

Site Selection: a Case Study in the Identification of Optimal Cysteine Engineered Antibody Drug Conjugates

L. Nathan Tumey, Fengping Li, Brian Rago, Xiaogang Han, Frank Loganzo, Sylvia Musto, Edmund I. Graziani, Sujiet Puthenveetil, et al.

The AAPS Journal

An Official Journal of the American Association of Pharmaceutical Scientists

e-ISSN 1550-7416

AAPS J

DOI 10.1208/s12248-017-0083-7



Your article is protected by copyright and all rights are held exclusively by American Association of Pharmaceutical Scientists. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".

Research Article

Site Selection: a Case Study in the Identification of Optimal Cysteine Engineered Antibody Drug Conjugates

L. Nathan Tume^{1,9}, Fengping Li,² Brian Rago,³ Xiaogang Han,⁴ Frank Loganzo,⁵ Sylvia Musto,⁵ Edmund I. Graziani,³ Sujiet Puthenveetil,⁶ Jeffrey Casavant,³ Kimberly Marquette,² Tracey Clark,³ Jack Bikker,⁷ Eric M. Bennett,² Frank Barletta,³ Nicole Piche-Nicholas,² Amy Tam,² Christopher J. O'Donnell,³ Hans Peter Gerber,⁸ and Lioudmila Tchistiakova²

Received 9 March 2017; accepted 3 April 2017

Abstract. As the antibody drug conjugate (ADC) community continues to shift towards site-specific conjugation technology, there is a growing need to understand how the site of conjugation impacts the biophysical and biological properties of an ADC. In order to address this need, we prepared a carefully selected series of engineered cysteine ADCs and proceeded to systematically evaluate their potency, stability, and PK exposure. The site of conjugation did not have a significant influence on the thermal stability and *in vitro* cytotoxicity of the ADCs. However, we demonstrate that the rate of cathepsin-mediated linker cleavage is heavily dependent upon site and is closely correlated with ADC hydrophobicity, thus confirming other recent reports of this phenomenon. Interestingly, conjugates with high rates of cathepsin-mediated linker cleavage did not exhibit decreased plasma stability. In fact, the major source of plasma instability was shown to be retro-Michael mediated deconjugation. This process is known to be impeded by succinimide hydrolysis, and thus, we undertook a series of mutational experiments demonstrating that basic residues located nearby the site of conjugation can be a significant driver of succinimide ring opening. Finally, we show that total antibody PK exposure in rat was loosely correlated with ADC hydrophobicity. It is our hope that these observations will help the ADC community to build “design rules” that will enable more efficient prosecution of next-generation ADC discovery programs.

KEY WORDS: antibody drug conjugate; hydrophobicity; linker stability; PK exposure; plasma stability.

Electronic supplementary material The online version of this article (doi:10.1208/s12248-017-0083-7) contains supplementary material, which is available to authorized users.

¹ Present Address: Binghamton University, School of Pharmacy and Pharmaceutical Sciences, P.O. Box 6000, Binghamton, New York 13902-6000, USA.

² Biomedicine Design, Pfizer, Inc., Cambridge, Massachusetts 06379, USA.

³ Worldwide Research and Development, Pfizer, Inc., 445 Eastern Point Road, Groton, Connecticut 06379, USA.

⁴ PKDM, Amgen, Inc., 360 Binney Street, AMA 1, Cambridge, Massachusetts 02142, USA.

⁵ Oncology Research and Development, Pfizer, Inc., 401 N. Middletown Rd., Pearl River, New York 10965, USA.

⁶ AbbVie, Inc., 100 Research Dr, Worcester, Massachusetts 01605, USA.

⁷ International Flavors and Fragrances, 521 West 57th Street, New York, New York 10019, USA.

⁸ Maverick Therapeutics, Inc, 3260 Bayshore Blvd, Brisbane, California 94005, USA.

⁹ To whom correspondence should be addressed. (e-mail: ltume@binghamton.edu)

INTRODUCTION

Investment in antibody drug conjugate (ADC) technology has rapidly increased in response to the FDA approval of Adcetris® and Kadcyla® for the treatment of Hodgkin's lymphoma and human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer, respectively. This investment has resulted in a burgeoning clinical ADC pipeline now consisting of a variety of antibody targets and cytotoxic payloads (1). However, in spite of the advances in targets and payloads, the vast majority of the ADCs in the clinic continue to utilize conjugation technology that results in a drug product consisting of a heterogeneous mixture of ADC species. Over the past 5–10 years, preclinical research in the ADC field has gradually moved away from heterogeneous ADC preparations towards homogeneous site-specific conjugates. The resulting ADCs have been frequently shown to result in improved safety (2,3), exposure (4), and stability (5,6). In addition to their improved pharmacological properties, site-specific ADCs offer potential advantages in terms of regulatory burden due to the simplified bioanalytical strategy

as compared to traditional lysine and hinge cysteine conjugates (7). In response, a variety of site-specific conjugation technologies have been developed in recent years. Principally, these technologies fall into three broad categories: Incorporation of an unnatural amino acid via expansion of the genetic code (8), enzymatic approaches that exploit short peptide sequences that are typically involved in posttranslational modification (9), and engineering of a novel cysteine residue into the antibody backbone (2). While success has been realized using each of these three methods, few reports have emerged that describe a systematic approach for identifying the optimal site(s) of conjugation for a given system. In this report, we will describe the initial identification of a series of engineered cysteine antibodies and the subsequent work that went into understanding the subtle differences in biophysical and biochemical properties of the resulting ADCs. We believe that the methodology outlined herein may help to establish a framework for the optimization of site of conjugation for a variety of site-specific bioconjugate programs.

A typical IgG1 has approximately 16 endogenous disulfides that help to stabilize the protein tertiary and quaternary structure. This large number of disulfides is likely a key driver behind the high thermal stability and resistance towards proteolysis that is often typical for these proteins. While the majority of these disulfides are in buried regions of the protein that are inaccessible to bulk solvent, four of them are located in more flexible and exposed regions that render them easily cleaved by exogenous reducing agents. Selective reduction of these four disulfides has been widely exploited in the generation of ADCs, such as Adcetris®. However, the resulting ADCs consist of a stochastic mixture of species that have been shown to have distinct biological properties. In particular, the higher loaded species have been shown to be cleared faster (10) and to have a higher propensity to aggregate (11) while the lower loaded species have slightly reduced potency due to the delivery of a smaller amount of active payload (12). Two approaches have been used to decrease the heterogeneity of these cysteine conjugates: 1) replacement of one or more cysteine residues with serine (or other amino acid) in order to purposefully create unpaired cysteine residues at specific sites (13) and 2) insertion of a defined cysteine residue within the constant domain of the antibody. This latter approach was first pioneered in 1990 for the generation of site-specific radio-immuno-conjugates (14). A variety of research groups have built upon this early work leading to numerous reports of engineered-cysteine bioconjugates that have advanced through preclinical testing (2,3,5).

One focus of the ADC strategy at Pfizer has been the identification of structurally and mechanistically differentiated payloads for use on next-generation ADC programs (6,15,16). Like others in the field, however, we quickly realized that the resulting hinge-cysteine conjugates often resulted in suboptimal *in vivo* properties (6,17). As a result, we invested significant efforts in the identification of engineered site-mutants that would result in ADCs with improved novelty, stability, and exposure. The present discussion focuses on the strategy that was employed for the identification and selection of cysteine variants for use in up-and-coming ADC programs. The widely studied anti-HER2 trastuzumab framework was employed as a model antibody for these efforts.

Experimental Procedures

Molecular Modeling Used to Identify Sites of Interest

Initial modeling on the Fc domain of human IgG1 (PDB code 3DO3, [10.2210/pdb3do3/pdb](https://www.rcsb.org/structure/10.2210/pdb3do3/pdb)) (18–20) was performed in Side Chains with Rotamer Library (SCWRL) and Discovery Studio (BIOVIA, San Diego, CA). Individual Cys mutant structures were generated for all sites using SCWRL. These mutant structures were prepared following the default Discovery Studio protocol, and the side chain solvent accessibility of the mutated residue was calculated using the method of Spassov and Yan (21). For mutations in the CH1 and CL domains, we made the same measurements on multiple structures (2R8S, 1N8Z, and one unpublished proprietary structure, all having kappa light chains), giving preference to sites with consistent metrics on multiple structures. Sites were triaged to avoid mutants that may interfere with functional epitopes of the IgG1 including complement determining regions (CDRs), the neonatal Fc receptor (FcRn) binding domain, the Fc-gamma receptor (FcγR) binding domain, and the protein A binding domain. A subset of the remaining sites was selected for protein expression using the method outlined below.

Identification of nearby side chains that might affect reactivity of E380C was done by first mutating E380 to Cys, based on PDB entry 1L6X after preparation in the Schrodinger Protein Preparation tool, and attaching a linker fragment from the maleimide through the amino group on the N-terminal side of the Val residue. A 10,000-step mixed LMCS/MCMM conformational search in MacroModel was used to identify low energy conformations of the Cys/Linker and nearby side chains Lys248, Glu382, and Asn389, while the remainder of the atoms were frozen. To investigate kinetic effects on ring opening by introducing charge mutations near sites such as E388C, including the possibility of water-mediated effects, a 100-ns solvated MD simulation was carried out in Desmond using the OPLS3 force field and default settings. For the MD simulation, application of crystal symmetry operators generated an intact Fc from PDB entry 1L6X, the maleimide was attached as noted above, and nearby side chains were mutated to Arg and/or Lys as appropriate.

Production of Proteins of Interest

Single reactive cysteine residues were introduced into the trastuzumab IgG1 and the human kappa constant regions at the 11 positions listed in Table I using a standard PCR site-directed mutagenesis method. Overlapping PCR mutagenesis was performed as detailed in the “Experimental Procedures” section. In short, sense and anti-sense mutagenic oligonucleotides harboring the individual cysteine mutations as well as forward and reverse human IgG1 and kappa constant region flanking primers were synthesized. Two separate PCR amplification reactions were performed by using the sense flanking and antisense mutagenic primers for one and the sense mutagenic and corresponding anti-sense flanking primers for the second reaction, which generated two overlapping sequences containing the desired mutation. A final PCR amplification reaction was done using only the flanking primers with the overlapping PCR products

Identification of Optimal Cysteine-Engineered Conjugation Sites

Table I. *In Vitro* Cytotoxicity of vc0101 ADCs

Mutant location	N87 IC ₅₀ (ng/mL) (HER2+++)	BT-474 IC ₅₀ (ng/mL) (HER2+++)	MDA-MB-468 IC ₅₀ (ng/mL) (HER2-)
A114C	28.3 (±16.3)	16.7 (±9.3)	23,600 (±12,200)
L443C	36.0 (±20)	19.9 (±7.8)	29,300 (±17,300)
Q347C	42.2 (±31.4)	24.0 (±17.1)	14,000 (±14,500)
Kappa-K183C	25.4 (±7.1)	23.1 (±4.7)	31,200 (±16,400)
E388C	44.9 (±23)	25.9 (±13.9)	23,900 (±4600)
N421C	41.7 (±16.2)	19.5 (±2.6)	9500 (±86,800)
K392C	35.0 (±7.4)	21.2 (±7)	39,900 (±21,300)
K290C	43.5 (±14.3)	22.0 (±7.3)	25,800 (±13,700)
K334C	44.9 (±12.4)	21.8 (±8.7)	32,100 (±18,800)
S375C	56.3 (±4.1)	16.6 (±1.0)	18,700 (±2690)
E380C	63.2 (±37.7)	33 (±25.5)	12,300 (±10,900)

Results are the mean IC₅₀ (ng/mL antibody) from multiple determinations ± standard deviation

containing the mutation as the template resulted in the full-length sequence. These mutated constant regions were then joined to the appropriate trastuzumab heavy or light chain variable region and sub-cloned into an expression vector. The plasmid sequence was confirmed prior to transient expression in HEK-293 cells to generate the protein of interest harboring the individual mutations. Upon harvesting the conditioned medium, transient expression levels were quantitated using a total human IgG ELISA and the antibody variants were purified using a two-step method: protein A capture followed by preparative SEC to remove any HMMS. The resultant antibody variants containing the individual engineered reactive cysteine were characterized by SEC and LCMS.

Conjugation of the Engineered Antibodies

The engineered antibodies were prepared at 5 mg/mL in PBS (pH 7.4) containing 50 mM EDTA and were treated with 50–100 M equivalents of TCEP (as a 20 mM stock solution). After allowing the reaction to stand at 37°C for 1–2 h, the antibody was buffer exchanged into PBS (ultrafiltration or gel filtration) in order to remove the excess TCEP (note that the 334C and 290C mutant required a 12–18-h incubation in order to achieve complete uncapping). The resulting antibody was resuspended in PBS/EDTA and treated with a freshly prepared 50 mM solution of dehydroascorbate (final concentration = 1–4 mM). After standing at 4°C overnight, the antibody was buffer exchanged into PBS/EDTA (ultrafiltration or gel filtration) and treated with a 8-fold molar excess of mcValCitPABC_PF-06380101 (hereafter referred to as “vc0101,” Table I, top) as a solution in DMA. After 1–2 h at rt, the reaction was buffer exchanged into PBS and purified by size-exclusion chromatography (SEC) in order to remove aggregate and excess vc0101. The final material was characterized by analytical SEC, LCMS, and hydrophobic interaction chromatography (HIC) as previously reported (6). The HIC relative retention time (rrt) was calculated by dividing the absolute retention time of the ADC by the retention time of a mAb reference standard (unmodified trastuzumab). The characterization of the ADCs is shown in Table S1.

In Vitro Cytotoxicity of Site-Specific ADCs

The cytotoxicity of the engineered ADCs was evaluated as previously described (6). ADCs were screened against HER2 high expressing (N87 and BT474) and non-expressing (MB-468) cell lines. A summary of the results is shown in Table I (relative HER2 expression levels are in parentheses).

Differential Scanning Calorimetry (DSC) of ADCs and mAbs

The thermal stability of the antibodies and ADCs were evaluated using capillary VP-DSC equipped with an auto-sampler (Malvern, Westborough, MA). In order to determine if the uncapping procedure (reduction/re-oxidation) resulted in loss of stability, both the naked mAb and the reduced/re-oxidized mAb were evaluated alongside the corresponding ADC. Samples, normalized to 0.02 mM in PBS, pH 7.2 buffer, were heated from 10 to 100°C at a scan rate of 100°C per hour. The resulting heat capacity thermogram was baseline-corrected by subtracting against a PBS buffer scan and fitted to three non-2 state transitions using the Origin7.0 software to generate the melting temperature (T_m) values. Figure 1a illustrates representative DSC data for the 334C and 392C mutants while Fig. 1b shows the complete dataset (note that data was obtained for only 9 out of the 11 mutants). Tabular results are shown in Supplemental Table S2.

Cathepsin-B Cleavage of Site-Specific ADCs

Cathepsin B (Calbiochem, cat#219362) was activated using 6 mM dithiothreitol (DTT) in 150 mM sodium acetate, pH 5.2 for 15 min at 37°C. The activated cathepsin-B (50 ng) was mixed with 20 µL of 1 mg/mL of ADC at a final concentration of 2 mM DTT and 50 mM sodium acetate (pH 5.2). Following incubation at 37°C for 20 min, 1 h, 2 h, and 4 h, reactions were quenched using 10 µM E-64 cysteine protease inhibitor in 250 mM borate buffer (pH 8.5). Samples were reduced with TCEP and analyzed by LC/MS in order to determine the amount of linker cleavage that had taken place. Data is reported in Fig. 2.

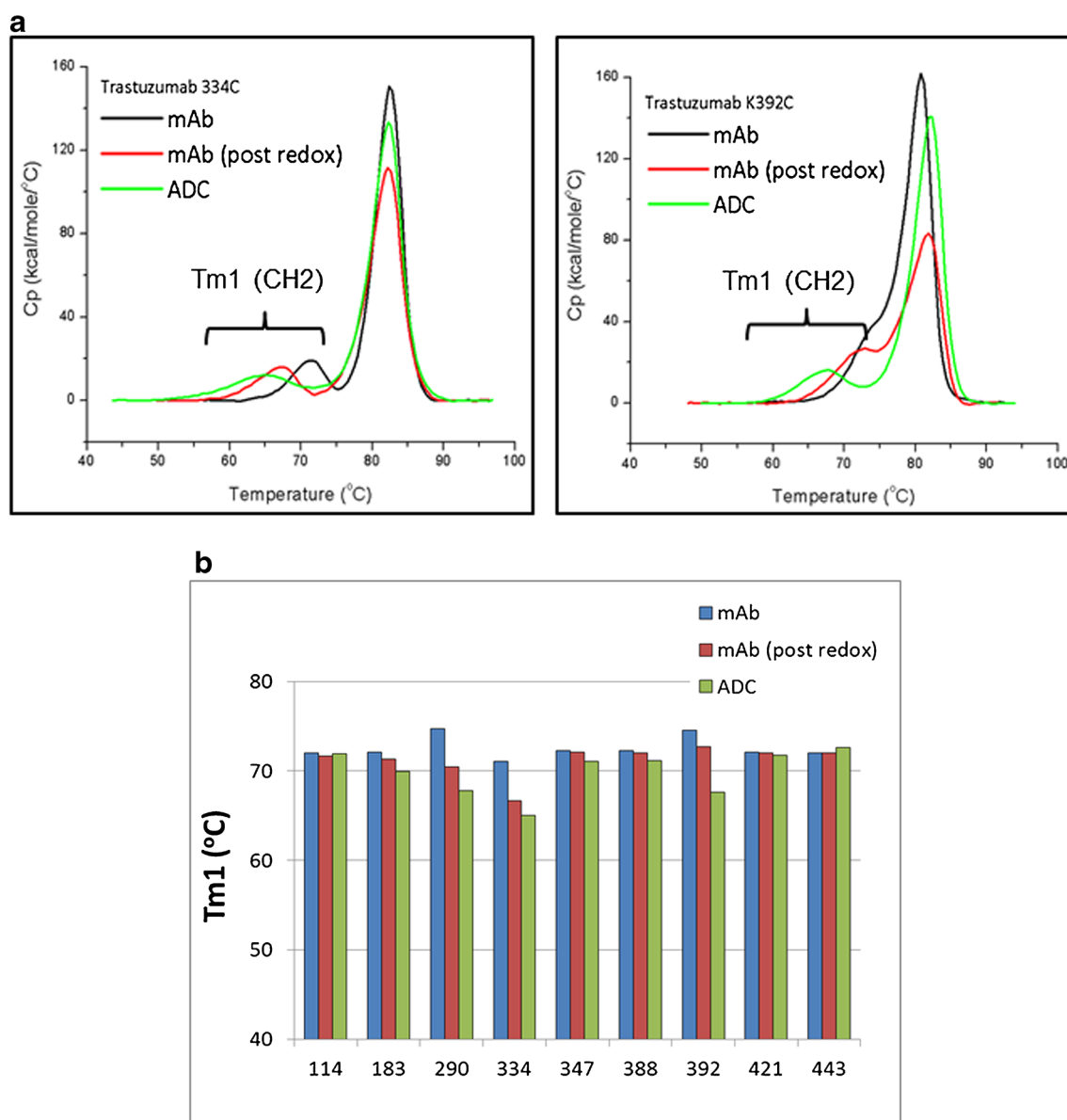


Fig. 1. **a** Sample DSC plots for 334C and 392C engineered ADCs. **b** Summary of T_{m1} (CH2 domain) for key mutants of interest

Plasma Stability of Site-Specific ADCs

The stability of each ADC (50 μ g/mL) was evaluated in heparin-treated plasma collected from male CD-1 mice. A zero time point was removed and stored at -80°C immediately after addition of the ADC. After 3 days (72 h), an additional aliquot was removed and stored at -80°C until analysis. The drug-antibody ratio (DAR) and the %hydrolysis were determined by immunocapture LCMS as previously described (6). Data is summarized in Fig. 3.

Glutathione-Mediated Forced Deconjugation of Site-Specific ADCs

Each ADC (0.25 mg) was combined with PBS and glutathione (GSH) such that the final GSH concentration in the resultant reaction mixture was 0.49 mM and the final

protein concentration was 1.5 mg/mL. Samples were incubated at 37°C , and aliquots were removed at time zero and at 6 days. LCMS analysis was performed using an Aquity H-class UPLC connected to a Xevo G2-XS TOF mass spectrometer as previously described (6). In order to determine the extent of succinimide hydrolysis, samples were treated with 1.5 μ L of Fabricator solution (Genovis, 67 units/ μ L) and incubated at 37°C for 1.5 h. Samples were reduced with TCEP immediately prior to analysis. The separation was performed using a BEH-C18 column (2.1 μ m x 50 mm, P/N 186002350) at 80°C . A gradient from 10% acetonitrile to 95% acetonitrile in water (+0.1% formic acid) was performed and MS data was collected from 700–1900 m/z (positive mode). The entire protein peak was selected for deconvolution using MaxEnt software. Typical injection size is 0.1 μ g. The amount of ring hydrolysis was determined using the +18 peak. Examples of typical LCMS traces are shown in Supplemental Fig. S1. Data is summarized in Fig. 3.

Identification of Optimal Cysteine-Engineered Conjugation Sites

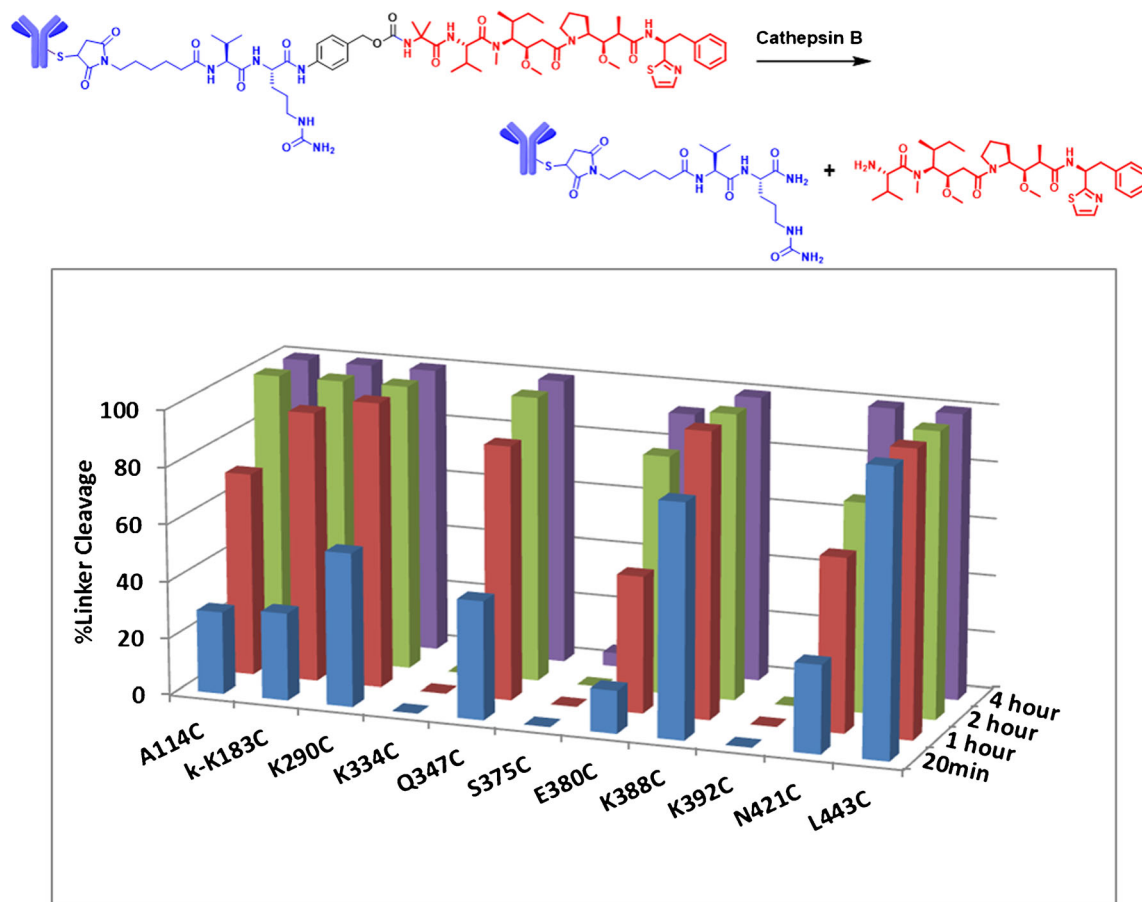


Fig. 2. The rate of linker cleavage by cathepsin B is highly dependent upon site of conjugation

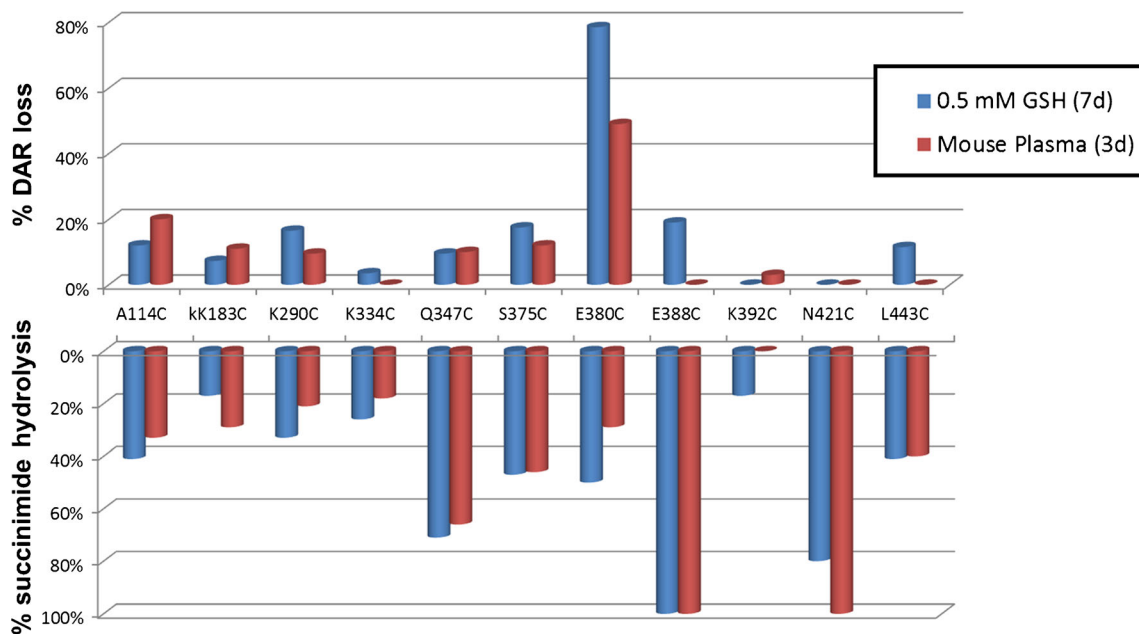


Fig. 3. DAR loss (top) and %succinimide hydrolysis (bottom) observed for ADCs of interest after incubation in mouse plasma for 3 days or 500 μ M glutathione for 7 days (at 37°C)

Table II. Total Antibody (t_{Ab}) and ADC Pharmacokinetics Following a 10 mpk IV Administration of ADCs in Male SD rats

	Total Ab AUC (0–336 h) ($\mu\text{g}\cdot\text{h}/\text{mL}$)	ADC AUC (0–336 h) ($\mu\text{g}\cdot\text{h}/\text{mL}$)	Ab C_{max} ($\mu\text{g}/\text{mL}$)	ADC/Ab
T-IgG1	24,900	NA	256	NA
T-k183C-vc0101	25,400	20,300	258	80%
T-290C-vc0101	17,400	15,900	192	91%
T-334C-vc0101	17,000	15,600	204	92%
T-392C-vc0101	16,500	17,800	168	108%
T-443C-vc0101 ^a	11,000	8680	208	79%

^a AUC = 0–316 h*Pharmacokinetic Evaluation of ADCs in rat*

Five ADCs of particular interest (shown in Table II) were evaluated for pharmacokinetic exposure in male SD rats from Charles River Labs (MA, USA) that were 8–10 weeks old or 300–350 g. The in-life portion of the rat PK study was conducted at Pfizer, Inc., in compliance with the NIH guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee. Rats were randomized in groups were then administered the ADC in PBS at 10 mg/kg as a single intravenous dose via the tail vein. After dosing, rats ($n = 4$ for k183C, 290C, 334C, and 392C and $n = 5$ for 443C) were anesthetized with isoflurane inhalation and blood (200 μL) was collected from the jugular vein at 0.083, 6, 24, 48, 72, 168, and 336 h (or 0.083, 6, 24, 48, 72, 96, 168, and 312 h for the 443C ADC). Blood was transferred to serum tubes and immediately placed on ice until centrifugation for collection of serum samples. Total mAb and ADC levels were determined by a Gyrolab (NJ, USA) LBA platform as previously described (22).

FcRN Binding Chromatography

ADCs of interest were evaluated for binding to immobilized human FcRN. Prior studies on antibody therapeutics has shown that increased relative retention time on an immobilized FcRN column showed strong correlation with poor human clearance. The extent of such correlation has yet to be established for ADCs. The human FcRN affinity column was prepared as described by Schlothauer and colleagues (23). To determine the human FcRN column retention time, 50 μg of ADC sample was injected onto the FcRN coated streptavidin agarose column (GE Healthcare, Marlborough, MA) and a linear pH gradient from pH 5.5 (MES) to 8.8 (Tris) in the presence of 150 mM NaCl was applied for elution of sample. The FcRN column data is reported as a relative elution time where the elution time of sample is subtracted against a control antibody to normalize variability within assay. Data is summarized in Fig. 4.

Affinity-Capture Self-Interaction Nanoparticle Spectroscopy (AC-SINS) (24)

ADCs were screened for self-association using gold nanoparticles precoated with an anti-Fc mAb. Briefly, 20-nm gold nanoparticles (Ted Pella, Reading, CA) were coated with a mixture of 80% anti-human goat IgG Fc and 20% nonspecific goat polyclonal antibodies (Jackson

ImmunoResearch, West Grove, PA). The nanoparticles were then incubated with the ADCs of interest for 2 h at room temperature, and the sample absorbance was measured using the Tecan Infinite M1000 spectrophotometer (Morrisville, NC). Using the Linest function in Excel, the absorbance data was smoothed and fitted to a second-order polynomial to determine the delta shift in maximum wavelength absorbance relative to a PBS, pH 7.2 blank sample. Data is reported in Fig. S2.

RESULTS

The sites of interest for the present study were initially identified by molecular modeling based upon reported crystal structures of IgG1 Fc and Fab domains. Building on the method reported by Voynov (25), we identified a series of potential sites that exhibited partial solvent exposure and lack of involvement in key structural motifs. We specifically avoided sites with high solvent exposure due to possible

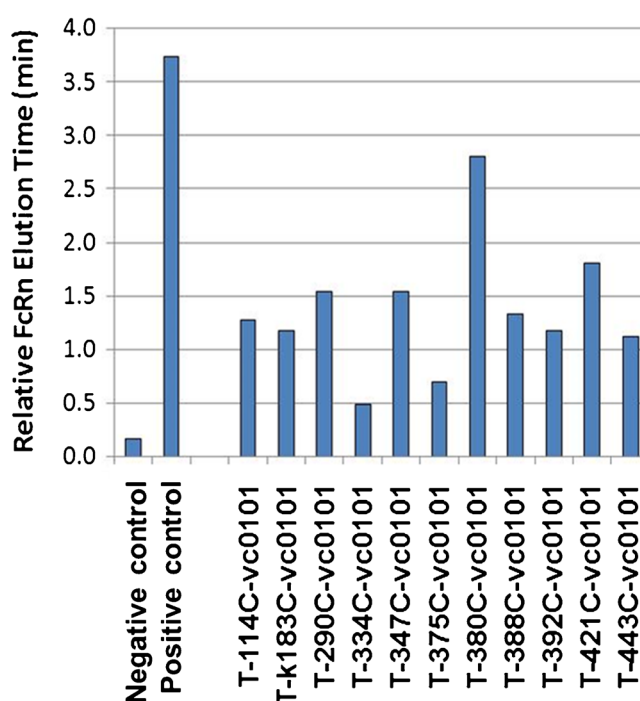


Fig. 4. Relative elution time from a column coated with immobilized human neonatal Fc receptor (FcRN). Antibodies with known fast and slow human clearance were used as negative and positive controls (respectively)

Identification of Optimal Cysteine-Engineered Conjugation Sites

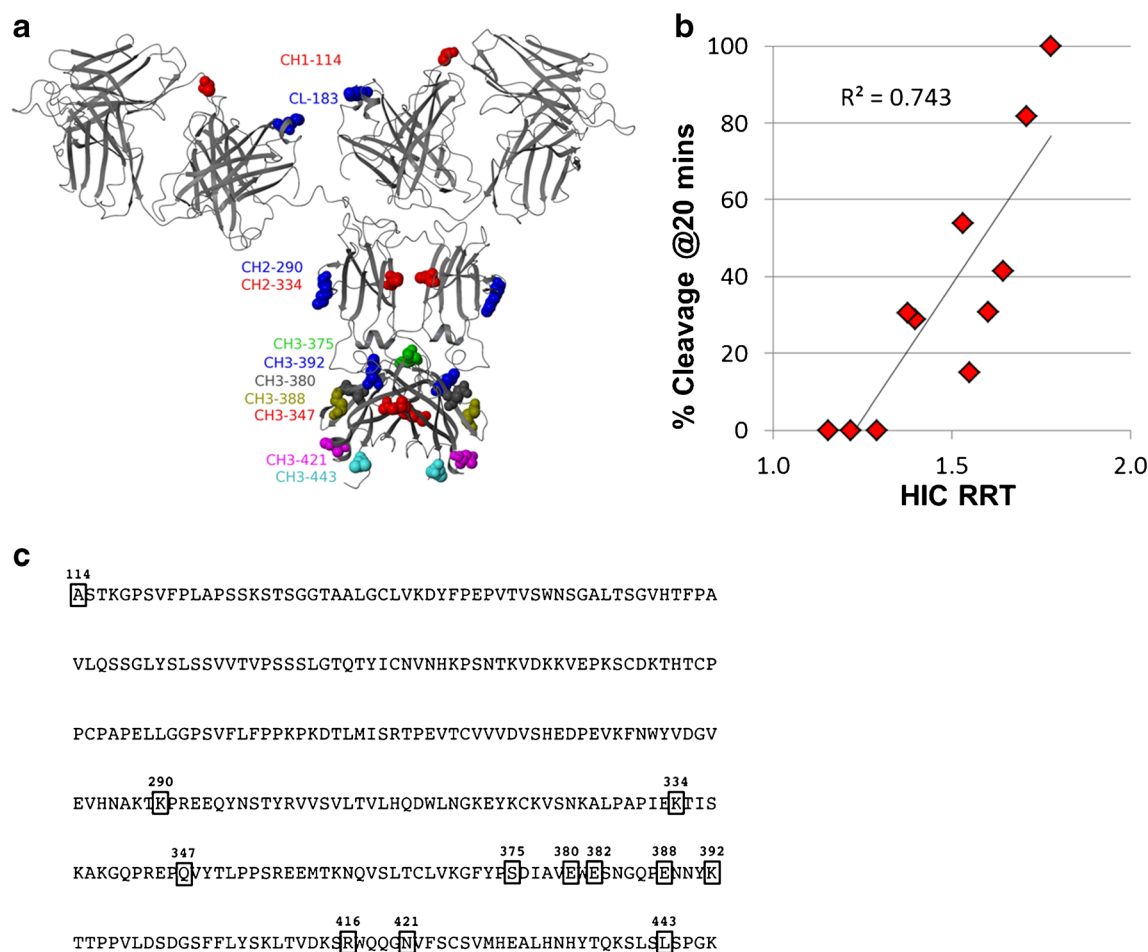


Fig. 5. **a** Structural location of the engineered sites from Table 1, illustrated using PDB entry 1HZH. The two Fab arms are at the top of the picture, the CH2 domains are in the middle, and the CH3 domains are on the *bottom*. Native amino acids are shown in space filling representation on both copies of each chain, with coloring identical to the matching labels, which indicate the domain and site number. **b** The rate of linker cleavage is correlated with the ADC hydrophobicity. **c** Sequence of the constant region of the wild type heavy chain. Due to varying literature precedents, some sites are numbered using differing methods; this panel defines the mapping of site numbers used herein onto the linear sequence

propensity towards aggregation. Amino acids in the CDR region were avoided in order to maintain antigen binding and residues involved in the protein A binding domain were avoided in order to facilitate protein purification. Moreover, residues known to interact with FcRN and Fc-gamma were avoided in order to maintain PK exposure and antibody-dependent cell-mediated cytotoxicity (ADCC), respectively. A subset of the remaining sites was selected for transient expression in COS-1 cells.

The expression and purification processes of the engineered antibodies were comparable with native antibodies. A total of 11 engineered antibodies were isolated: nine of the antibody variants incorporated cysteines into the Fc domain, one onto the HC-Fab domain (3), and one onto the kappa (light) chain. The antibodies were each conjugated to an auristatin payload (Aur-0101) with a valine-citrulline-PABC linker (mcValCitPABC_PF-06380101, or hereafter called “vc0101”). As has been previously reported, the engineered cysteine residues were found to be capped with a disulfide (cysteine or glutathione) and therefore required a reduction/re-oxidation procedure in order to free the newly

incorporated thiol for reaction with electrophiles (3). Interestingly, the 334C and 290C mutants required an extended incubation with TCEP in order to achieve complete uncapping. While not apparent at the time, the slower reaction rate is likely due to the fact that these sites are located in regions of the antibody that are sterically occluded from bulk solvent (*vide infra*). Conjugation with vc0101 provided the 11 corresponding site-specific conjugates as shown in Table S1. The conjugates generally exhibited good loading (approaching a DAR of 2.0 in most cases) and showed the proper mass shift expected for the addition of vc0101. Evaluation by hydrophobic interaction chromatography (HIC) showed that the 11 conjugates exhibited distinct retention times that are presumably correlated with the steric accessibility of the linker-payload to the HIC stationary phase. Consistent with our previous study of site-specific tubulysin ADCs, conjugates at the 334C and 392C sites displayed particularly short HIC retention times (rrt = 1.15 and 1.29) while conjugates at the 443C and 388C sites displayed particularly long HIC retention times (rrt = 1.79 and 1.71) (6).

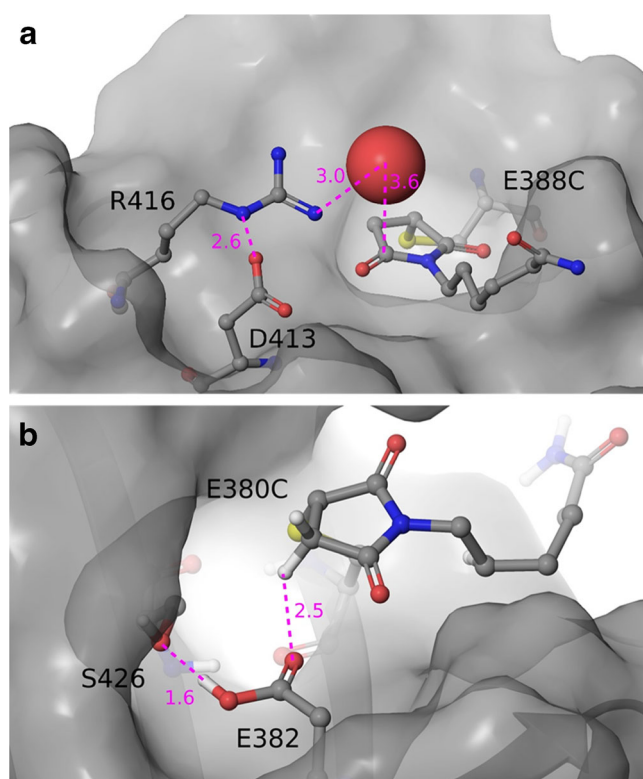


Fig. 6. **a** A snapshot from an MD simulation of a linker fragment attached to the E388C site, showing an Arg416 conformation which might activate a water (large red sphere) to attack the imide. Distances (in Å, purple dashed lines) are between heavy atoms. **b** A low-energy conformation of the E380C site showing the proximity of E382 in a conformation compatible with accelerating a retro-Michael reaction. Distances are between oxygen and hydrogen atoms

The ADCs were evaluated for cytotoxicity in high antigen expressing cell lines (N87 and BT474) and found to have activity $\sim 20\text{--}40$ ng/mL (or $\sim 0.25\text{--}0.50$ nM, based on bound payload concentration). As expected, the ADCs were ~ 1000 -fold less active in a non-HER2 expressing cell line (MDA-MB-468). Consistent with previous reports (3,6), we observed little or no dependence of *in vitro* cytotoxicity on the site of conjugation.

The thermal stability of the conjugates was assessed using DSC in order to determine if any destabilization of the protein structure had occurred as a result of the conjugation. The residues selected for mutation were specifically chosen in order to minimize the impact on the tertiary and quaternary structure of the IgG1. Not surprisingly, therefore, the naked mAbs (Fig. 1b, blue bars) exhibited T_{m1} values typical for IgG1 ($\sim 70^\circ\text{C}$). Six of the nine conjugates showed little or no decrease in melting temperature as compared to the naked mAb. However, three ADCs exhibited a decrease in T_{m1} ($\Delta \sim -7^\circ\text{C}$) that is suggestive of structural destabilization. In two of these (290C and 334C), the decrease in T_{m1} occurred both in the conjugate and also in the reduced/re-oxidized antibody. This suggests that the antibody processing (prior to conjugation) plays a role in the destabilization. Perhaps related to this observation, these same two sites (290C and 334C) were shown to require a long period of uncapping in

the presence of TCEP in order to have optimal loading (see above). While a variety of explanations could be entertained, an obvious hypothesis is that the extensive period of uncapping perhaps resulted in the cleavage and/or shuffling of disulfides that are involved in the maintenance of the tertiary protein structure. As such, the observed destabilization for these sites may be alleviated by employing a more mild reduction protocol, such as the enzymatic approach described by Duppatla (26).

ADCs that employ a proteolytically cleavable linker, such as vc0101, are presumed to undergo antigen-mediated internalization and lysosomal processing resulting in the intracellular release of the payload. While the specific lysosomal enzymes responsible for cleavage have not been identified, the optimization of cleavable linkers has frequently relied upon the fact that cathepsin B is a protease found in abundance in the lysosome (27). As would be expected, the rate of cleavage is highly dependent upon the sequence of the peptidic linker. However, to our knowledge, no one has reported whether cathepsin-mediated linker cleavage is dependent upon site of conjugation. Thus, in order to see if the various site-specific conjugates were efficiently cleaved by cathepsin B, each ADC was treated with cathepsin and the amount of linker cleavage was monitored by LCMS (Fig. 2). Because the *in vitro* potency of the ADCs was not dependent upon site (Table I), we anticipated only minor differences in the cleavage rates for the various mutants. However, we were surprised to find that the rate of linker cleavage was heavily influenced by the site of conjugation. Under the given conditions, several sites (392C, 334C, and 375C) exhibited little or no linker cleavage over the course of 2 h while other sites (443C and 388C) were cleaved almost immediately under identical conditions.

The site dependence upon cleavage shown in Fig. 2 is in apparent disagreement with the recent report by Gikanga showing that the rate of cleavage of vcMMAE conjugates is *not* dependent upon drug location. However, the report by Gikanga focuses only on hinge-cysteines—all of which are located in highly flexible and solvent accessible regions of the antibody. In contrast, many of the sites in the present study are located in relatively hindered regions of the Fc domain (Fig. 5a). As such, it might be expected that some sites would be more prone towards cleavage than others. In fact, a recent report by our group illustrates that esterase mediated metabolism of a tubulysin ADC is highly site-dependent and that the rate of cleavage is closely correlated with relative hydrophobicity of the conjugate (6). Likewise, Fig. 5b illustrates that the amount of ValCit linker cleavage at 20 min (Fig. 2) shows a strong dependency upon the HIC retention of the corresponding conjugate (Table S1). Based on this and our prior work, we believe that a consistent theme is emerging that ADC hydrophobicity is predictive of susceptibility to enzymatic metabolism. It is important to note, however, that increased rates of ValCit cleavage are not associated with increased *in vitro* cytotoxicity. This is likely due to a combination of two factors. First, the lysosome employs a wide variety of catabolic enzymes which degrade the entire IgG1 framework into short peptides thereby obviating the relative accessibility of the ValCit linker. Second, the enzymatic study in Fig. 2 took place over only 2 h while the cytotoxicity study takes place over the course of 4 days.

Identification of Optimal Cysteine-Engineered Conjugation Sites

Table III. Amount of DAR Loss and Succinimide Hydrolysis Observed After Incubation with 0.5 mM Glutathione for 7 days at 37°C

ADC	%Loss of DAR (day 7)	%Succinimide hydrolysis (day 7)
E388C-vc0101	20%	85%
E388C+R416Q-vc0101	15%	44%
N421C-vc0101	20%	88%
N421C+R416Q-vc0101	20%	27%
E380C-vc0101	75%	38%
E380C+E382K-vc0101	75%	100%
E380C+E382R-vc0101	70%	100%
E380C+E382S-vc0101	65%	51%

The increased rate of cathepsin-mediated linker cleavage at sites such as 443C and 388C does not result in decreased plasma stability (Fig. 3, red bars). In fact, no linker cleavage was observed in mouse plasma for any of the 11 mutants evaluated in this study. Rather, the loss of DAR in mouse plasma for various mutants was associated solely with retro-Michael mediated loss of the entire linker-payload. Most engineered antibodies exhibited <15% loss of DAR over the course of 7 days. However, the 380C antibody lost ~50% of its payload via retro-Michael deconjugation over the same period. Treatment of the ADCs with 500 μ M glutathione elicited similar levels of DAR loss (Fig. 3, blue bars), thus confirming that the loss of linker-payload in plasma is largely driven by retro-Michael elimination. Unlike the rate of linker cleavage, the rate of deconjugation was not correlated with ADC hydrophobicity.

We and others have previously shown that the retro-Michael process can be blocked by hydrolytic succinimide ring opening (28). In fact, a report by Genentech based on a limited number of mutants suggested that the rate of ring hydrolysis is a primary determinant of the stability of ADCs generated from cysteine engineered antibodies (5). However, the data shown in Fig. 3 clearly indicates that lack of hydrolysis does not directly lead to ADC instability. For example, two of the most stable ADCs (392C-vc0101 and 334C-vc0101) exhibit little or no hydrolysis during the course of the incubation. This suggests that the retro-Michael process for succinimides is likely driven by the local environment around the conjugation site. It is important to note that this work does not downplay the importance of succinimide hydrolysis. Multiple reports have shown that succinimide hydrolysis blocks retro-Michael mediated deconjugation. We do not disagree with this assertion. Rather, data presented in Fig. 3 indicates that a *lack* of succinimide hydrolysis does not necessarily imply ADC instability.

We performed molecular modeling on several of the mutants in order to better understand the role and mechanism of succinimide hydrolysis. The 388C and 421C antibodies were specifically selected for evaluation due to their propensity for ring hydrolysis (Fig. 3). The succinimide ring and a short alkyl chain were modeled (with both R and S chirality) onto the cysteine site in order to better understand the local environment around the conjugation site. Interestingly, the succinimide ring at both the 388 and 421 sites was found to be in close proximity (≤ 8 Å) to arginine 416.

Molecular dynamics simulations suggested this Arg could activate a water molecule to attack the imide linkage, in a conformation where Asp413 helps position the Arg (Fig. 6a). Conversely, a model of the 392C ADC (which exhibited little or no ring hydrolysis) has no nearby basic residue. We speculated that the R416 residue may facilitate a base-promoted addition of water to the succinimide thereby resulting in the rapid ring hydrolysis observed for the 388C and 421C ADCs. In order to test this “neighboring group” hypothesis, we generated mutants in which the basic 416 arginine residue was changed to a neutral glutamine residue. Encouragingly, both resulting ADCs exhibited significantly lower levels of ring hydrolysis (Table III). The addition of the R416Q mutant to the E388C-vc0101 reduced the level of ring opening from 85 to 44%. Likewise, the incorporation of the R416Q mutant reduced the level of ring hydrolysis from 88 to 27% in the 421C-vc0101 ADC. Interestingly, the level of DAR loss remained unchanged in both cases (~15–20% DAR loss over 7 days) irrespective of the 416 mutant. Once again, this demonstrates that a lack of succinimide hydrolysis does not necessarily imply ADC instability.

We also made attempts to better understand rapid GSH-mediated loss of payload at the 380C site. Once again, molecular modeling with a succinimide ring and short alkyl chain was performed in order to identify nearby residues that might be involved in the payload loss. We speculated that a nearby glutamate residue may help to stabilize the negative charge on the thiol leaving group, and modeled the nearby E382 in the protonated state to investigate this possibility. However, the simulation (Fig. 6b) suggested that E382 was more likely to adopt a conformation compatible with extracting a proton from the CH2 of the ring as opposed to protonating the thiol. In any event, we attempted to introduce neutral and positively charged residues in place of the glutamic acid in order to determine if this residue played a role in the instability of the 380C-vc0101 ADC. Unfortunately, none of the mutants resulted in improved stability (Table III, bottom three rows). We were excited to note, however, that mutations incorporating a basic residue (E382K and E382R) resulted in nearly complete ring hydrolysis. The ring hydrolysis presumably took place at a slower rate than the retro-Michael deconjugation, and thus, there was no improvement in the DAR loss for these ADCs. The fact that we were unable to improve the stability of the

380C antibody by manipulating the E382 residue suggests that acid-promoted elimination does not play a major factor in the deconjugation that is occurring at this site (note that there are no other nearby acidic residues).

Finally, the pharmacokinetics of five of the 11 ADCs were evaluated in Sprague-Dawley rats (Table II). The total-antibody (tAb) concentration was determined using a ligand-binding assay (LBA) with an anti-human Fc antibody. Likewise, the total ADC concentration was determined using an LBA with an anti-payload antibody. As anticipated based on the plasma stability studies, the ADC/tAb ratio was very high (80–108%) suggesting that little or no payload was being lost from the ADC during the PK study. However, only the kappa-183C-vc0101 conjugate exhibited a tAb level comparable to the naked antibody. In fact, the exposure of the 443C conjugate (11,000 µg-h/mL) was less than half of the exposure of the naked mAb (24,900 µg-h/mL). Attempts to correlate this observation with changes in FcRN binding (Fig. 4) and increased aggregation propensity (Supplemental Fig. S2) were unsuccessful. However, Fig. 7 shows that there is a weak correlation between tAb exposure in rat and ADC hydrophobicity as measured by HIC retention. A similar correlation was recently reported by Lyon (29). Interestingly, and for reasons that are unclear at the moment, the correlation between tAb exposure and hydrophobicity is not observed in mice.

DISCUSSION

The design of ADCs is a complex and multifaceted endeavor that requires the optimization of potency, stability, PK, and safety. These factors, in turn, rely on the careful selection and optimization of the antibody target, linker, payload, and site of conjugation. The systematic study of these factors often gets bogged down in a large combinatorial assortment of species in which it is difficult to determine the specific feature that may be driving a pharmaceutical property of interest. For this reason, we targeted a simple assortment of ADCs which employed a common antibody framework (trastuzumab) and a common linker-payload (vc0101) but which employed a variety of conjugation sites. This assortment of ADCs was then used in order to better understand the role that site of conjugation plays in the biophysical and biological properties of an ADC.

Engineered cysteine residues have been extensively used for the site-specific incorporation of tags into proteins and peptides. As the ADC field has moved away from heterogeneous lysine conjugates and hinge-cysteine conjugates, there have been numerous reports of engineered cysteine antibodies being used for the preparation of site-specific conjugates. However, few, if any, of these studies have systematically studied the specific impact that the site of conjugation has on the biophysical properties of the resulting ADC. In this study, we generated a series of ADCs in which the linker-payload and antibody-framework were maintained while we systematically varied the site of conjugation.

An analysis of 14 internal and literature sites identified the predicted solvent-accessible surface area (SASA) as a

property which distinguished site behavior (data not shown). Five poorly behaved sites (that exhibited conjugation or aggregation issues) all had SASA values $>60 \text{ \AA}^2$, while seven of the nine well-behaved sites had lower SASA. No minimum SASA cutoff could be identified from this dataset. Based on this preliminary work, further molecular modeling of the Fc domain was used to identify 11 sites which, when mutated to Cys, exhibited desirable surface exposure and lack of involvement in key structural and functional elements of the antibody. The resulting antibodies maintained natural interactions with antigen, Fc-gamma, FcRN, and protein A. Conjugation of these antibodies resulted in a series of ADCs which were evaluated in various biophysical and biological assays. We demonstrate that the site of conjugation has little impact on the thermal stability or the *in vitro* cytotoxicity of the ADCs (Fig. 1 and Table I, respectively). In spite of having similar cytotoxicity in HER2 expressing cell lines, the rate of payload release in the presence of cathepsin B was found to vary significantly between sites (Fig. 2). Consistent with our recently published work on tubulysin ADC metabolism (6), the rate of payload cleavage by cathepsin was found to be closely correlated with the hydrophobicity of the conjugate (Fig. 5). Conjugation at highly exposed sites such as 443C and 388C results in ADCs in which the linker-payload readily interacts with the HIC stationary phase (resulting in long retention times) and, likewise, is readily accessible by cathepsin B. Conversely, conjugation at more hindered sites such as 334C and 392C results in ADCs in which the linker-payload does not readily interact with the HIC stationary phase (resulting in short retention times) and, likewise, is not readily accessible by cathepsin B.

The correlation between ADC hydrophobicity and the propensity for enzymatic modification is now established quite firmly. The identification of biophysical properties that are associated with a meaningful biological outcome is an important step in moving away from strictly empirical testing. In combination with our previous report (6), we believe that this work establishes ADC hydrophobicity as a useful indication of the solvent accessibility of the linker-payload. ADCs with high HIC retention may be appropriate for particular applications that demand a high solvent exposure for the linker-payload. For instance, sites with high solvent exposure are likely to be advantageous in applications such as extracellular drug conjugates (30) (EDCs) that employ payloads that interact with extracellular and cell surface partners. Additional applications in which high solvent exposure may be beneficial include ADCs which are designed to release payload in the extracellular matrix or in the early endosome. More traditional ADCs that rely on lysosomal catabolism, on the other hand, will likely favor sites with low solvent exposure (low HIC retention) in order to maximize stability in plasma and to minimize the possibility of adverse effects on tAb PK.

Rapid cleavage by cathepsin B does not necessarily imply instability in plasma, as the data in Fig. 3 clearly shows. Conjugates at the 443C and 388C site (two of the most rapidly cleaved mutants by cathepsin B) exhibit little or no DAR loss when incubated in mouse plasma for 3 days. In fact, no linker cleavage was noted for any of the 11 mutants evaluated in this study. Rather, the only mechanism by which payload was lost from the ADCs was via retro-Michael elimination. Previous

Identification of Optimal Cysteine-Engineered Conjugation Sites

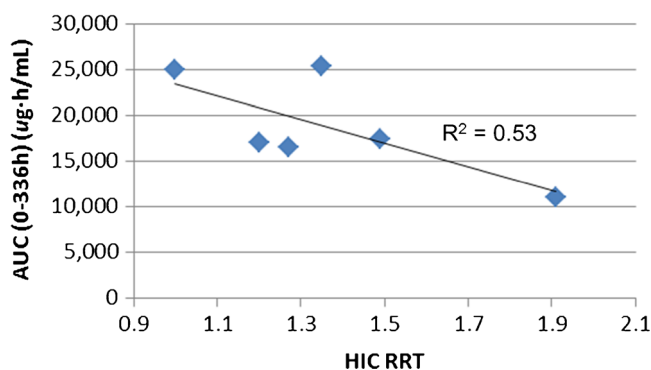


Fig. 7. Correlation of HIC RRT (*i.e.*, ADC hydrophobicity) with rat tAb exposure

reports have clearly demonstrated that retro-Michael loss of DAR can be mitigated by succinimide hydrolysis (5,28). Indeed, those mutants that exhibited rapid ring-opening (388C and 421C) were quite resistant to deconjugation both in plasma and in the presence of glutathione. Molecular modeling (Fig. 6a) suggested that arginine 416 may be involved in the rapid ring hydrolysis observed for both the 388C and 421C ADCs. Consistent with this hypothesis, the rate of ring hydrolysis was reduced when this residue was mutated to a non-basic glutamine moiety (Table III). Conversely, we hypothesized that introduction of an arginine nearby to a conjugation site should accelerate succinimide ring hydrolysis. Indeed, we found that introducing an arginine (E382R) nearby to the 380C conjugation site resulted in increased rates of ring hydrolysis (Table III). The concept of introducing a basic residue nearby to the conjugation site in order to induce ring-hydrolysis has been previously reported by Seattle Genetics. Their work, published in 2014, demonstrated that introducing a basic amine in the linker (nearby to the succinimide) results in spontaneous ring-hydrolysis (31). Our work shows that a similar concept can be employed by exploiting nearby basic residues in the antibody backbone rather than in the linker. This would allow for the flexibility of using the myriad of maleimide-based linker-payloads that have been developed in recent years without resorting to re-engineering of the linker.

In spite of the importance of succinimide hydrolysis, our results strongly suggest that this factor alone is not the sole determinant of ADC stability. Several sites were shown to result in very stable conjugates even though only minor amounts of ring hydrolysis were observed (392C, 443C, and 334C). In these cases, linker or antibody engineering in order to affect the ring hydrolysis would be expected to have little or no impact on conjugate stability.

For reasons that are yet unclear, the 380C site was shown to be particularly susceptible to retro-Michael mediated deconjugation. We hypothesized that an acidic residue nearby to the succinimide ring at the 380C position may accelerate the retro-Michael deconjugation process. However, our mutational studies do not support this assertion. The E380C+E382X mutants in Table III were shown to undergo deconjugation at approximately the same rate as the parent molecule. Thus, the underlying chemical drivers behind retro-Michael mediated deconjugation of sites such as 380C remain unknown at this time.

In vitro measurements of stability, while important, are only a tool used to triage compounds prior to *in vivo*

evaluation. As such, five of the most promising ADCs were selected for PK evaluation in rats. All five conjugates exhibited a high ADC/tAb, as our *in vitro* plasma stability and forced deconjugation assays predicted (Fig. 3), thus indicating that little or no payload was being lost either by retro-Michael deconjugation or by premature linker cleavage. However, the five conjugates had significantly different tAb exposure. Only the kappa-183C conjugate was found to have comparable exposure to that of the naked mAb. In fact, a conjugate near the C-terminus of the Fc domain (443C-vc0101) exhibited less than half of the exposure of the parent antibody (Table II). These results are quite comparable to a study by Strop in which an ADC with the payload at the C-terminus of the Fc domain had less than half of the tAb exposure of an analogous light-chain conjugate (4). There have been reports that ADC hydrophobicity is closely correlated with tAb exposure and our results partially support this. Interestingly, we found that the C-terminal heavy chain conjugate reported by Strop was found to have a very high HIC shift ($rrt = 1.8$) compared to the corresponding C-terminal light chain ADC ($rrt = 1.3$). Similarly, the 443C conjugate (reduced exposure) exhibited a HIC rrt of 1.79 while the corresponding kappa-183C conjugate (higher exposure) exhibited a HIC rrt of 1.38. While this preliminary correlation was encouraging, a more broad evaluation of other site-specific conjugates suggested that the correlation between hydrophobicity and exposure was rather weak, as illustrated in Fig. 7.

While the poor exposure of the 443C conjugate was correlated with its high hydrophobicity, this is not an explanatory mechanism. Reduced tAb PK can be associated with perturbations in the FcRN recycling mechanism, either through decreased FcRN affinity at low pH or increased FcRN affinity at neutral pH. However, we did not observe a discernable difference between the 443C and kappa-183C conjugates in the elution time on an immobilized FcRN column (Fig. 4). This suggests that the difference between these conjugates may not lie with FcRN binding. Likewise, no increase in self-association was observed for the 443C conjugate in a gold nanoparticle affinity-capture assay (AC-SINS, Fig. 6). Thus, while there is a weak correlation of ADC hydrophobicity with reduced tAb exposure, a causative explanation remains elusive at this time. However, a consistent set of data now clearly illustrates that the C-terminus of the heavy chain is a problematic point of attachment for ADC payloads.

CONCLUSIONS

The selection of an optimal site of conjugation, of course, depends on the overall objectives to be achieved. This report describes a series of lessons learned through the evaluation of a carefully selected set of bioconjugates. We demonstrate that cytotoxicity is not dependent upon site of conjugation. We show that the rate of ValCit linker cleavage can be tuned by careful selection of site and that this tuning can be informed by the hydrophobicity of the conjugate. This correlation adds to a growing body of evidence that ADC hydrophobicity plays an important role in conjugate stability. We demonstrate that careful selection of site can result in conjugates that exhibit no loss of thermal stability, protein A binding, and FcRN binding. We demonstrate that

succinimide hydrolysis can be induced by the careful placement of a basic amino acid in close proximity to the site of conjugation. Finally, we show that total antibody exposure varies with the site of conjugation and that this may be partially correlated with ADC hydrophobicity, thereby confirming recent reports of this phenomenon.

While these studies were generated using a model antibody (trastuzumab) and a model linker-payload (vc0101), we believe that the results will translate to therapeutic programs and will help teams to make more informed decisions about the optimal site of conjugation. Most importantly, this report will help teams to know what factors can and cannot be tuned by manipulating the site of conjugation. It is our hope that other teams will report systematic studies such as this so that the ADC community can continue the process of building “design rules” for the next generation of ADC programs.

ACKNOWLEDGEMENTS

We would like to gratefully acknowledge the work of Russell Dushin, Dahui Zhou, Matthew Doroski, Alex Porte, Hud Risley, Zecheng Chen, and Andreas Maderna for the preparation and identification of mcValCitPABC_06380101. Dosing of animals was performed by Judy Lucas and Christine Hosselet.

REFERENCES

1. Thomas A, Teicher BA, Hassan R. Antibody-drug conjugates for cancer therapy. *Lancet Oncol.* 2016;17(6):e254–62.
2. Panowski S, Bhakta S, Raab H, Polakis P, Junutula JR. Site-specific antibody drug conjugates for cancer therapy. *MAbs.* 2014;6(1):34–45.
3. Junutula JR, Raab H, Clark S, Bhakta S, Leipold DD, Weir S, Chen Y, Simpson M, Tsai SP, Dennis MS, Lu Y, Meng YG, Ng C, Yang J, Lee CC, Duenas E, Gorrell J, Katta V, Kim A, McDorman K, Flagella K, Venook R, Ross S, Spencer SD, Wong WL, Lowman HB, Vandlen R, Sliwkowski MX, Scheller RH, Polakis P, Mallet W. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat Biotechnol.* 2008;26:925–32.
4. Strop P, Liu S-H, Dorywalska M, Delaria K, Dushin RG, Tran T-T, Ho W-H, Farias S, Casas MG, Abdiche Y, Zhou D, Chandrasekaran R, Samain C, Loo C, Rossi A, Rickert M, Krimm S, Wong T, Chin SM, Yu J, Dilley J, Chaparro-Riggers J, Filzen GF, O'Donnell CJ, Wang F, Myers JS, Pons J, Shelton DL, Rajpal A. Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. *Chem Biol (Oxford, U K).* 2013;20:161–7.
5. Shen B-Q, Xu K, Liu L, Raab H, Bhakta S, Kenrick M, Parsons-Reponte KL, Tien J, Yu S-F, Mai E, Li D, Tibbitts J, Baudys J, Saad OM, Scales SJ, McDonald PJ, Hass PE, Eigenbrot C, Nguyen T, Solis WA, Fuji RN, Flagella KM, Patel D, Spencer SD, Khawli LA, Ebens A, Wong WL, Vandlen R, Kaur S, Sliwkowski MX, Scheller RH, Polakis P, Junutula JR. Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat Biotechnol.* 2012;30:184–9.
6. Tumey LN, Leverett CA, Vetelino B, Li F, Rago B, Han X, Loganzo F, Musto S, Bai G, Sukuru SC, Graziani EI, Puthenveetil S, Casavant J, Ratnayake A, Marquette K, Hudson S, Doppalapudi VR, Stock J, Tchistiakova L, Bessire AJ, Clark T, Lucas J, Hosselet C, O'Donnell CJ, Subramanyam C. Optimization of tubulysin antibody-drug conjugates: a case study in addressing ADC metabolism. *ACS Med Chem Lett.* 2016;7:977–82.
7. Xu K, Liu L, Saad OM, Baudys J, Williams L, Leipold D, Shen B, Raab H, Junutula JR, Kim A, Kaur S. Characterization of intact antibody-drug conjugates from plasma/serum in vivo by affinity capture capillary liquid chromatography-mass spectrometry. *Anal Biochem.* 2011;412:56–66.
8. Axup Jun Y, Bajjuri Krishna M, Ritland M, Hutchins Benjamin M, Kim Chan H, Kazane Stephanie A, Halder R, Forsyth Jane S, Santidrian Antonio F, Stafin K, Lu Y, Tran H, Seller Aaron J, Biroc Sandra L, Szydluk A, Pinkstaff Jason K, Tian F, Sinha Subhash C, Felding-Habermann B, Smider Vaughn V, Schultz PG. Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proc Natl Acad Sci U S A.* 2012;109:16101–6.
9. Rashidian M, Dozier JK, Distefano MD. Enzymatic labeling of proteins: techniques and approaches. *Bioconjug Chem.* 2013;24:1277–94.
10. Hamblett KJ, Senter PD, Chace DF, Sun MMC, Lenox J, Cervený CG, Kissler KM, Bernhardt SX, Kopcha AK, Zabinski RF, Meyer DL, Francisco JA. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin Cancer Res.* 2004;10:7063–70.
11. Adem YT, Schwarz KA, Duenas E, Patapoff TW, Galush WJ, Esue O. Auristatin antibody drug conjugate physical instability and the role of drug payload. *Bioconjug Chem.* 2014;25:656–64.
12. Zhao RY, Wilhelm SD, Audette C, Jones G, Leece BA, Lazar AC, Goldmacher VS, Singh R, Kovtun Y, Widdison WC, Lambert JM, Chari RVJ. Synthesis and evaluation of hydrophilic linkers for antibody-maytansinoid conjugates. *J Med Chem.* 2011;54:3606–23.
13. Stimmel JB, Merrill BM, Kuyper LF, Moxham CP, Hutchins JT, Fling ME, Kull Jr FC. Site-specific conjugation on serine right-arrow cysteine variant monoclonal antibodies. *J Biol Chem.* 2000;275:30445–50.
14. Lyons A, King DJ, Owens RJ, Yarranton GT, Millican A, Whittle NR, Adair JR. Site-specific attachment to recombinant antibodies via introduced surface cysteine residues. *Protein Eng.* 1990;3:703–8.
15. Puthenveetil S, Loganzo F, He H, Dirico K, Green M, Teske J, Musto S, Clark T, Rago B, Koehn F, Venezia R, Falahatisheh H, Han X, Barletta F, Lucas J, Subramanyam C, O'Donnell CJ, Tumey LN, Sapra P, Gerber HP, Ma D, Graziani EI. Natural product splicing inhibitors: a new class of antibody-drug conjugate (ADC) payloads. *Bioconjug Chem.* 2016;27:1880–8.
16. Maderna A, Doroski M, Subramanyam C, Porte A, Leverett CA, Vetelino BC, Chen Z, Risley H, Parrisi R, Pandit J, Varghese AH, Shanker S, Song C, Sukuru SCK, Farley KA, Wagenaar MM, Shapiro MJ, Musto S, Lam M-H, Loganzo F, O'Donnell CJ. Discovery of cytotoxic Dolastatin 10 analogues with N-terminal modifications. *J Med Chem.* 2014;57:10527–43.
17. Wei C, Zhang G, Clark T, Barletta F, Tumey LN, Rago B, Hansel S, Han X. Where did the linker-payload go? A quantitative investigation on the destination of the released linker-payload from an antibody-drug conjugate with a maleimide linker in plasma. *Anal Chem (Washington, DC, U S).* 2016;88:4979–86.
18. Krivov GG, Shapovalov MV, Dunbrack Jr RL. Improved prediction of protein side-chain conformations with SCWRL4. *Proteins.* 2009;77:778–95.
19. Ye JD, Tereshko V, Frederiksen JK, Koide A, Fellouse FA, Sidhu SS, Koide S, Kossiakoff AA, Piccirilli JA. Synthetic antibodies for specific recognition and crystallization of structured RNA. *Proc Natl Acad Sci U S A.* 2008;105:82–7.
20. Cho HS, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney Jr DW, Leahy DJ. Structure of the extracellular region

Identification of Optimal Cysteine-Engineered Conjugation Sites

- of HER2 alone and in complex with the Herceptin Fab. *Nature*. 2003;421:756–60.
21. Spassov VZ, Yan LA fast and accurate computational approach to protein ionization. *Protein Sci*. 2008;17:1955–70.
22. Rago B, Clark T, King L, Zhang J, Tumey LN, Li F, Barletta F, Wei C, Leal M, Hansel S, Han X. Calculated conjugated payload from immunoassay and LC-MS intact protein analysis measurements of antibody-drug conjugate. *Bioanalysis*. 2016;8:2205–17.
23. Schlothauer T, Rueger P, Stracke JO, Hertenberger H, Fingas F, Kling L, Emrich T, Drabner G, Seeber S, Auer J, Koch S, Papadimitriou A. Analytical FcRn affinity chromatography for functional characterization of monoclonal antibodies. *MAbs*. 2013;5:576–86.
24. Tessier PM, Jinkoji J, Cheng YC, Prentice JL, Lenhoff AM. Self-interaction nanoparticle spectroscopy: a nanoparticle-based protein interaction assay. *J Am Chem Soc*. 2008;130:3106–12.
25. Voynov V, Chennamsetty N, Kayser V, Wallny HJ, Helk B, Trout BL. Design and application of antibody cysteine variants. *Bioconjug Chem*. 2010;21:385–92.
26. Duppatla V, Gjorgjevikj M, Schmitz W, Kottmair M, Mueller TD, Sebald W. Enzymatic deglutathionylation to generate interleukin-4 cysteine muteins with free thiol. *Bioconjug Chem*. 2012;23:1396–405.
27. Dubowchik GM, Firestone RA, Padilla L, Willner D, Hofstead SJ, Mosure K, Knipe JO, Lasch SJ, Trail PA. Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. *Bioconjug Chem*. 2002;13:855–69.
28. Tumey LN, Charati M, He T, Sousa E, Ma D, Han X, Clark T, Casavant J, Loganzo F, Barletta F, Lucas J, Graziani EI. Mild method for succinimide hydrolysis on ADCs: impact on ADC potency, stability, exposure, and efficacy. *Bioconjug Chem*. 2014;25:1871–80.
29. Lyon RP, Bovee TD, Doronina SO, Burke PJ, Hunter JH, Neff-LaFord HD, Jonas M, Anderson ME, Setter JR, Senter PD. Reducing hydrophobicity of homogeneous antibody-drug conjugates improves pharmacokinetics and therapeutic index. *Nat Biotechnol*. 2015;33:733–5.
30. Marshall DJ, Harried SS, Murphy JL, Hall CA, Shekhani MS, Pain C, Lyons CA, Chillemi A, Malavasi F, Pearce HL, Thorson JS, Prudent JR. Extracellular antibody drug conjugates exploiting the proximity of two proteins. *Mol Ther*. 2016;24:1760–70.
31. Lyon RP, Setter JR, Bovee TD, Doronina SO, Hunter JH, Anderson ME, Balasubramanian CL, Duniho SM, Leiske CI, Li F, Senter PD. Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates. *Nat Biotechnol*. 2014;32:1059–62.