity.

With over 100 ADCs now in clinical trials and 12 FDA-approved, the field is rapidly maturing. Where do you envision your specific technologies making the biggest clinical impact in the next 5-10 years, and are there any cancer types or disease areas you're particularly excited to explore?

Our stable EGCit linker technology is designed to significantly enhance the safety profile while maintaining, or even improving, antitumor efficacy. Many approved ADCs are still limited by adverse events, so improving linker stability and selectivity has a direct impact on therapeutic index and quality of life. My hope is that this platform will enable best-in-class ADCs that deliver strong efficacy with fewer dose-limiting toxicities.

Our flexible dual conjugation platform allows us to generate a variety of dual-payload ADCs with relatively little additional process optimization. That makes it realistic to tailor payload combinations to address tumor heterogeneity and resistance mechanisms, which could lead to first-in-class ADCs for patients who are not well served by existing single-payload agents. I am particularly interested in applying this to intractable cancers such as pancreatic cancer, CNS malignancies, and AML.

Looking a bit further ahead, the same dual conjugation chemistry is not limited to cytotoxic payloads. We are actively exploring ways to incorporate other functional molecules, with the goal of creating new ADC-like modalities with novel mechanisms of action.

As artificial intelligence (AI) shows great potential in drug design and many biopharma companies have applied AI tools in ADC structural design, do you have any plans to explore this powerful technique in your future ADC research?

I have recently started to learn more about AI and its potential for ADC development, and I do see several areas where it can be very powerful. For example, AI-based tools can help analyze and predict how the properties of each structural component (namely antibody, linker, and payload) influence target specificity, stability, and potency, and can accelerate SAR exploration far beyond what we can do manually. I also think AI will be very useful for pathway analysis and patient stratification, for example, by linking target expression, mutational status, and microenvironment features to the probability of response to a given ADC design.

At the same time, I am cautious about expecting AI to fully predict the behavior of a final ADC construct. An ADC is a very complex molecular system, and its efficacy and toxicity depend on many interconnected steps, including target binding, internalization, trafficking, linker cleavage, payload release, tissue distribution, and excretion. Going forward, my plan is to integrate AI where it clearly adds value, especially in the design of each structural component and data analysis, while still relying on careful chemistry and biology to validate and refine the most promising ADC candidates.

You've mentioned on LinkedIn that you never imagined receiving this level of recognition when you started your lab. What advice would you give to young investigators just starting in the ADC field or considering the transition from synthetic chemistry to therapeutic development?

My advice is to invest time in learning the true unmet needs on the biology and clinical side, rather than only asking, "What can I do with the techniques I already have?" It feels risky, because you may discover gaps that are outside your comfort zone, but I encourage you to look for problems that biologists tend to describe as "very difficult or not realistic, but fantastic if addressed," and then ask whether, from a chemist's perspective, they are actually solvable. Those interface problems, where one side sees a wall and the other side sees a toolbox, often point to the most impactful opportunities and lead to innovative work.

In my case, when I started my lab, I tried to fully understand what was actually needed and not yet fulfilled in the ADC field, especially around linker and conjugation chemistry. Then I gradually moved to deeper cancer biology questions and critical issues in cancer clinical management while evolving our ADC chemistry platform. Although I was not 100% sure that my research would achieve huge success in future clinical applications, I was sure that I was working on truly important and unaddressed questions.

Finally, I suggest you try to enjoy the whole process. For me, watching our research evolve from the first linker ideas to dual-drug constructs and now new modalities has felt a bit like growing plants in a garden. You plant something, you adjust conditions, you learn from failures, and over time, you see the system mature. If you can enjoy that long arc, it is much easier to stay motivated in a complex field like ADCs.

Interview conducted by **Róisín Murtagh**, *Wiley*

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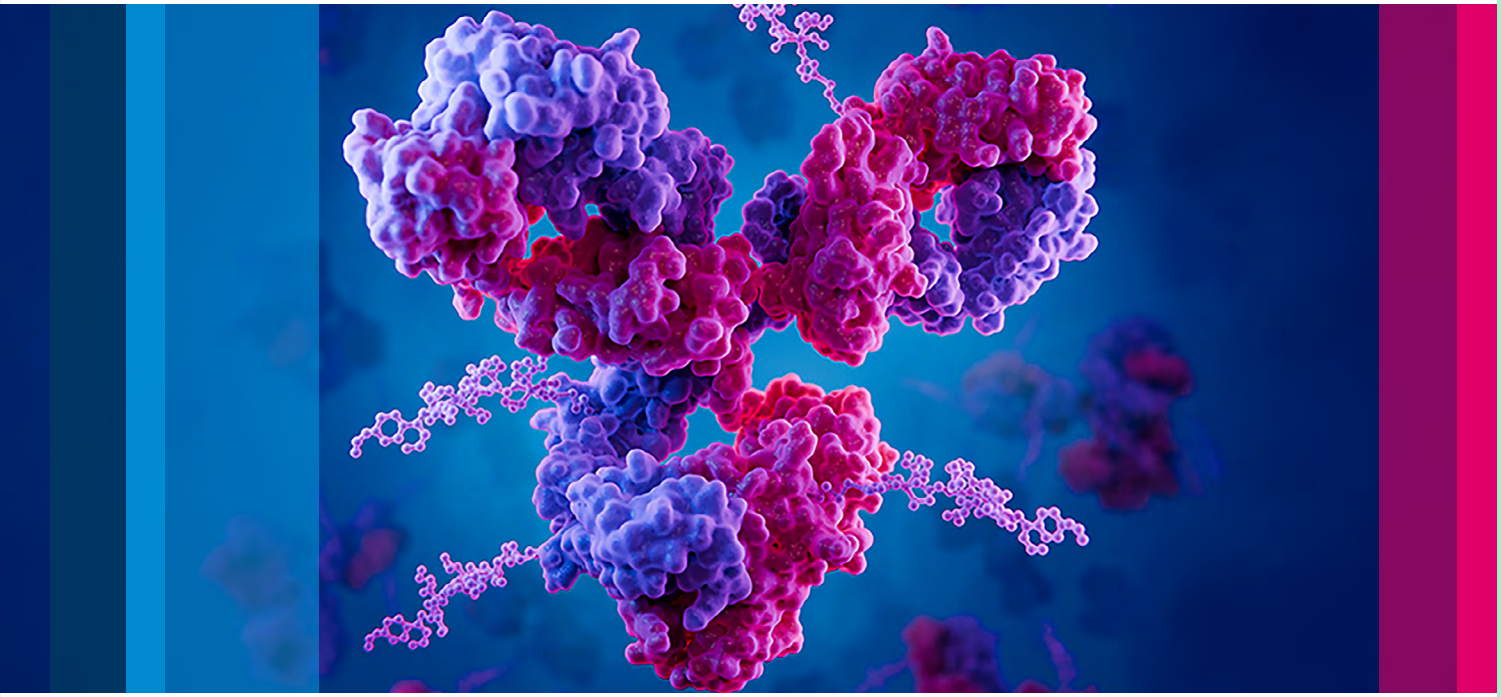

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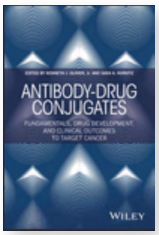

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