

Resin-Supported Site-Specific Antibody Conjugation Method Leads to Antibody-Drug Conjugates with Retained Efficacy and Improved Stability

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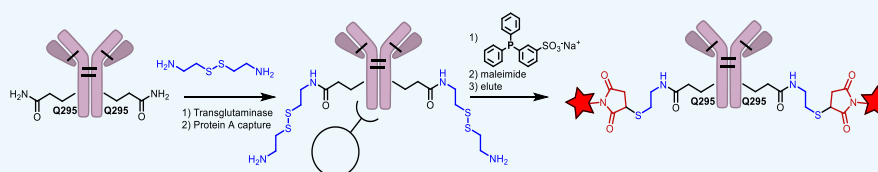
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ABSTRACT: Herein, we describe an optimized method for the generation of “thiolated Q295” site-specific antibody-drug conjugates (ADCs) with drug-to-antibody ratio (DAR) 2 from nonengineered IgG1 antibodies. Traditional ADCs take advantage of the 4 intrachain disulfide residues as the sites of attachment. While operationally simple to prepare, ADCs that rely on attachment to these endogenous cysteine residues suffer from heterogeneity arising from stochastic mixtures of differently loaded species. Our team recently reported a site-specific thiolation method targeting the conserved Q295 residue in deglycosylated antibodies. This approach involves deglycosylation of Q297 (using PNGase F) to eliminate steric hindrance from the N-glycan, followed by introducing a thiol-containing small molecule, cysteamine, at Q295, using microbial transglutaminase (mTGase). Our original method employed a global reduction/reoxidation to liberate the Q295 thiol for conjugation. However, this process was challenging due to competing reoxidation of the newly introduced Q295 thiol. In order to overcome this issue, we systematically explored various reducing agents and conditions, ultimately resulting in a new process that avoids the need for reduction/reoxidation. This resin-supported method, which is suitable for high-throughput synthesis, relies on the selective reduction of the engineered disulfide by sterically hindered phosphine, monosulfonated triphenylphosphine (TPPMS). Relying on this optimized methodology, we studied a small set of tubulysin ADCs showing that the resulting Q295-conjugated ADCs have favorable biophysical and biological properties as compared to traditional stochastic conjugation.

INTRODUCTION

An antibody-drug conjugate (ADC) is a type of therapeutic agent that uses the targeting ability of an antibody to selectively deliver a small molecule drug to antigen-expressing cells. ADCs consist of a monoclonal antibody and a small molecule therapeutic agent that are attached via a chemical linker. While ADCs are being explored for a variety of therapeutic applications,^{1–6} the primary focus of ADC research has been on the selective delivery of cytotoxic agents for the treatment of cancer. In this scenario, the monoclonal antibody binds to a selectively expressed tumor antigen, undergoes clathrin-mediated uptake and lysosomal localization, and finally is degraded by proteases, thereby releasing the cytotoxic payload. There are currently 14 ADCs that are FDA approved for marketing, and more than 80 in clinical trials.

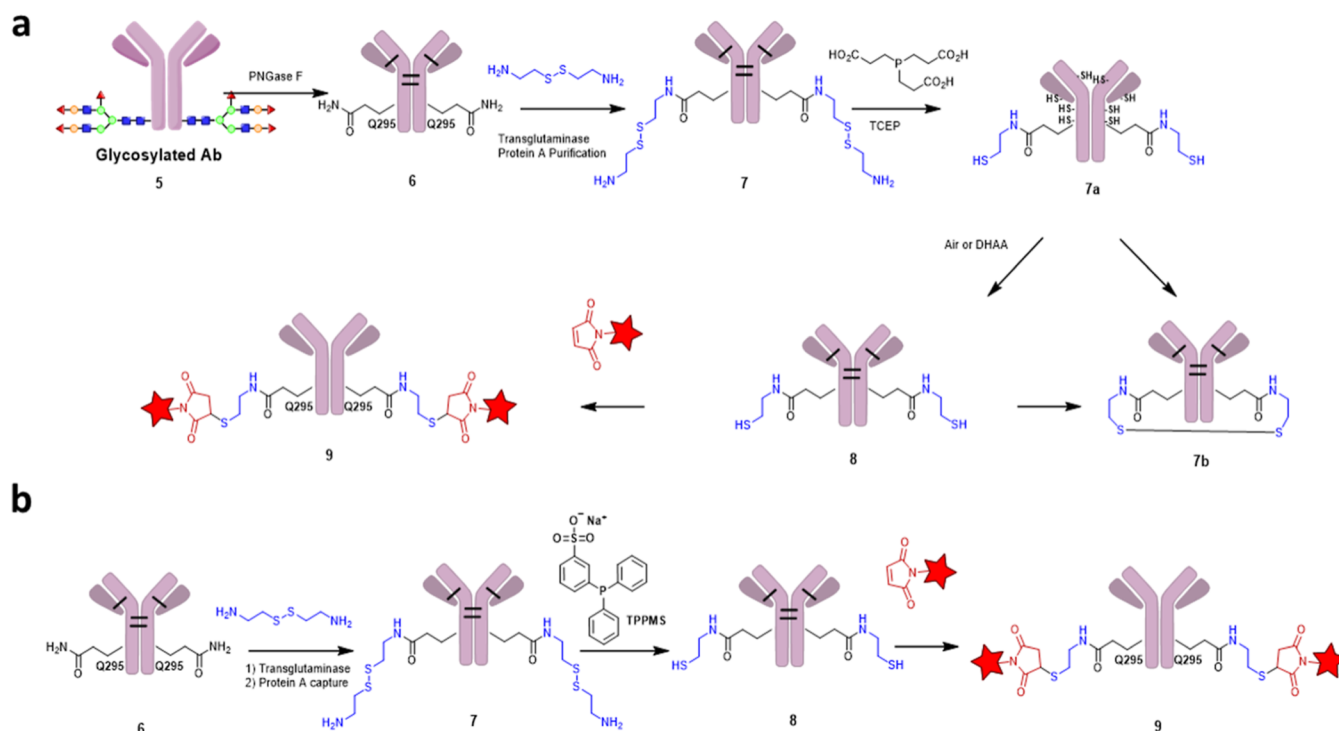
One of the key design criteria for ADCs is the site of conjugation. Traditional ADCs, including most of those on the market today, frequently take advantage of the 4 intrachain disulfide residues as attachment sites. Selective reduction of these disulfides liberates up to 8 thiol residues that can readily

react with electrophilic linker-payloads, frequently using maleimide chemistry or bromoacetamide chemistry. While operationally simple to prepare, ADCs that rely on attachment to these endogenous cysteine residues suffer from heterogeneity arising from stochastic mixtures of differently loaded species. More importantly, the cysteine residues involved in the intrachain disulfides are often highly solvent-exposed, thereby resulting in retro-Michael-mediated deconjugation, premature linker cleavage by serum proteases, and rapid clearance due to uptake by Kupffer cells.^{7–10} For this reason, the past 10 years have seen a significant push toward optimizing site-specific bioconjugation approaches that “hide” the payload from bulk

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Scheme 1. (a) Previous Reported Q295 Methodology with Non-Specific Reduction and Nonreactive Byproduct, (b) Current Report with Optimized Reduction and Streamlined High Throughput “Solid Phase” Conjugation



solvent, thereby minimizing plasma clearance and protecting the ADC from premature linker cleavage.

Typically, site-specific conjugation technology relies on the introduction of a cysteine, an unnatural amino acid, or a short peptide sequence into the antibody backbone. Recently, there have been a handful of approaches reported that do not require re-engineering of the antibody backbone. These methods employ selective chemical or biochemical reagents to attach a linker-payload at a “native” (conserved) residue in the IgG backbone. Site-specific conjugation to native (unmodified) antibodies has the advantage of being able to rapidly conjugate therapeutic agents to readily available “off the shelf” antibodies without the need for time-consuming and expensive re-engineering.

We recently reported a two-step bioconjugation procedure for the site-specific attachments of payloads to the conserved Q295 residue that is located in a protected hydrophobic pocket adjacent to the N297 glycosylation site.¹¹ This method involves a four-step process in which mTGase is used to attach a short disulfide-containing stub to a deglycosylated antibody (Scheme 1). Upon reduction by tris(2-carboxyethyl)phosphine (TCEP) and subsequent reoxidation of the intrachain disulfides, the Q295-thiol is available for conjugation to typical maleimide-containing linker-payloads. As we previously demonstrated, this process results in ADCs with a drug-to-antibody ratio (DAR) of ~ 2 . More importantly, we showed that the hydrophobic pocket surrounding the Q295 site shields the linker-payload from metabolism and protects the payload from exposure to bulk solvent. Moreover, we demonstrated that this method was particularly advantageous for the conjugation of very hydrophobic linker-payloads that traditionally resulted in aggregation and poor loading. Despite these advantages, one of the limitations of our method was the rather cumbersome 3-step method that required multiple buffer exchanges and time-

sensitive reaction steps. Indeed, the selectivity of the entire process was driven by the air reoxidation of 7a to 8 (Scheme 1), a reaction that at times proved difficult to control, because 7a can also be oxidized to form 7b, thus resulting in low yields.

Herein, we report a series of significant improvements to this methodology that allow for rapid preparation of Q295-modified ADCs using a solid-phase conjugation approach that can be easily conducted in parallel. A key discovery that enabled this improved methodology was the identification of a sterically hindered phosphine, monosulfonated triphenylphosphine (TPPMS), that rapidly reduces the Q295 disulfide without impacting the intrachain disulfides. This discovery has significantly improved the site-specificity and ease of preparation of Q295-conjugated ADCs across a range of therapeutic antibodies. As compared to ADCs that employ traditional stochastic conjugation, ADCs prepared using our new Q295 methodology demonstrate improved biophysical stability, improved plasma stability of a metabolically labile payload, and comparable pharmacokinetic exposure.

RESULTS AND DISCUSSION

As ADC technology has matured, site-specific conjugation has become an increasingly important approach for mitigating problems associated with hydrophobic and metabolically labile payloads. A variety of site-specific conjugation approaches have been described in the literature, including engineered cysteine residues, incorporation of unnatural amino acids, and enzyme-mediated protocols. Importantly, most of these approaches employ changes in the antibody sequence that require antibody engineering. While these methods are useful, there has been significant advancements made in site-specific conjugation approaches that do not require antibody engineering. These so-called “native site-specific” conjugation methods employ

Table 1. Results of Screening for Optimal Conditions for Phosphines to Specifically Reduce Q295 Modified Cysteine^a

entry	antibody	linker-payload	phosphine, eq	incubation temperature	time (h)	pH	DAR
1	trastuzumab	mcGly-Tub	diPPBS 10	37 °C	3	7.4	0.6
2	trastuzumab	mcGly-Tub	diPPBS 10	37 °C	24	7.4	1.1
3	trastuzumab	mcGly-Tub	diPPBS 10	37 °C	48	7.4	1.2
4	trastuzumab	mcGly-Tub	diPPBS 40	37 °C	3	7.4	0.6
5	trastuzumab	mcGly-Tub	diPPBS 40	37 °C	24	7.4	1.3
6	trastuzumab	mcGly-Tub	diPPBS 40	37 °C	48	7.4	1.5
7	trastuzumab	mcGly-Tub	diPPBS 160	37 °C	16	7.4	0.7
8	trastuzumab	mcGly-Tub	diPPBS 10	37 °C	6	5	1.2
9	trastuzumab	mcGly-Tub	diPPBS 10	37 °C	16	5	1.4
10	trastuzumab	mcGly-Tub	diPPBS 40	37 °C	6	5	1.4
11	trastuzumab	mcGly-Tub	diPPBS 40	37 °C	16	5	1.6
12	trastuzumab	mcGly-Tub	TPPMS 10	37 °C	1	7.4	2.2
13	trastuzumab	mcGly-Tub	TPPMS 10	37 °C	2	7.4	2.7
14	trastuzumab	mcGly-Tub	TPPMS 10	37 °C	24	7.4	3.8
15	trastuzumab	mcGly-Tub	TPPMS 6	RT	1	7.4	1.7
16	rituximab	mcGly-Tub	TPPMS 6	RT	1	7.4	1.7
17	trastuzumab	mcGly-Tub	TPPMS 10	RT	1	7.4	2
18	rituximab	mcGly-Tub	TPPMS 10	RT	1	7.4	2
19	trastuzumab	mcMMAF	TPPMS 4	37 °C	1	7.4	2
20	trastuzumab	mcMMAF	TPPMS 4	RT	1	7.4	2
21	trastuzumab	mcMMAF	TPPMS 6	37 °C	1	7.4	2.6
22	trastuzumab	mcMMAF	TPPMS 6	RT	1	7.4	2.1
23	rituximab	mcMMAF	TPPMS 4	37 °C	1	7.4	2
24	rituximab	mcMMAF	TPPMS 4	RT	1	7.4	2
25	rituximab	mcMMAF	TPPMS 6	37 °C	1	7.4	2.4
26	rituximab	mcMMAF	TPPMS 6	RT	1	7.4	2
27	trastuzumab	vcMMAE	TPPMS 6	37 °C	1	7.4	2.5
28	rituximab	vcMMAE	TPPMS 6	37 °C	1	7.4	2.4
29	trastuzumab	vcMMAE	TPPMS 4	37 °C	1	7.4	2
30	rituximab	vcMMAE	TPPMS 4	37 °C	1	7.4	2

^aTwo tri-aryl phosphines were employed for the optimization: 2-(diphenylphosphino)benzenesulfonic acid (diPPBS), triphenylphosphine-3-sulfonic acid monosodium salt (TPPMS).

selective chemistry or enzymatic methods to attach payloads to specific residues of nonmodified antibodies.¹²

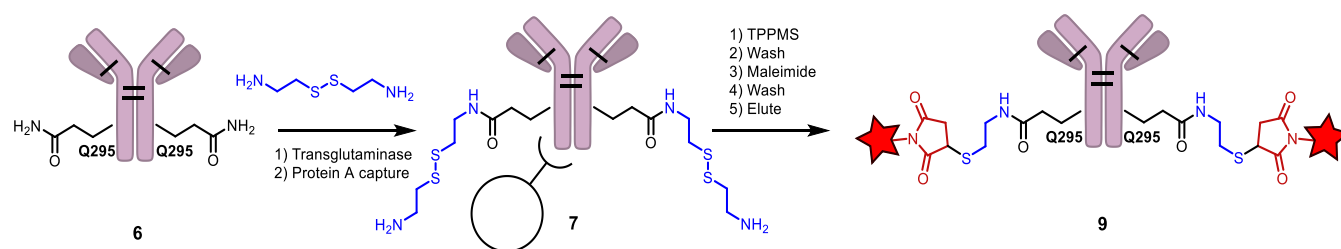
For example, AJICAP, a site-specific conjugation technology developed by Ajinomoto Bio-Pharma Services, enables the precise introduction of bio-orthogonal functionalities, such as thiol groups, to two specific lysine residues in the antibody's Fc region, without the need for genetic engineering or enzymatic methods. The specificity is achieved using a peptide with high affinity for the Fc-domain nearby to the Lys248 and Lys288 region. The peptide is linked to a disulfide spacer, which is functionalized with an NHS ester for conjugation, thus enabling the addition to alkyl-thiol residues selectively to these sites.^{13–16}

A number of native-site specific conjugation approaches involve mTGase-mediated modification of the conserved Q295 residue. Due to the steric hindrance from the N-glycan at Asn297, conjugation with this method typically requires deglycosylation. However, researchers at Northeastern University introduced an approach in which the glycans are "trimmed" using EndoS2, thus allowing mTGase to selectively modify Q295 without deglycosylation.¹⁷ A related approach was reported by Wehrmüller et al., this time promoting transamidation at the Q295 position without glycan trimming or deglycosylation. Their method uses short, positively charged lysine-containing peptides in the linker to achieve native conjugation. By conjugating lysine-rich peptides bearing azide or thiol functional groups, the technology then utilizes

biorthogonal conjugation of payloads to achieve multiple DAR values.¹⁸

Building on these strategies, our team recently developed a site-specific thiolation method targeting the conserved Q295 residue in deglycosylated antibodies. This approach involves deglycosylation of Q297 (using PNGase F) to eliminate steric hindrance from the N-glycan, followed by introducing a thiol-containing small molecule, cysteamine, at Q295, using mTGase. A global reduction and subsequent reoxidation prepare this site for efficient conjugation of maleimide-containing linker-payloads. Beyond resolving steric issues, deglycosylation also creates a hydrophobic pocket that enhances payload stability.¹¹

As we previously demonstrated, one of the key advantages of our approach is the ability to "hide" hydrophobic payloads within the pocket formed between the two CH₂ domains.¹¹ We showed that this shielding reduced the overall hydrophobicity of the resulting ADCs and reduced protease-mediated linker metabolism. Building on these findings, our team began working with an amine-modified tubulysin reported by Pfizer in 2016.⁹ Tubulysins are a class of antimitotic tetrapeptides produced by myxobacteria. Unlike most auristatin derivatives, tubulysins are resistant to MDR efflux pumps, making them an attractive target for ADC designs. Indeed, numerous tubulysin ADCs have been explored clinically and preclinically.^{12,19–24} The particular tubulysin derivative reported by Pfizer in 2016 contains a pharmacologically essential ester moiety that was shown to be metabolically labile (mcGly-Tub, Scheme 3 and Figure 5b).

Scheme 2. High Throughput Conjugation Protocol Employing Protein A Resin for the TPPMS Reduction and Maleimide Conjugation

Cleavage of the ester by plasma esterases results in rapid inactivation of the ADC. As such, we hypothesized that attachment of this linker-payload to the Q295 site would impede the cleavage and result in sustained activity of the ADC. Simultaneously, we wanted to employ this linker-payload to optimize the conjugation efficiency, hoping to overcome some of the aforementioned operational challenges associated with our previously reported reduction/reoxidation protocol.

Our initial efforts to reproduce the 3-step synthesis of the linker-payload reported in 2016 were stymied by poor yields and purification difficulties. In order to address this issue, we developed an optimized synthetic route for mcGly-Tub that resulted in improved overall yield and efficiency (see experimental details, Scheme 3). The previously reported strategy for making mcGly-Tub involved a 3-step process in which tubulysin was coupled with preactivated N-Boc glycine, followed by deprotection of the Boc group and coupling with a maleimide caproyl succinimide ester. The overall yield was reported to be 26%. To enhance efficiency, we implemented a two-step synthesis wherein glycine was precoupled with maleimide caproyl succinimide ester, and the resulting mcGly-OH was directly conjugated to the tubulysin derivative in a single step. This method greatly simplified the purification of the final product and significantly improved the overall yield (70%).

Screening of Optimal Conditions for Q295 Methodology. Our previously reported Q295 conjugation strategy relied on global disulfide reduction using TCEP, followed by reoxidation of intrachain disulfides to expose the thiol at the Q295 site (Scheme 1a). However, overoxidation of the key intermediate resulted in the formation of a nonreactive byproduct (3b), which decreased the overall DAR (Scheme 1). In the hopes of avoiding this problematic oxidation, we envisioned a selective reduction of compound 7 in which the cystamine disulfide would be reduced while the intrachain disulfides would remain unaffected (Scheme 1b). Inspired by the work of Coumans et al.,²⁵ we began investigating hindered triaryl phosphine reducing agents. We evaluated two triaryl phosphines: 2-(diphenylphosphino)benzenesulfonic acid (diPPBS) and triphenylphosphine-3-sulfonic acid monosodium salt (TPPMS). These water-soluble phosphines are commonly used in homogeneous catalysis due to their solubility in aqueous media and unique electronic properties, making them valuable for bioconjugation applications.

Using these three reducing agents, we systematically evaluated various conjugation conditions, specifically focusing on equivalents, time, temperature, and pH (Table 1). Per our aforementioned interest in tubulysin, our initial optimization was focused around mcGly-Tub (entries 1–20) while later optimization also included mcMMAF and vcMMAE (entries 21–32). The report by Coumans showing that diPPBS could be used to selectively reduce the disulfide of an engineered cysteine

in the Fab domain (HC-41C) inspired our initial focus on employing this reducing agent. Accordingly, we explored various equivalents of diPPBS (10–160×) at 37 °C (Table 1, entries 1–7). Upon reduction under the shown conditions, the antibody was quickly buffer-exchanged into PBS and treated with an excess of the shown linker-payload. While the initial results were modestly encouraging, we were unable to achieve full loading onto the HC (DAR 2). Attempts to perform the reaction at a lower pH seemed to accelerate the reaction, but we were still unable to achieve full loading (Table 1, entries 8–11). Moving from the monosulfate to the corresponding trisulfate (TPPTS) resulted in reduced loading. Encouragingly, however, moving the monophosphate from the ortho position (diPPBS) to the meta position (TPPMS) resulted in increased reactivity, as seen in entries 12–14. However, the increase in reactivity resulted in small amounts of interchain disulfide cleavage, thus resulting in overloading. Excitingly, by lowering the temperature (to RT) and slightly reducing the equivalents of TPPMS, we were able to achieve nearly complete loading with minimal to no cleavage of interchain disulfides (entries 15–18).

Having achieved the desired loading with the mcGly-Tub linker-payload, we next turned our attention to two more widely employed linker-payloads, mcMMAF and vcMMAE. Excitingly, the same conditions resulted in nearly complete HC loading on both trastuzumab and rituximab (entries 22 and 26). Once again, performing the reduction at 37 °C resulted in slight overloading, while the same reaction at RT resulted in clean DAR 2 product formation. However, selective loading at 37 °C was enabled by further reduction in the amount of TPPMS (to 4 equiv), as evidenced for both mcMMAF and vcMMAE (entries 19, 23, 29, and 30).

High Throughput Conjugation Using Protein A Capture. Having identified optimal conditions for selective reduction of the Q295 cystamine disulfide, we next turned our attention to increasing the throughput of this reaction by performing the entire conjugation process on a protein A solid support. Antibody capture on the resin is achieved via noncovalent, yet highly specific, affinity interactions between protein A and the Fc region of the antibody. This widely used chromatographic technique allows for efficient and selective immobilization of antibodies. Importantly, the solid-phase format facilitates thorough removal of excess small molecules, including unreacted linker-payloads, reagents, and enzymes through repeated washing steps, thereby streamlining purification.

As shown in Scheme 2, our solid-phase methodology begins with deglycosylation of the antibody followed by the addition of cystamine (shown in blue) and mTGase. Upon completion of the reaction, the antibody is captured onto a protein A resin. Extensive washing removes excess mTGase and cystamine. Rather than eluting from the protein A column, the resin-bound

antibody is then treated with TPPMS (10 equiv) and vortexed briefly to ensure homogeneity. After 2 h at RT, the resin is washed extensively and subsequently treated with 10 eq of a maleimide-containing linker-payload. After overnight incubation at RT, the resin is again washed with PBS, and the final ADC is eluted with a low pH buffer. The final ADC, ready for biological evaluation, is obtained after neutralization and buffer exchange into PBS.

Using the optimized solid-phase conjugation method, we prepared a series of mcMMAF conjugates from a panel of 13 commercially available IgG1 antibodies (Table 2). Encourag-

Table 2. The Characteristics of Various ADCs Synthesized Using mcMMAF Payload via Enhance Q295 Methodology^a

antibody	DAR	observed Δ mass (expected Δ = 925)	yield (%)	agg. (%)
trastuzumab (anti-HER2)	2	927	72	2
rituximab (anti-CD20)	2	928	58	<1
adalimumab (anti-TNF)	2	926	64	<1
coltuximab (anti-CD19)	2	927	72	2
efalizumab (anti-CD11a)	2.1	919	53	3
tocilizumab (anti-IL6R)	1.9	918	84	<1
daratumumab (anti-CD38)	2	919	47	5
ramucirumab (anti-VEGFR2)	1.8	920	86	<1
NMK2045 (ant-GCC)	2.2	924	62	<1
sacituzumab (anti-TROP2)	2.2	921	67	<1
palivizumab (anti-RSV)	2.5	924	66	<1
polatuzumab (anti-CD79b)	1.7	923	74	3
anti-MSR1	1.9	927	52	<1

^aNew method resulted in increased reliability, broad applicability, and homogeneous product formation.

ingly, we observed high conjugation yields, typically ranging from 50% to 80%, and consistent DAR values of $\sim$ 2.0. Mass spectrometry (LC–MS) analysis revealed excellent agreement between the expected and observed mass shifts. Only minimal aggregation was observed, typically below 1–2%.

Antibody-Drug Conjugates Prepared Using Thiolated Q295 Retain Antigen-Mediated Cytotoxicity. Having established the methodology for efficient preparation of Q295-thiolated conjugates, we next determined to prepare a model set of mcGly-Tub conjugates for various biophysical and biological evaluations. As such, a small set of matched Q295 conjugates and stochastic cysteine conjugates was prepared as shown in Table 3. In short, Q295 conjugation was performed as described above, while endogenous cysteine conjugates were prepared using TCEP-mediated disulfide reduction. To confirm functional activity of the conjugates, a HER2+ breast cancer cell line (SKBR3) was evaluated in the presence of the trastuzumab (anti-HER2) ADCs, while two CD79b+ lymphoma cell lines (SUDHL2 and GRANTA519) were evaluated in the presence of the polatuzumab (anti-CD79b) ADCs (Figure 1). Appropriate isotype controls were included in each assay. As anticipated, the endogenous cysteine conjugate and the Q295 conjugates exhibited comparable cytotoxicity while the isotype controls

Table 3. Preparation of Various mcGly-Tub Conjugates for Biophysical and Biological Evaluations

antibody	conjugation type	DAR	expected Δ mass (Da)	observed Δ mass (Da)	agg (%)
trastuzumab (anti-HER2)	Q295	1.9	992	994	<1
trastuzumab (anti-HER2)	Cys	4.3	992	993	<1
polatuzumab (anti-CD79b)	Q295	1.8	992	995	<2.5
polatuzumab (anti-CD79b)	Cys	3.8	992	1052	<4.9
rituximab (anti-CD20)	Q295	2	992	994	<1
rituximab (anti-CD20)	Cys	3.7	992	995	<1
palivizumab (anti-RSV)	Cys	3.2	992	1002	<1

had little or no effect on cell growth. As many have previously noted,¹⁰ the difference between a DAR 2 and DAR 4 ADCs typically has little or no impact on cytotoxicity. Notably, the anti-HER2 ADCs exhibited $\sim$ 500 $\times$ better activity than the anti-CD79b ADCs, likely due to the well-known high expression of HER2 on SKBR3 cells.

While the Q295-conjugated ADCs clearly retain antigen-mediated cytotoxicity, we hypothesized that the required deglycosylation may impact the Fc γ R binding affinity needed for antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cell-mediated phagocytosis (ADCP). To assess this possibility, we performed a series of SPR experiments wherein a His-tagged Fc γ R was immobilized on the surface of a gold-coated plate, and the test ADC (or mAb) was passed over the sample to ascertain binding. The results of this experiment (Figure S1) showed that the Q295 conjugation chemistry (namely the deglycosylation) reduced the binding to Fc γ RI by a factor of $\sim$ 12 (6.02–74.2 nM) and completely ablated the binding to Fc γ RIIb (302–>8000 nM).

Fc γ R binding (and the associated effector function) is speculated to be an important driver of off-target toxicity for many ADCs. Thus, minimizing binding to these receptors may be a favorable design feature. For example, a recent study demonstrated that deglycosylated radioimmunoconjugates not only have a decreased affinity for Fc γ RI but also reduced off-target exposure.²⁶ On the other hand, reduced Fc γ R binding can impact therapeutic efficacy in some cases, as it is crucial for immune functions such as ADCC and ADCP, which complement payload-mediated cytotoxicity to enhance tumor killing. For example, Li et al. showed that the loss of Fc γ RI engagement reduced ADC efficacy in xenograft models of an anti-CD30 MMAE conjugate. This loss of efficacy was attributed to reduced uptake into tumor associated macrophages (TAMs), thereby illustrating the importance of understanding the impact of Fc γ R-mediated uptake for specific therapeutic applications.²⁷

Importantly, deglycosylation is generally not associated with changes to FcRn binding and is not anticipated to impact ADC pharmacokinetics (PK) (vide infra), as the FcRn interaction site is spatially distinct from the glycosylation site.^{28,29} Both glycosylated and deglycosylated IgGs have been shown to bind equally to FcRn and generally show comparable PK in preclinical models.³⁰ Furthermore, deglycosylated antibodies typically show comparable serum half-life, clearance, and other key PK parameters to their glycosylated comparators.³¹

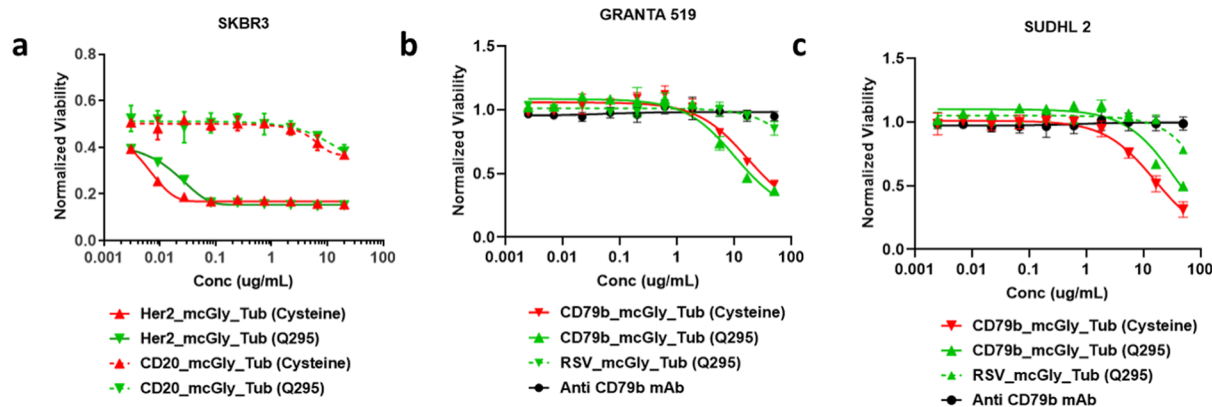


Figure 1. Cytotoxicity of Q295 and cysteine conjugates against various cell lines.

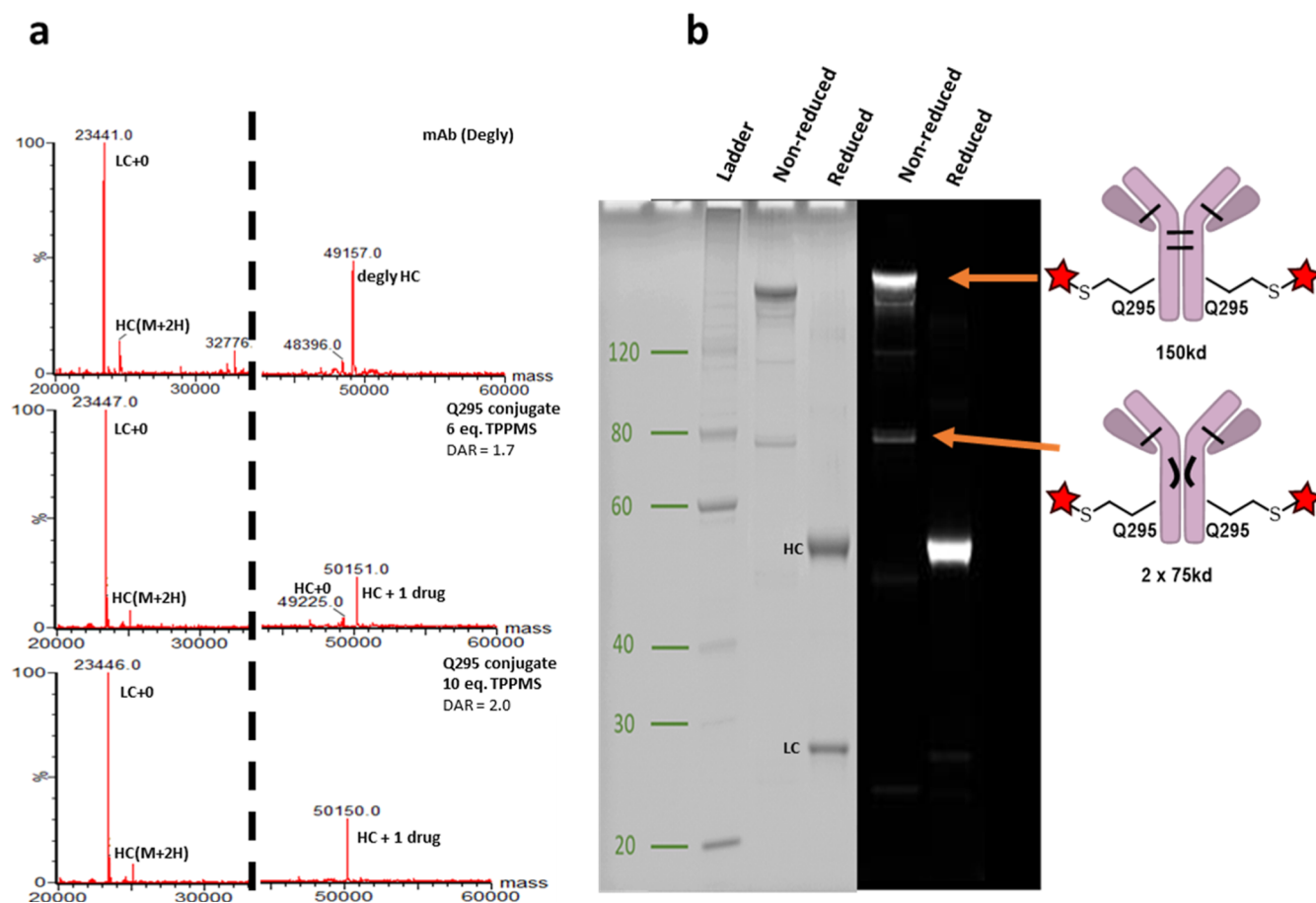


Figure 2. (a) LCMS showing clean addition to HC on trastuzumab; (b) gel electrophoresis of TxRed conjugates showing clean loading onto the HC along with the presence of ~12% of a "half-mer" species presumably arising from disulfide bond rearrangement as shown.

Selective Reduction with TPPMS Results in Minimal Disulfide Shuffling. Conjugation using the optimized protocol generally resulted in selective loading onto the HC with a DAR of 1.7–2.0. As seen in Figure 2a, no LC loading or HC + 2 loading was observed in conjugations of trastuzumab with mcGly-Tub, strongly suggestive of site-specific attachment to the thiolated Q295 residue (note that the small peak to the right of the LC0 peak is not a loaded LC. It is the doubly charged M + 2H of the unloaded HC). To further verify the specificity of loading, we employed our Q295 thiolation chemistry to attach a maleimide-functionalized Texas Red (Mal-TxRed) fluorophore to trastuzumab. SDS polyacrylamide gel electrophoresis was

performed under both reducing and nonreducing conditions to assess the conjugation efficiency (Figure 2b). Under reducing conditions, the TxRed was observed only on the HC band. Under nonreducing conditions, a weak ~75 kDa band was observed (~12% fluorescence intensity) in addition to the primary 150 kDa band. Prior studies have noted that small amounts of this "half-mer" antibody can occur naturally, but levels often increase due to disulfide scrambling that occurs during reduction-reoxidation protocols.^{32,33}

In order to more fully quantify the disulfide shuffling, we turned to a higher resolution separation method, capillary electrophoresis. Under reducing conditions, the stochastic

cysteine conjugate (mcGly-Tub, DAR ~4) displayed a single LC and HC band, practically identical to the naked mAb (Figure 3a). As anticipated, the loaded HC of the cysteine conjugate

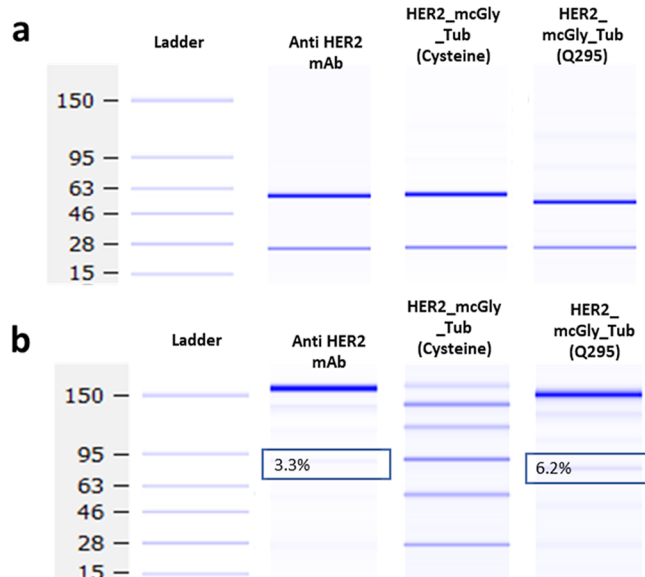


Figure 3. Capillary electrophoresis of a Q295 conjugate and stochastic conjugate demonstrate minimal disulfide rearrangement takes place during the TPPMS reduction process.

exhibited a negligible shift as compared to the unloaded HC band. The HC of the Q295 conjugate exhibited a slightly reduced shift, reflective of the loss of the N297 glycosylation

inherent in this conjugation approach. Under nonreducing conditions, the major species observed for the naked mAb and the Q295 conjugate was the full 150 kDa protein. In contrast, the nonreduced analysis of the cysteine conjugate revealed a plethora of various HC/LC species, as anticipated based on the partial cleavage of the intrachain disulfides. Interestingly, we detected a small ~75 kDa band in the nonreduced analysis of both the naked mAb (~3.3%) and the Q295 conjugate (~6.2%). This band likely reflects the aforementioned isomer in which the hinge disulfides form an intrachain bond instead of the usual interchain bond. The slight increase in this species observed upon Q295 conjugation (3.3–6.2%) is likely reflective of a small amount of disulfide shuffling that is taking place during the TPPMS reduction. Despite this minor increase in the 75 kDa half-mer, the capillary electrophoresis results provide important evidence for the homogeneity of the Q295 conjugate.

Q295-Conjugated Antibody-Drug Conjugates Exhibit Improved Stability Compared to Cysteine Conjugates.

Having demonstrated that the endogenous intrachain disulfide residues remain intact during the Q295 conjugation, we went on to assess the thermal stability of Q295-conjugated ADCs as compared to stochastic cysteine conjugates using DSF. DSF is a widely used technique for assessing the thermal stability of antibodies and ADCs³⁴ based on the fluorescence of an environmentally sensitive dye that binds to hydrophobic regions of proteins as they are exposed during the denaturation process. The fluorescence of ADCs (or mAbs) mixed with SYPRO dye was assessed while heating in a thermal cycler from 25 to 95 °C (Figure 4). Interestingly, the melting analysis revealed that deglycosylation decreases stability, as evidenced by the decreased melting point (69 °C vs 61.9 °C). Upon conjugation,

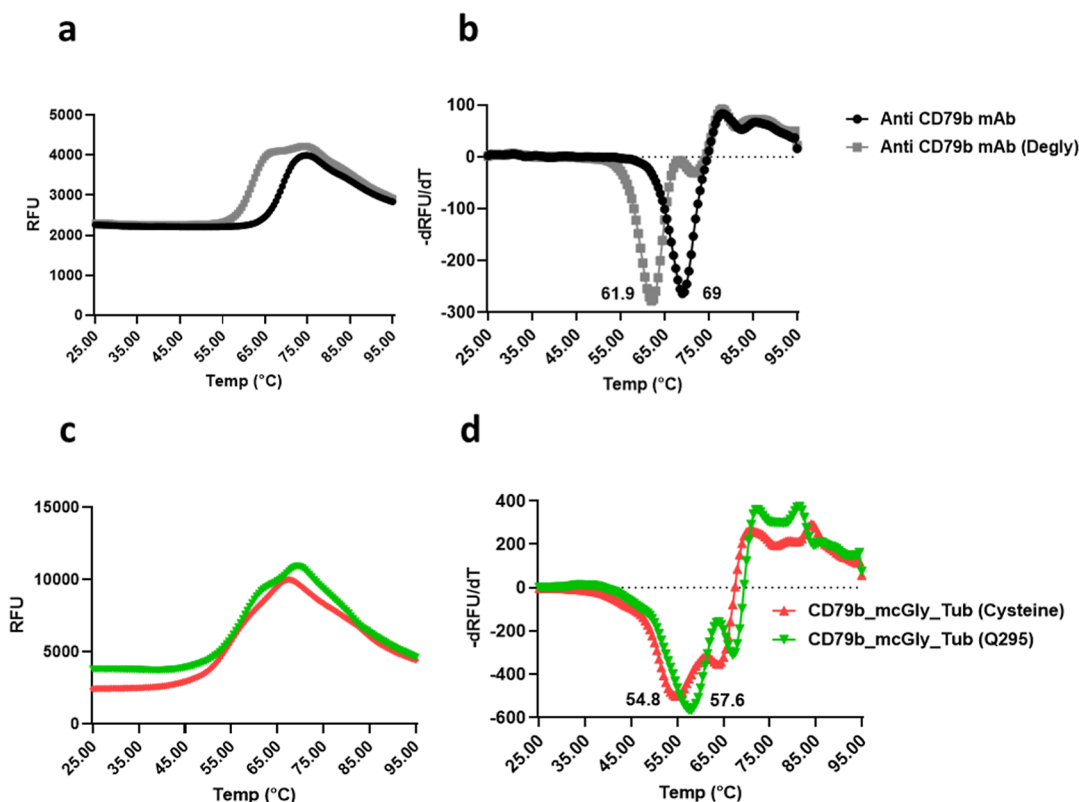


Figure 4. DSF to assess the stability of glycosylated and deglycosylated polatuzumab (a,b) as compared to a cysteine and Q295 conjugate of the same antibody (c,d).

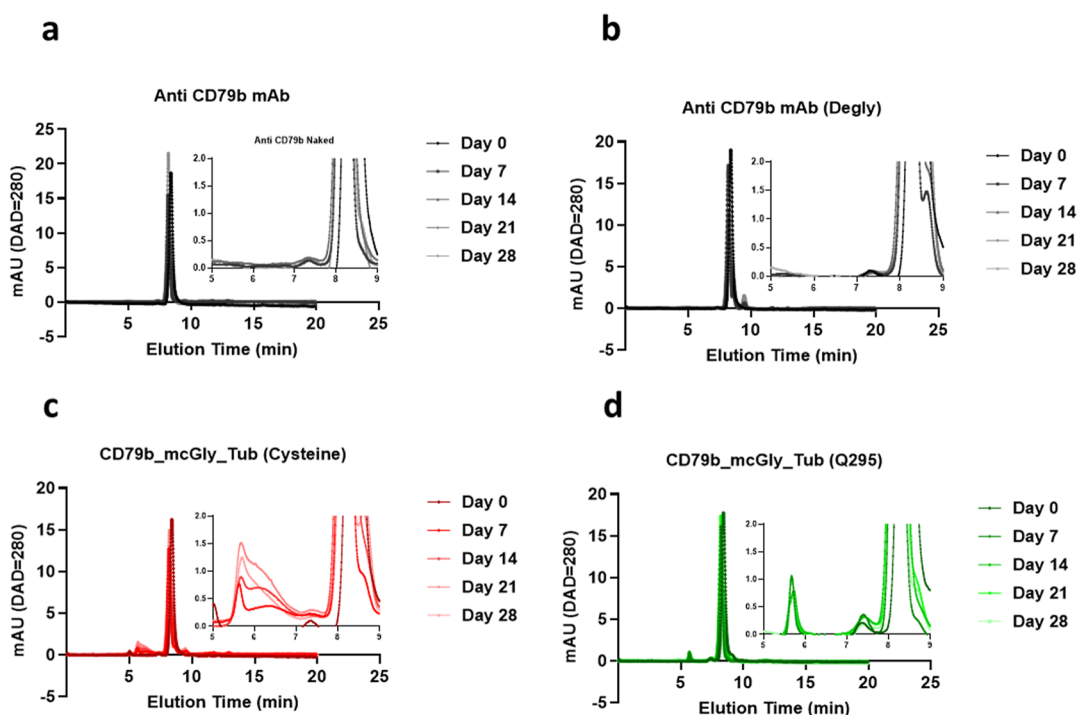


Figure 5. Thermal stress stability as assessed by SEC of glycosylated polatuzumab (a), deglycosylated polatuzumab (b), cysteine-conjugated polatuzumab (c), and Q295 conjugated polatuzumab (d).

the melting point of the cysteine-based conjugate (glycosylated) decreases by 14.2 °C—from 69 °C (mAb) to 54.8 °C. In contrast, conjugation to the Q295 site only decreases the melting point by 4.3 °C (61.9–57.6 °C) (Figure 4). This is likely due to the retention of the interchain disulfides in the Q295 conjugate, in contrast to the cysteine conjugate, in which the interchain disulfides are broken to facilitate payload attachment.

Building on the DSF findings, we proceeded to evaluate the long-term ADC stability under thermal stress conditions. The same four samples as above were incubated at 37 °C over a period of 28 days, and the aggregation of the samples was monitored by SEC. As expected, both glycosylated and deglycosylated polatuzumab exhibited minimal and comparable aggregation for up to 28 days (Figure 5a,b and Table 1). As anticipated, the cysteine conjugate exhibited progressive unfolding, reaching ~19% aggregation by the 28th day (Figure 5c). In contrast, the Q295 conjugated ADC demonstrated superior stability, only reaching ~5% aggregation over the same time period (Figure 5d). Interestingly, the aggregation increased from 1% to ~5% over the first 7 days and then remained constant at ~5% for the remainder of the study. This suggests that perhaps a minor species (overloaded material or disulfide-shuffled material) is aggregating rapidly while the bulk Q295 conjugate is highly resistant to aggregation. Overall, these results complement the DSF results, further strengthening the hypothesis that Q295 conjugates offer improved stability as compared to cysteine conjugation, likely due to a combination of retention of endogenous disulfides and shielding offered by the hydrophobic pocket (see Table 4).

Having evaluated the biophysical stability of the Q295 conjugated material in contrast to the endogenous cysteine conjugate, we next proceeded to study the biological stability. When we initiated this work, we hypothesized that the Q295-conjugated ADCs would offer superior payload shielding and encapsulation within the hydrophobic pocket, thus minimizing

Table 4. Summary of Thermal Stability Analysis Using SEC^a

sample	day 0	day 7	day 14	day 21	day 28
	aggregation (%)				
Pola		<1	<1	<1	<1
Deg Pola		<1	<1	<1	<1
cysteine	4	9	13	17.5	18.7
Q295	1.3	5	4.7	5.5	4.5

^a(a,b) Glycosylated and deglycosylated antibodies (polatuzumab), (c,d) CD79b_mcGly_Tub ADCs cysteine and Q295.

hydrophobicity-driven aggregation and also protecting the payload from enzymatic degradation. As described above, the Tubulysin derivative employed for these studies possesses an acetyl group that is highly susceptible to esterase-mediated degradation (Figure 6b). Importantly, as we demonstrated in 2016,⁹ cleavage of this ester results in the formation of an inactive metabolite. With this in mind, we incubated an anti-HER2 Q295 and an anti-HER2 cysteine conjugate in mouse serum for 72 h. Mouse serum is widely known to contain esterases that rapidly cleave many ester-containing linker-payloads.^{5,6} Due to the analytical challenges associated with the detection of the loss of acetate by LCMS, we instead opted to perform a functional evaluation of the conjugate over time. Based on our prior report,⁹ loss of acetate results in nearly complete inactivation of the ADC. Thus, plasma aliquots at various time points were serially diluted into SKBR3 (HER2+) cells, and dose–response curves were generated for each time point, as illustrated in Figure 6. Consistent with our hypothesis, the endogenous cysteine conjugate lost ~20-fold activity during the 72 h incubation (IC₅₀ shifting from 0.029 µg/mL to 0.63 µg/mL) while the Q295 conjugate lost only about 4-fold activity (IC₅₀ shifting from 0.024 µg/mL to 0.096 µg/mL) (Figure 6). These results clearly indicate that Q295-conjugated ADCs exhibit greater protection against esterase-mediated metabolism

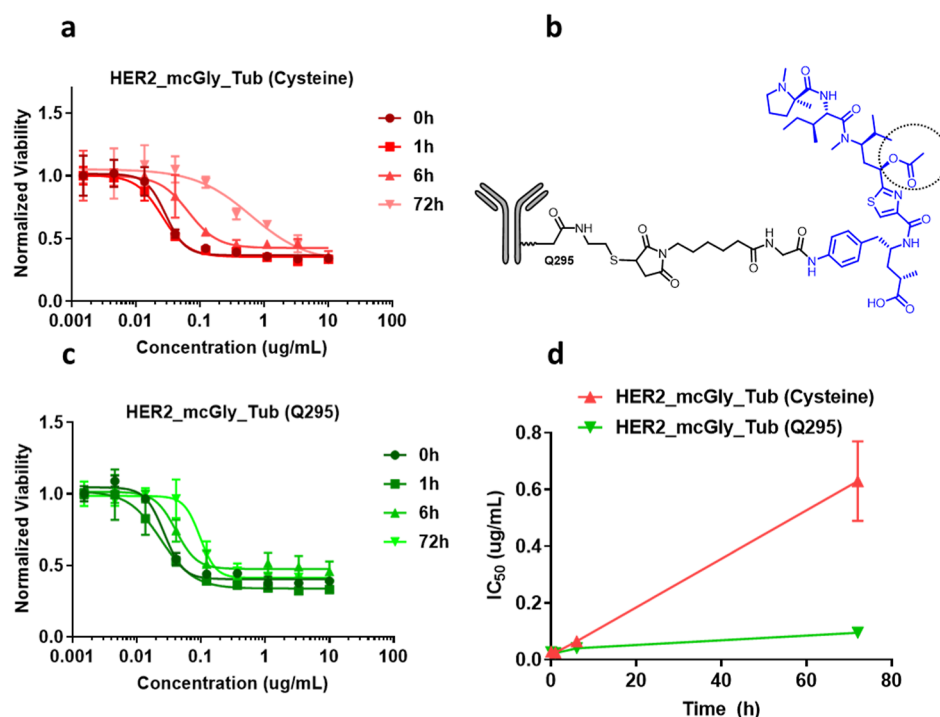


Figure 6. Q295 thiol conjugation shields the tubulysin from esterase-mediated metabolism in mouse plasma, as evidenced by the shift in IC₅₀ in SK-BR3 cells.

compared to cysteine-conjugated ADCs. This is consistent with our prior report showing that Q295 ADCs protected a related vcMMAE ADC against CatB-mediated linker cleavage.

Q295 Conjugated Tubulysin Antibody-Drug Conjugates Exhibit Comparable Pharmacokinetics to Unmodified Antibodies. It is widely known that site of conjugation can impact ADC PK. Attachment of hydrophobic payloads to “privileged” sites is a common strategy to address stability and safety concerns. As a preliminary assessment of the suitability of the Q295 conjugation technology for in vivo applications, we performed a pharmacokinetic study in both mice and rats (Figure 7 and Table S). Encouragingly, we found that the terminal half-life of the Q295 conjugate in mice (150 h) was comparable to the cysteine conjugate (176 h) and to the naked antibodies (~170 h). The overall exposure (AUC) of the Q295 conjugate in mice was modestly higher than the corresponding cysteine conjugate (9500 vs 13,800 h μg/mL). In rats, however, we observed that the terminal half-life of the Q295 conjugate (255 h) and the corresponding cysteine conjugate (300 h) were lower than the naked mAb (573 h). For reasons that are unclear, deglycosylation of the naked mAb resulted in more rapid clearance in rat ($T_{1/2}$ = 131 h). However, this trend was not observed in mice. The C_{max} was generally in the range of 100–150 μg/mL in mice and 40–50 μg/mL in rats, generally consistent with the corresponding naked antibodies. Overall, the results of the PK study in Figure 7 and Table S demonstrate acceptable PK parameters and support the further advancement of these ADCs in preclinical animal models.

DISCUSSION AND CONCLUSION

The improved methodology for Q295 conjugation technology described in this study represents a significant advancement in our ability to rapidly transform nonengineered antibodies into site-specific ADCs. By leveraging a sterically hindered phosphine (TPPMS), this method achieves selective reduction

of the Q295 cystamine without disrupting interchain disulfides. Interestingly, prior reports by Coumans et al.²⁵ (and unpublished work by our team) show that this reducing agent was not suitable for the selective reduction of engineered cysteine residues. Thus, TPPMS seems to be uniquely suitable for the reduction of disulfides within the hydrophobic pocket adjacent to the Q295 site. This selective reduction is crucial for the success of this conjugation approach as it maintains the structural integrity of the antibody while allowing for precise conjugation of the drug payload. Specifically, we demonstrated that very little “disulfide shuffling” takes place during the conjugation reaction and no reoxidation step is needed. In addition to the selectivity of this methodology, integration of the solid-phase “capture and release” approach simplifies the conjugation process by eliminating the need for multiple buffer exchanges, thus making the method more efficient and scalable. We demonstrated broad applicability of this approach, as seen in the consistent DAR (1.8–2.0), high yield (70%+), and low aggregation (<1%) when conjugating to a panel of 13 commercially available IgG1 antibodies.

In order to further probe the utility of this technology, we assessed a set of tubulysin ADC in “head-to-head” studies with a matched cysteine conjugate. The stability of this pair of ADCs was compared using DSC (to assess changes in T_m) and SEC (under thermal stress). Encouragingly, we found that conjugation to the Q295 site enhanced the biophysical stability as reflected both in an increased T_m and in reduced stress-promoted aggregation. Further, we found that the Q295 site, located in a protected hydrophobic pocket, effectively shielded the tubulysin from enzymatic degradation in mouse plasma. This is a particularly beneficial property for payloads with metabolically labile functional groups. Finally, PK studies in mice and rats revealed that Q295 ADCs exhibit favorable profiles, with comparable or superior exposure as compared to the matched cysteine conjugate and naked mAb. Taken

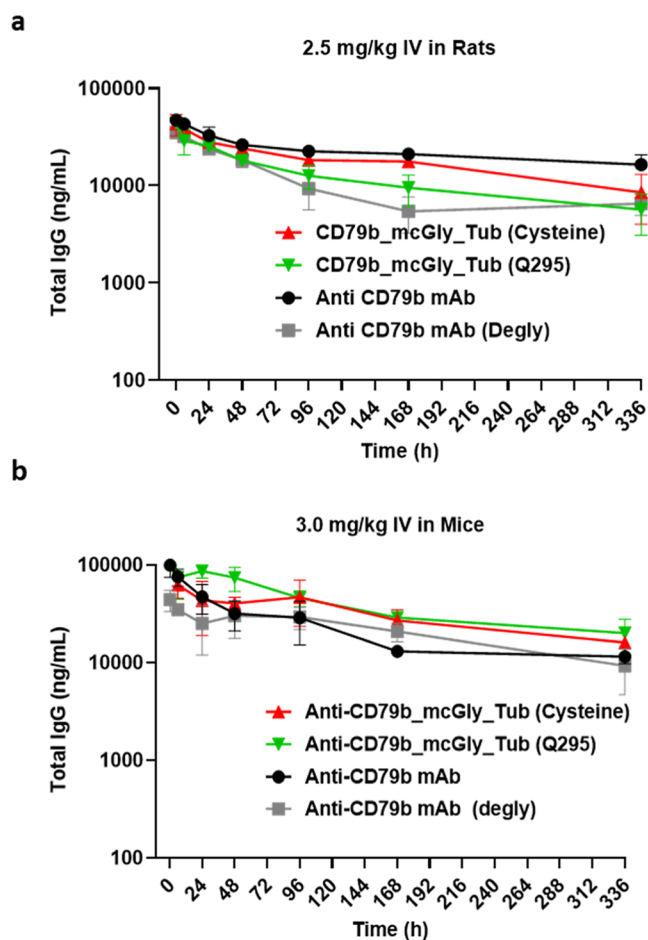


Figure 7. PK studies in mice and rats were conducted showing that both the cysteine conjugate and the Q295 conjugate have comparable PK to naked mAb.

together, these findings underscore the potential of the Q295 thiolation method to produce robust and reliable ADCs without the need for complex antibody engineering.

In summary, we developed a solid-phase site-specific conjugation method for the preparation of site-specific ADCs without the need for antibody engineering. The core innovation in this method involves a selective reduction protocol employing a sterically hindered phosphine reducing agent, TPPMS, thereby addressing the limitations of our previously reported methodology. The method is operationally simple and does not rely on proprietary technology or antibody sequence manipulation. The resulting ADCs exhibit excellent serum stability, biophysical stability, and pharmacokinetic exposure, thus highlighting the potential of this method for next-generation site-specific ADCs.

We believe that this method opens the door for novice teams to rapidly generate high-quality site-specific ADCs from “off the shelf” antibodies.

EXPERIMENTAL METHODS

General Experimental Information. All chemical reagents were purchased commercially and were used without further purification. The identity and purity of all compounds were determined by LCMS (see methodology below), and the final linker-payload was confirmed by comparison with a previously synthesized and published compound from our earlier work. The tubulysin (Tub) payload (Scheme 3) was generously donated by Pfizer. Antibodies were obtained from SydLabs or MedChemExpress.

Synthesis of Tubulysin Linker-Payload (mcGly_Tub).

Step 1: Synthesis of mcGly. *N,N*-Dimethylformamide (DMF) (0.5 mL) was added to a vial containing 2,5-dioxypyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanoate (20 mg, 65 μ mol) and glycine (6.3 mg, 84 μ mol). Then *N,N*-diisopropylethylamine (DIPEA) (13 mg, 97 μ mol) was added to the above mixture. After stirring for 2 h at room temperature (RT) the reaction mixture was purified directly using high-performance liquid chromatography (HPLC) equipped with C18 chromatography (10% acetonitrile/90% H₂O for 5 min, then 10% acetonitrile to 95% acetonitrile in H₂O over 18 min, each solvent containing 0.02% trifluoroacetic acid (TFA)) to yield the desired intermediate (9 mg, 50%) as a brown colored solid.

Step 2: Synthesis of mcGly_Tub. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (2 mg, 0.01 mmol), and (6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-hexanoyl)glycine (9 mg, 0.03 mmol) were transferred to a vial with a magnetic stir bar. Then DMF (25 μ L) was added and the linker was solubilized for 5 min under stirring. Tub (5 mg, 7 μ mol), (100 μ L, DMF solution) was added under stirring and left to react at RT. The reaction was completed in less than 30 min while monitoring via liquid chromatography–mass spectrometry (LC–MS). After purification by preparative HPLC (as described above), the desired product (4.7 mg, 70%) was obtained as a yellowish solid that was shown to be identical to an authentic standard provided by Pfizer.

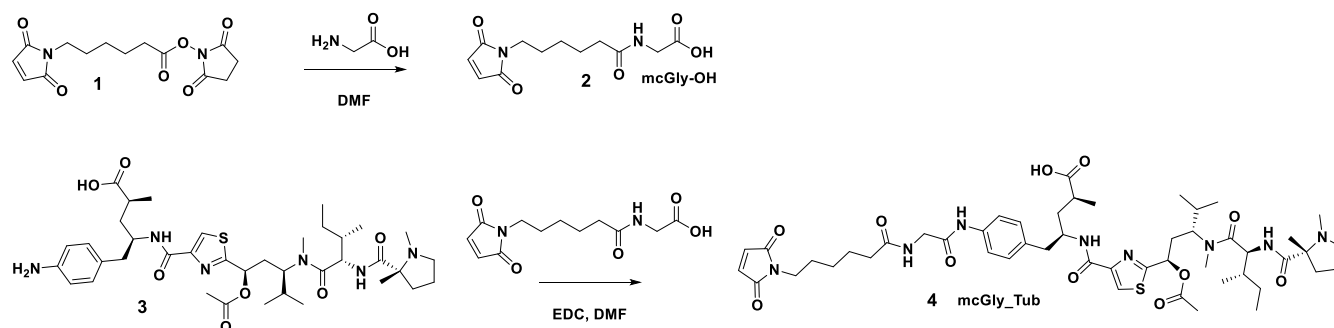
General Preparation of Antibody-Drug Conjugates Using Q295 Methodology. The antibody deglycosylation and conjugation procedure was performed in the following steps:

- 1. Deglycosylation:** the antibody (5 mg) was treated with 3.5 μ g of PNGase F (Bulldog Bio) and diluted to a final concentration of 10 mg/mL in PBS. The mixture was incubated overnight at 37 °C, and reaction completion was confirmed by LC–MS.

Table 5. Plasma Pharmacokinetic Parameters of Anti-CD79b ADCs in Mouse and Rat Following Intravenous Administration

ADC name	species	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (μ g/mL)	AUC(0–336 h) (h μ g/mL)	V (mL/kg)	Cl (mL/h/kg)
anti-CD79b Tub (cysteine)	mice	176 $\pm$ 47	0.25	124 $\pm$ 11	9499 $\pm$ 3014	56 $\pm$ 15	0.221 $\pm$ 0.043
	rat	255 $\pm$ 187	2.16 $\pm$ 2.71	43.9 $\pm$ 7.36	4850 $\pm$ 1105	75.9 $\pm$ 11.3	0.336 $\pm$ 0.194
anti-CD79b Tub (Q295)	mice	150 $\pm$ 39	0.25	167 $\pm$ 45	13,756 $\pm$ 972	35 $\pm$ 7	0.164 $\pm$ 0.012
	rat	300 $\pm$ 219	0.25	43.6 $\pm$ 2.69	4034 $\pm$ 710	138 $\pm$ 58.7	0.421 $\pm$ 0.172
anti-CD79b mAb	mice	176 $\pm$ 78	0.25	99 $\pm$ 20	7578 $\pm$ 1427	71 $\pm$ 26	0.287 $\pm$ 0.039
	rat	573 $\pm$ 252	2.16 $\pm$ 2.71	47.6 $\pm$ 5.10	7540 $\pm$ 537	93.3 $\pm$ 7.05	0.132 $\pm$ 0.046
anti-CD79b mAb (degly)	mice	170 $\pm$ 87	0.25	46 $\pm$ 6	7181 $\pm$ 1232	71 $\pm$ 19	0.334 $\pm$ 0.108
	rat	131 $\pm$ 9.57	0.25	35 $\pm$ 2.21	3403 $\pm$ 412	102 $\pm$ 8.99	0.536 $\pm$ 0.018

Scheme 3. Improved Synthesis of mcGly_Tub



- Cystamine conjugation:** the fully deglycosylated antibody was diluted with 1.2 mL of 50 mM phosphate buffer (pH 6) and treated with 100 μ L (100 equiv) of 30 mM aqueous cystamine solution, followed by 350 mg of mTGase powder (Ajinomoto Activa). The reaction mixture was vortexed thoroughly until the mTGase powder dissolved completely and then incubated at 37 $^{\circ}$ C for 48 h. After incubation, the reaction was centrifuged to remove insoluble material, and the supernatant was collected and diluted 1:1 with PBS.
- Protein A purification:** a pierce protein A column (12–19 mg capacity) was prepared by equilibrating with 5 mL of PBS. The reaction mixture was applied to the column and allowed to flow completely into the resin bed. The column was washed three times with 5 mL of PBS.
- TPPMS modification:** a 10 mM solution of TPPMS (10 equiv) was added to the column and incubated at room temperature for 2 h. The column was capped and vortexed extensively to ensure thorough mixing.
- Payload conjugation:** the column was drained, and a 10 mM solution of maleimide payload (mc_MMAF) was added, diluted with PBS and 10% DMA to a final volume of 800 μ L. The mixture was vortexed thoroughly and incubated at room temperature overnight. Excess linker payload was removed by washing with 15 mL of PBS.
- Elution and buffer exchange:** conjugated antibodies were eluted with 5 mL of glycine buffer (pH 2.7) and collected in 1 mL fractions. The eluate was immediately neutralized by the addition of 100 μ L of Tris buffer (pH 9.0). The sample was buffer-exchanged into PBS using a 30 kDa MWCO column and subsequently filter-sterilized.

Antibody-Drug Conjugate Concentration Determination and Recovery Estimation. ADC concentrations were measured using a NanoDrop One spectrophotometer (Thermo Scientific), employing the Protein A280 method with the appropriate IgG subclass selected from the instrument's database. Baseline correction was set at 340 nm to account for background absorbance. Recovery (or yield) was then estimated by comparing the final ADC concentration to the starting concentration of the unconjugated IgG prior to conjugation and purification.

LCMS and Size Exclusion Chromatography Characterization of Antibody-Drug Conjugates. DAR estimation of mAbs and ADCs was evaluated using a Waters Acquity UPLC equipped with a TUV detector and Xevo TQD mass spectrometer (MS). 10 μ L of a 1 mg/mL sample was mixed with 5 μ L of 500 mM TCEP, vortexed, and 15 μ L was injected into above MS. The separation was achieved using a Sepax proteomics RP-1000 5 μ m column (1000 $\text{\AA}$ and 2.1×100 mm)

at 80 $^{\circ}$ C, using the gradient below. Solvent A was water with 0.1% formic acid (FA), and solvent B was acetonitrile with 0.1% FA. The typical injection size was 5–10 μ L. A gradient elution was performed (10% $\rightarrow$ 90%B), and the eluent was monitored by UV (220, 260, and 280 nm) and by ESI mass spectroscopy (TQD, ES+). The charge envelope ($\sim$ 700–1700 m/z) was deconvoluted using Maxent1.

Aggregation levels were assessed via size exclusion chromatography (SEC) on an Agilent 1260 infinity II with a TUV detector using a TSKGel 3000SW (7.5 mm $\times$ 30 cm) column. 10 μ L of 1 mg/mL samples were injected. Analysis was performed at room temperature using a 20 min isocratic gradient of phosphate buffer (50 mM, pH 7.4) containing 10% acetonitrile at 1.00 mL/min. The eluent was monitored by UV at 220, 254, and 280 nm. Under these conditions, the antibody eluted at $\sim$ 8.4 min, and any aggregate material eluted at $\sim$ 7.2 min. Excess small molecules eluted at approximately 11.3 min.

Gel Electrophoresis Analysis. ADC and mAb samples were prepared under reducing (Laemmli loading buffer) and nonreducing (loading buffer) conditions. For antibody samples, 6 μ L of $\sim$ 35 μ g/mL antibody or ADC was mixed with 2 μ L PBS and 2 μ L of either loading buffer or Laemmli loading buffer, yielding a final concentration of 1.5 μ M. The protein ladder was diluted 1:3 and loaded. After diluting with the respective buffers, samples (10 μ L final) were heated at 95 $^{\circ}$ C for 5 min (the protein ladder excluded from heating) and immediately loaded onto the gel. Electrophoresis was run at 80 mA for $\sim$ 1 h. The gel was imaged using Amersham Typhoon (Cy5 setting), then stained with Coomassie Blue on a rocking device at medium speed. The stained gel was stored at 4 $^{\circ}$ C before destaining and reimaging on the Azure system under visible gel settings. ImageQuant software was used to quantify protein band volumes from FLR and Coomassie images, calculating the ratio of 75–150 kDa protein in unreduced lanes.

Capillary Electrophoresis. Capillary electrophoresis was performed using an Agilent 2100 bioanalyzer, generally following the manufacturer's suggested protocol. In short, mAb and ADC samples were prepared under reducing (Laemmli loading buffer) and nonreducing (loading buffer) conditions. For sample preparation, the denaturing solution and Protein 230 ladder were equilibrated at room temperature for 10 min. Each protein sample (1 mg/mL, 4 μ L) was mixed with 2 μ L of denaturing solution in a 0.5 mL tube, spun down, and heated at 95–100 $^{\circ}$ C for 5 min, followed by dilution with 84 μ L of deionized water. The gel-dye mix was equilibrated for 30 min before being pipetted into designated wells on a protein chip. Each sample and ladder (6 μ L) was loaded into the respective wells, ensuring the run was started within 5 min on the Agilent 2100 bioanalyzer.

Melting Point Determination. The melting temperature of antibodies and ADCs was measured by differential scanning fluorimetry (DSF) using a BIO-RAD CFX96 real-time PCR detection system with C1000 touch thermal cycler. All samples were prepared in Tempassure 0.2 mL PCR pull-apart tube strips with optical flat cap strips and maintained on an ice bath until loading. 2 μ L of each antibody (1–4 mg/mL) was diluted with 15.5 μ L of PBS and treated with 2.5 μ L SYPRO dye (50 $\times$), giving a total volume of 20 μ L. Samples were heated from 25 to 95 $^{\circ}$ C with 0.5 $^{\circ}$ C increments every 10 s. DSF data (RFU vs Temp) was analyzed using the Web site “gestwickilab.shinyapp.io/dsfworld/”.

Forced Aggregation Stability Studies. Each ADC was prepared as a 500 μ L dilution of 0.5 mg/mL in PBS. The samples were incubated at 45 $^{\circ}$ C in a shaking incubator, with tubes sealed with parafilm and covered with aluminum foil. Aggregation was analyzed via SEC (10 μ L injection) at days 0, 7, 14, 21, and 28. The SEC was performed as described above.

Mouse Serum Stability and Cytotoxicity in SKBR3 Cells. ADCs were incubated in 1.5 mL of mouse serum, diluted with 500 μ L PBS to a final concentration of 100 μ g/mL, and maintained at 37 $^{\circ}$ C under 5% CO₂ for 7 days. At 0, 1, 2, and 7 days, 300 μ L aliquots were collected and stored at –80 $^{\circ}$ C. SKBR3 cells were cultured in RPMI 1640 medium with 10% FBS, harvested during the exponential growth phase using 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) (0.53 mM EDTA), and seeded at 0.25 million cells/mL into 96-well plates. A 3 $\times$ serial dilution of the serum-incubated ADCs was performed, and the resulting solutions were spiked into cells, reaching a maximum concentration of 10 μ g/mL. Cells were incubated at 37 $^{\circ}$ C under 5% CO₂ for 72 h, followed by XTT assay (Biotium) for cytotoxicity assessment. Absorbance was measured at 450 and 630 nm using a Varioskan LUX microplate reader, and IC₅₀ values were calculated using GraphPad Prism 7, applying a log(inhibitor) vs response model with a variable slope (three or four parameters) fit.

Cytotoxicity Studies of Her2_{mcGly}_Tub Antibody-Drug Conjugates in SKBR3 Cells. SKBR3 cells (ATCC) were cultured and harvested as described above. To prepare cells for the assay, a cell suspension was prepared at 2.8×10^5 cells/mL to seed 60 wells per plate as required. Each well received 90 μ L of the cell suspension, ensuring uniform cell distribution. 3 $\times$ serial dilutions of control and test ADCs were prepared in PBS (10 $\times$ the final test concentrations), starting at 0.2 mg/mL, and 10 μ L of each diluted ADC was added to the corresponding wells. The plates were incubated at 37 $^{\circ}$ C under 5% CO₂ for 72 h. Following incubation, cytotoxicity was assessed using an XTT assay (Biotium). Finally, absorbance was measured (450 and 630 nm), and IC₅₀ values were calculated using GraphPad Prism 7, applying a log(inhibitor) vs response model with a variable slope (three or four parameters) fit.

Cytotoxicity Studies of CD79b_{mcGly}_Tubulysin Antibody-Drug Conjugates in GRANTA-519 and SUDHL-2 Cells. GRANTA-519 or SUDHL-2 (ATCC) cells were maintained in DMEM or RPMI-1640, respectively, media, supplemented with 10% FBS and 1 $\times$ penicillin/streptomycin. 90 μ L of cells were seeded in a 96-well plate at a concentration of 0.1 million cells/mL, approximately 9000 cells/well. A series dilution of each ADC was performed, resulting in stock solutions at 10 $\times$ the final test concentrations. The cells were allowed to acclimate to the plate for up to 4 h, then 10 μ L of 10 $\times$ ADC serial dilutions were added to the respective wells, and the plate was left to incubate for 96 h. After the incubation period, the plate was analyzed

using an MTS viability kit (Promega), wherein 20 μ L of MTS + 5% PMS solution was added to each well, then left to incubate for 2–4 h. At the end of the incubation period, the plate was read at 490 nm. Data was plotted and analyzed using Graphpad Prism 11.0 to determine the IC₅₀ values as described above.

In Vivo Pharmacokinetics Studies. Twelve male C57BL/6J mice (7–8 weeks old, strain #000664) and 12 male Sprague–Dawley rats (11–12 weeks old) were obtained from The Jackson Laboratory and Charles River Laboratories, respectively. The animals were acclimatized for 1 week prior to the study. Animals were maintained on a 12 h light/dark cycle with ad libitum access to standard chow and water. All animal procedures were conducted in accordance with the approved Institutional Animal Care and Use Committee (IACUC) protocols (#890-23 for mice and #868-21 for rats) from the Laboratory Animal Resources (LAR) at Binghamton University. ADCs were administered via tail vein injection at a dose of 3 mg/kg in mice and 2.5 mg/kg in rats. Blood samples were collected from the submandibular vein of mice and the saphenous vein of rats at multiple time points postdosing, specifically at 15 min, and 6, 24, 48, 96, 168, and 336 h. Plasma was separated by centrifugation at 4500 rpm for 8 min and stored at –80 $^{\circ}$ C until further analysis.

Total IgG1 Measurement by PIXI ELISA. The concentration of human IgG1 in mouse plasma was quantified using a PIXI ELISA kit (Correliabio). Each ADC was quantified using its standard curve to account for potential effects of conjugation or deglycosylation. This assay utilized PIXI cartridges precoated with antihuman IgG F(ab)₂ fragments, along with all required reagents, buffers, and consumables. Standard calibration curves were generated by plotting normalized absorbance values against IgG1 concentrations (ng/mL), as shown in Figure S2. The regression coefficient (R^2) for the calibration curve was greater than 0.99, confirming the assay's accuracy and linearity. Only the validated log–linear portion ($\sim 10^3$ – 10^5 ng/mL) of the 5-parameter logistic (SPL) curve was used for quantification. Samples near or below the LLOQ were flagged and reanalyzed at a lower dilution to ensure accurate measurement within the linear range. The concentration of human IgG1 in rat plasma was measured using Meso Scale Discovery (MSD) assay, following the manufacturer's protocol (Meso Scale Discovery, 2024). The established calibration standards, ranging from 1.95 to 1×10^3 ng/mL (Figure S3) were able to measure ADC concentrations.

Pharmacokinetic Analysis. The concentration–time data were analyzed by noncompartmental pharmacokinetic analysis (NCA) using Phoenix WinNonlin Version 8.4 (Certara, NJ, USA). The concentration–time data were plotted using GraphPad Prism. Clearance (CL) was calculated using the standard equation, $CL = \text{Dose}/AUC_{0-\infty}$, with doses of 2.5 mg/kg (rats) and 3 mg/kg (mice). AUC was determined using log–linear trapezoidal integration. Terminal elimination rate constant (λ_z) and half-life ($T_{1/2}$) were derived by linear regression of the log-transformed terminal phase.

Fc γ R Binding Studies by SPR. A high-capacity carboxyl sensor (Nicoya) was prepared by injecting a 50 mM solution of 1:1 NHS and EDC at 20 μ L/min (PBS, 25 $^{\circ}$ C). The AntiHis tag antibody was diluted to 6 μ g/mL in 200 μ L of pH 5.0 acetate buffer and was immobilized on Channel 2 of a Nicoya Open SPR instrument using a 20 μ L/min flow rate. Any remaining carboxyl groups were blocked with 1 mM ethanolamine. The appropriate histidine-tagged recombinant Fc γ R (Sino Biologics) was diluted in PBS to 4 μ g/mL and loaded onto the sensor through both

channels at 10 $\mu\text{L}/\text{min}$. The analytes, ADC and mAb, were diluted in running buffer (PBS) to a concentration correlating with the max binding concentration of a standard mAb to the individual Fc γ R (Fc γ RIa—300 nM, Fc γ RIIb 8000 nM). ADC and controls were injected at 20 $\mu\text{L}/\text{min}$, and at least 5 min were allowed for dissociation. A 10 mM HCl buffer (pH 1.5) was injected twice at 150 $\mu\text{L}/\text{min}$ to remove Fc γ R and analyte. The appropriate His-tagged receptor was injected again, and a new analyte was tested. Nicoya software was used to collect the raw binding data, and analysis was performed using Tracedrawer.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.5c00224>.

Additional experimental details and results, including raw SPR sensograms, ELISA calibration curves, HPLC purity traces, and LCMS characterization of the antibody conjugates (PDF)

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Notes

The authors declare no competing financial interest.

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