Bioconjugate Chemistry

Article

pubs.acs.org/bc

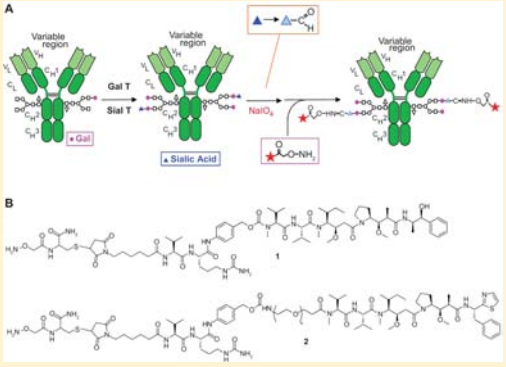
Site-Specific Antibody–Drug Conjugation through Glycoengineering

Qun Zhou,* James E. Stefano, Charlene Manning, Josephine Kyazike, Bo Chen, Diego A. Gianolio, Anna Park, Michelle Busch, Julie Bird, Xiaoyang Zheng, Helene Simonds-Mannes, Jennifer Kim, Rick C. Gregory, Robert J. Miller, William H. Brondyk, Pradeep K. Dhal, and Clark Q. Pan

Sanofi-Genzyme R&D Center, Genzyme Corporation, A Sanofi Company, Framingham, Massachusetts 01701, United States

Supporting Information

ABSTRACT: Antibody–drug conjugates (ADCs) have been proven clinically to be more effective anti-cancer agents than native antibodies. However, the classical conjugation chemistries to prepare ADCs by targeting primary amines or hinge disulfides have a number of shortcomings including heterogeneous product profiles and linkage instability. We have developed a novel site-specific conjugation method by targeting the native glycosylation site on antibodies as an approach to address these limitations. The native glycans on Asn-297 of antibodies were enzymatically remodeled *in vitro* using galactosyl and sialyltransferases to introduce terminal sialic acids. Periodate oxidation of these sialic acids yielded aldehyde groups which were subsequently used to conjugate aminooxy functionalized cytotoxic agents via oxime ligation. The process has been successfully demonstrated with three antibodies including trastuzumab and two cytotoxic agents. Hydrophobic interaction chromatography and LC-MS analyses revealed the incorporation of ~1.6 cytotoxic agents per antibody molecule, approximating the number of sialic acid residues. These glyco-conjugated ADCs exhibited target-dependent antiproliferative activity toward antigen-positive tumor cells and significantly greater antitumor efficacy than naked antibody in a Her2-positive tumor xenograft model. These findings suggest that enzymatic remodeling combined with oxime ligation of the native glycans of antibodies offers an attractive approach to generate ADCs with well-defined product profiles. The site-specific conjugation approach presented here provides a viable alternative to other methods, which involve a need to either re-engineer the antibody sequence or develop a highly controlled chemical process to ensure reproducible drug loading.



INTRODUCTION

Although current standard therapeutic interventions, including surgery, radiation, and chemotherapy, have prolonged survival time of many cancer patients, there is continuing need for more effective anti-cancer therapies. In particular, target-specific therapies with higher efficacy and a greater therapeutic index are most attractive. Antibody–drug conjugates (ADC) have exhibited clinically significant utility for the treatment of cancers. ADC technology exploits the specificity of monoclonal antibodies against tumor-specific antigens for the targeted delivery of highly potent cytotoxic agents. This approach enables one to use otherwise nonspecific cytotoxins with high dose-limiting toxicities. It has resulted in effective anti-cancer therapies with superior efficacy, lower side effects, and hence an improved quality of life.[1,2] In recent years, significant progress has been made in the area of ADC R&D including two newly approved products, brentuximab vedotin for the treatment of anaplastic large cell lymphoma and Hodgkin's lymphoma,[3,4] and trastuzumab emtansine for advanced Her2-positive breast cancer.[5,6] Thus, ADC technology has created a new paradigm for cancer treatment.

Current methods for the preparation of ADCs predominantly employ conventional bioconjugation strategies by targeting either amino groups of lysine residues or thiol groups (generated by the reduction of hinge region disulfides) as the sites for linking cytotoxins to the antibodies. Unfortunately, since there are ~30 surface-exposed lysines and 8 hinge cysteine residues in IgG1 antibodies, such conjugations can result in a diverse population of ADCs with a wide distribution of drugs per antibody.[7,8] Some of the undesired ADC subpopulation could lead to shorter circulation half-life, lower efficacy, and potentially increased off-target toxicity.[8,9] Therefore, development of synthetic methods for site-specific conjugation of cytotoxic agents to antibodies is highly desirable to overcome these clinically significant limitations. A variety of approaches have been developed in recent years for site selective ADC synthesis. They include incorporation of unpaired cysteines, glutamine tags, thiolfucose, and unnatural amino acids through mutagenesis.[9−13] Although nearly homogeneous product profiles have been obtained, these strategies require re-engineering of the antibody sequence and/or reoptimization of cell culture condition.

Received: October 29, 2013
Revised: February 14, 2014
Published: February 17, 2014

   dx.doi.org/10.1021/bc400505q | *Bioconjugate Chem.* 2014, 25, 510−520

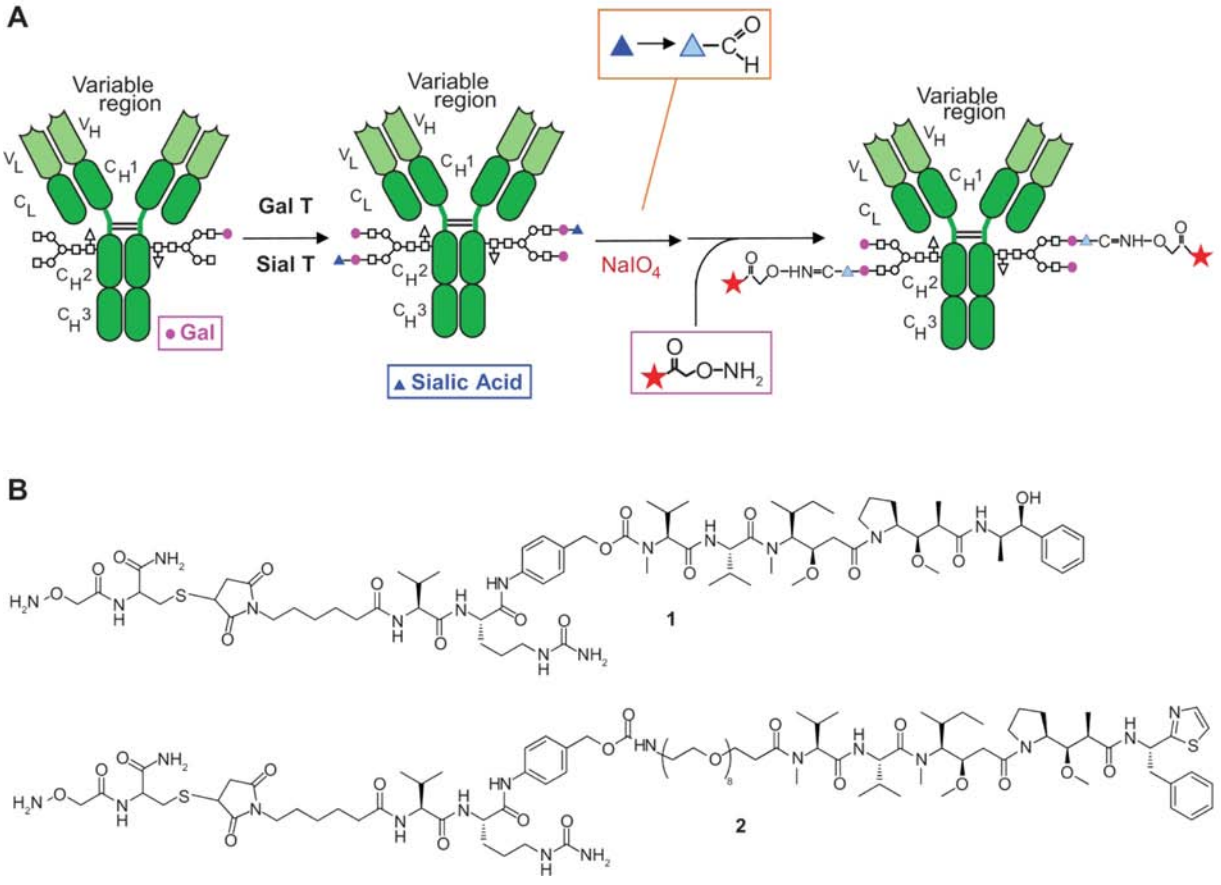
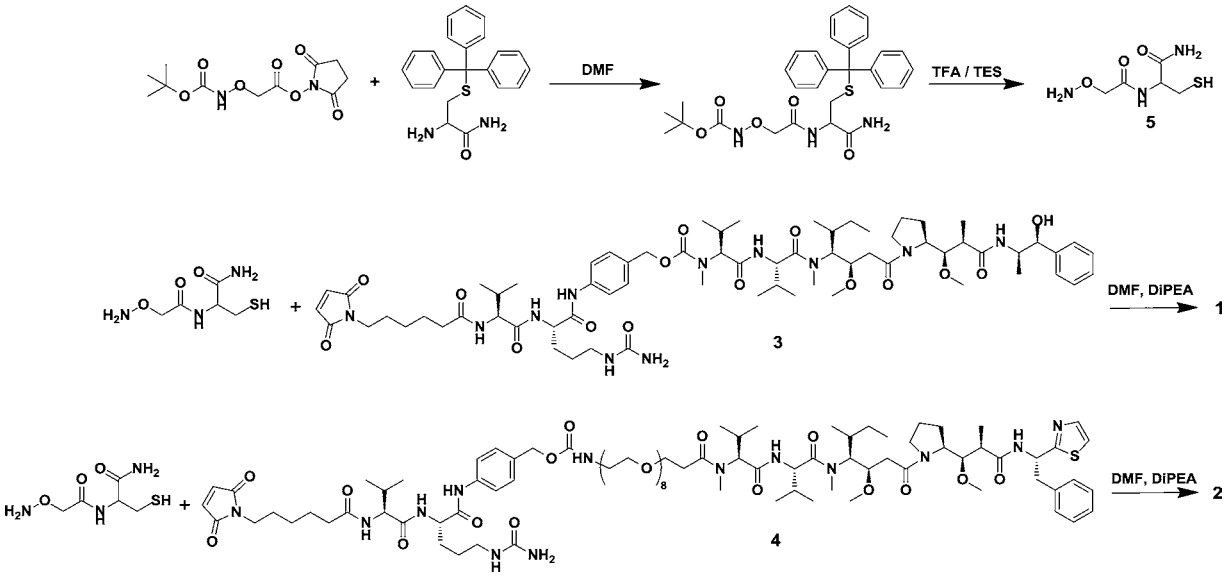


Figure 1. Site-specific glycoconjugation of antibody. (A) The conjugation scheme to generate Glyco ADCs. (B) Two aminooxy-derivatized drug-linkers for conjugation, aminooxy-Cys-MC-VC-PABC-MMAE and aminooxy-Cys-MC-VC-PABC-PEG8-Dol10, are shown as a star containing compound inside a purple box in panel A.

Scheme 1. Synthesis of Aminooxy-Cys-MC-VC-PABC-MMAE and Aminooxy-Cys-MC-VC-PABC-PEG8-Dol10



As an alternative, we have explored post-translational remodeling of the native glycans located at Asn-297 within the Fc domain as the site of conjugation via oxime ligation. It involves enzymatically introducing sialic acid moieties to the antibody, followed by mild oxidation of the former to introduce aldehyde groups. The resulting aldehyde functionalized antibodies can subsequently be conjugated with aldehyde-reactive aminooxy group containing drugs to form Glyco ADCs with drug linked through a stable oxime bond. By using this approach, site-specific conjugation to three antibodies using two different cytotoxins has been achieved. The *in vitro* antiproliferative activities and *in vivo* tumor regression efficacies

dx.doi.org/10.1021/bc400505q | *Bioconjugate Chem.* 2014, 25, 510−520

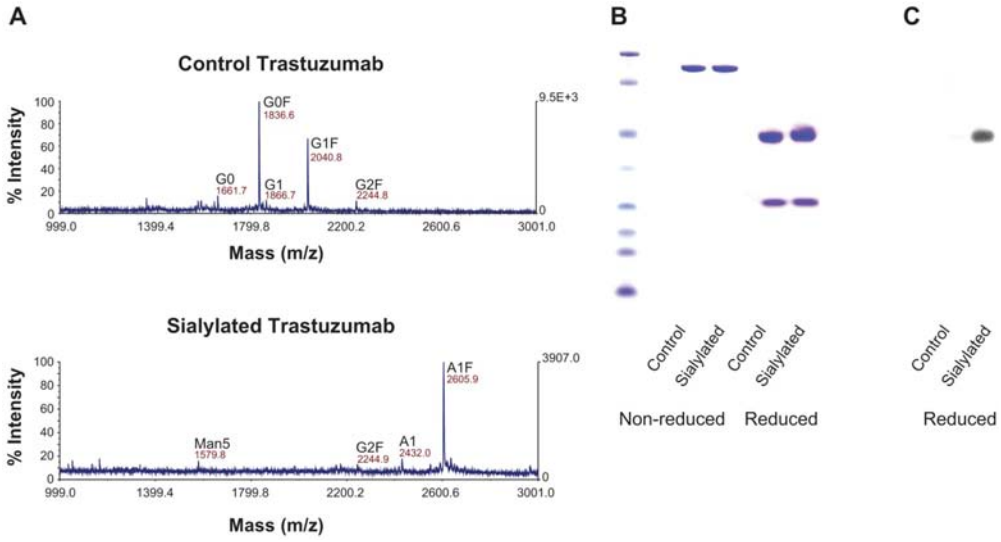


Figure 2. Characterization of sialylated trastuzumab. (A) MALDI-TOF analysis of permethylated *N*-glycan released with PNGase F. (B) SDS-PAGE and (C) SNA lectin blotting of control and sialylated antibodies. G0F represents biantennary glycan with core fucose but not galactose; G1F represents monogalactosylated biantennary glycan with core fucose; A1F refers as monosialylated biantennary glycan with core fucose.

of these Glyco ADCs attest to the utility of this approach as a modular method to prepare site-specific conjugates with nearly homogeneous composition directly from any existing monoclonal antibody.

RESULTS

Site-Specific Conjugation Strategy. Our approach for the preparation of site-specific Glyco ADC utilizes sialic acid directed oxime ligation by generating aldehyde functionalized antibody through mild periodate oxidation of the sialic acids, followed by reaction with aminooxy functionalized cytotoxic drug linker (Figure 1A). In order to allow direct comparison with the ADCs of the same antibodies obtained by thiol-mediated chemistry (generated from hinge disulfide reduction), two aminooxy drug-linkers, AO-Cys-MC-VC-PABC-MMAE (**1**) and AO-Cys-MC-VC-PABC-PEG8-Dol10 (**2**), were prepared (Figure 1B). These drug-linkers were synthesized by the introduction of aminooxy functional groups onto the corresponding maleimide forms of the drug-linkers (MC-VC-PABC-MMAE (**3**) and MC-VC-PABC-PEG8-Dol10 (**4**)), as shown in Scheme 1. This was accomplished by reaction of **3** or **4** with a thiol containing aminooxy reagent (**5**) to yield the desired compounds, **1** or **2** (Scheme 1). The syntheses of the thiol-reactive maleimide versions of the drug-linkers were carried out based on syntheses of previously described compounds.[14] The two cytotoxins employed in our studies were monomethylauristatin E (MMAE) and dolastatin 10 (Dol 10). MMAE is an inhibitor of microtubule assembly used in brentuximab vedotin and other ADCs currently in various stages of development.[14] Dol 10 is the first-in-the-class tubulin inhibitor isolated from the sea hare *Dolabella auricularia*. This linear pentapeptide was used as the model to design various microtubule assembly inhibitors including MMAE.[14] Although quite potent in our hands, the highly hydrophobic nature of Dol 10 was found to produce significant aggregation of ADCs prepared using it. Thus, an oligoethylene glycol (PEG8) spacer was introduced to improve its hydrophilicity, which has successfully overcome the aggregation issue (unpublished data). All these compounds also contained a cathepsin B-sensitive valine-citrulline-*p*-aminobenzyloxy carbonyl (VC-PABC) moiety to facilitate selective lysosomal release of the native drug after cellular internalization of the ADC.[14]

Preparation of Glyco ADCs. We have previously developed a highly effective and reproducible mild oxidation method for transforming protein bound sialic acid moieties of recombinant human lysosomal enzymes to the corresponding aldehyde form and their subsequent conjugation with different biologically important ligands via oxime ligation.[16] Although this sialic acid-mediated (SAM) chemistry produces highly defined and stable bioconjugates with glycoproteins, the process cannot be directly applied to the conjugation of drugs to IgGs, since the native glycans on the Asn-297 of the Fc region lack sialic acids. Thus, we incorporated sialic acid units onto the antibodies by an *in vitro* modification process as illustrated in Figure 1A. A mixture of β1,4-galactosyltransferase (Gal T) and α2,6-sialyltransferase (Sial T) was used to transfer galactose and sialic acid residues onto the native glycans of an antibody. As in the case of other glycoproteins, these sialic acid residues could be oxidized under mild condition using limiting concentrations of sodium periodate at low temperature to yield aldehyde functionalized antibodies. These aldehyde group-containing antibodies could be reacted with aminooxy drug-linkers to generate the desired ADCs linked through a stable oxime bond.

Enzymatic means to introduce sialic acids by using a combination of Gal T and Sial T had been previously demonstrated with a human polyclonal IgG (IVIG) and Fc fragments.[24,26] We therefore evaluated this process for the remodeling of monoclonal antibodies, which contain negligible amounts of sialic acid and particularly low levels of galactose, potentially making them poorer substrates. The native glycans from trastuzumab were released with PNGase F, permethylated, and analyzed using MALDI-TOF MS. The results showed the presence of mainly neutral biantennary complex-type glycans containing core fucose with zero or one galactose residue (G0F or G1F). When treated with both Gal T and Sial T, however, the *N*-glycans were converted to the monosialylated biantennary forms containing a core fucose A1F (>94%) (Figure 2A). Increasing concentrations of Sial T in the reaction mixture did not produce any further increase in sialylation as

dx.doi.org/10.1021/bc400505q | *Bioconjugate Chem.* 2014, 25, 510−520

evidenced by the near absence of disialylated biantennary structure (A2F). SDS-PAGE under both reducing and nonreducing conditions was unable to detect any difference in the polypeptide chain (Figure 2B). However, blotting with a lectin specific for α2,6-linked sialic acid (*Sambucus nigra agglutinin*, SAN) indicated the presence of α2,6-linked sialic acid specifically on the heavy chain of the treated antibody compared to unsialylated control (Figure 2C). This was confirmed by LC-MS analysis of partially reduced antibody, which was consistent with the presence of A1F glycan on each of the two heavy chains (data not shown). The sialic acid content in these antibodies was also determined by HPAEC-PAD (High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection), which showed the presence of 2.2 sialic acids per antibody in two preparations of sialylated trastuzumab (Table S1).

After establishing incorporation of sialic acid onto the antibodies, periodate mediated oxidation of these sialylated antibodies was explored. Oxidation of sialylated trastuzumab was carried out at low temperature using varying concentrations of periodate (0.25 to 2 mM) and the extent of oxidation was determined by measuring residual sialic acid contents. As shown in Figure S1A, as little as 0.5 mM of periodate was sufficient to quantitatively oxidize the sialic acids introduced by enzymatic remodeling. Since periodate treatment of antibodies also has the propensity to oxidize methionine residues in addition to sialic acid groups, it could potentially affect neonatal Fc receptor (FcRn) binding since a number of methionines are involved in the interaction with FcRn.[16,27,28] Therefore, we examined the impact of periodate oxidation on FcRn binding of trastuzumab. Following treatment with varying amounts of periodate, binding to mouse FcRn was determined by using surface plasmon resonance (SPR) (Figure S1B). At 1 mM periodate, 15% reduction in FcRn binding was observed. When the periodate concentration was increased to above 4 mM, there was a ~40% loss in FcRn binding compared to the trastuzumab control (Figure S1B). Interestingly, periodate treatment had a somewhat lower impact with sialylated trastuzumab compared to the unsialylated form (10% vs. 15%, Figure S1C). The extent of methionine oxidation was also determined by analyzing tryptic peptides derived from periodate oxidized antibody using LC-MS analysis. Treatment of the sialylated antibody with 1 mM periodate was found to result in oxidation of ~30% of Met-252 and <10% of Met-428 (Table S2). Thus, 1 mM periodate was used for preparation of Glyco ADCs for subsequent studies.

Sialylated trastuzumab as well as two different sialylated monoclonal IgG1 antibodies directed against fibroblast activation protein (FAP) were oxidized with sodium periodate. The resulting aldehyde functionalized antibodies were conjugated with the MMAE based aminooxy drug-linker (**1**). Hydrophobic interaction chromatography (HIC), which separates conjugates based on the number of attached hydrophobic drugs, indicated the presence of mostly one or two drugs per Glyco ADC (Figure 3A, C, and D) with an overall drug to antibody ratio (DAR) ranging from 1.3 to 1.9 (Table 1). Sialylated and oxidized trastuzumab was also conjugated with the corresponding aminooxy functionalized Dol10 drug-linker (**2**), yielding a Dol 10 containing Glyco ADC with a DAR of 1.6 (Figure 3B) (Table 1). The increased retention time of Dol10 conjugated sialylated trastuzumab (trastuzumab-AO-MC-PEG8-Dol10, Glyco Dol10) (Figure 3B) compared to its MMAE counterpart (trastuzumab-AO-MC-MMAE, Glyco MMAE) (Figure 3A) can be attributed to the greater hydrophobicity of Dol10. LC-MS analysis of the MMAE and Dol10 based trastuzumab Glyco ADCs showed DAR values of 1.7 and 1.5, respectively, which are consistent with HIC results (Table 1). In contrast, analyses of the thiol-maleimide based conjugates revealed the presence of a significantly more heterogeneous population of species carrying drug on either heavy or light chains with average DAR values ranging from 3.3 to 3.9 (Figure 3E and Table 1). Size-exclusion chromatography

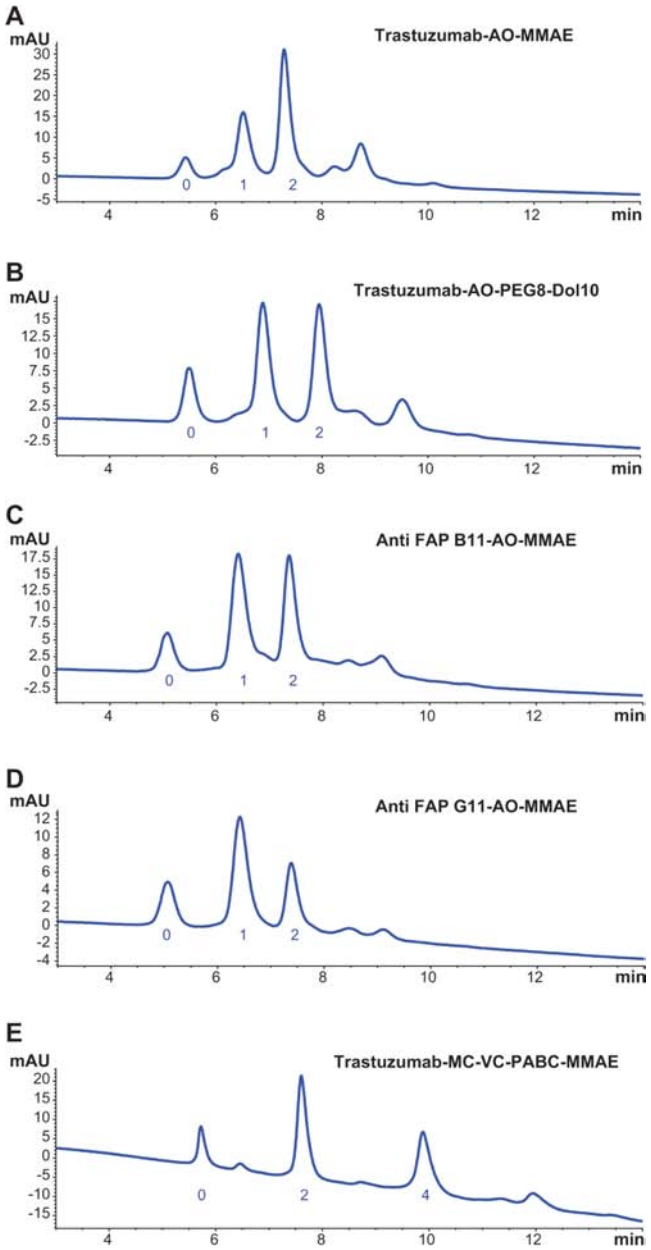


Figure 3. Hydrophobic interaction chromatography (HIC) of Glyco ADCs prepared with 3 different sialylated antibodies and 2 different aminooxy drug-linkers (AO-MMAE and AO-PEG8 Dol10). (A) Trastuzumab conjugated with AO-MMAE. (B) Trastuzumab conjugated with AO-PEG8-Dol10. (C) Anti-fibroblast activation protein (FAP) antibody B11 conjugated with AO-MMAE. (D) Anti-FAP antibody G11 conjugated with AO-MMAE. (E) Trastuzumab conjugated with MC-VC-PABC-MMAE (thiol conjugate). Numbers below each peak indicate the number of drugs conjugated. AO-MMAE: Aminooxy-Cys-MC-VC-PABC-MMAE; AO-PEG8-Dol10: Aminooxy-Cys-MC-VCPABC-PEG8-Dol10.

dx.doi.org/10.1021/bc400505q | *Bioconjugate Chem.* 2014, 25, 510−520

Table 1. Characterization of Trastuzumab Glyco and Thiol ADCs[a]

	DAR (HIC)	DAR (LC-MS/MS)	EC_{50} (ng/mL)	% monomer (SEC)	median survival (Day)	PR-CR/10
Vehicle	-	-	-	-	58	0–0
Trastuzumab	0	0	nd	99	73	0–0
Trastuzumab-AO-MC-MMAE (Glyco MMAE)	1.9	1.7	4.7	99	96	9–0
Trastuzumab-MC-MMAE (Thiol MMAE)	3.5	3.8	2.3	ND	>100	4–6
Trastuzumab-AO-MC-PEG8-Dol10 (Glyco Dol10)	1.6	1.5	0.97	99	79	1–0
Trastuzumab-MC-PEG8-Dol10 (Thiol Dol10)	3.9	3.9	0.45	98	96 (70)	8–0 (0–0)
Anti FAP B11-AO-MC-MMAE (B11 Glyco MMAE)	1.5	ND	682	93	ND	ND
Anti FAP B11-MC-PABC-MMAE (B11 Thiol MMAE)	3.3	ND	382	ND	ND	ND
Anti FAP G11-AO-MC-MMAE (G11 Glyco MMAE)	1.3	ND	ND	98	ND	ND

[a]ND is referred as "not done"; nd is referred as "value cannot be determined"; PR and CR represent the number of animal showing partial response and complete response, respectively, from 10 mice per the group; The last two columns showed the data from mice treated with trastuzumab or ADCs at 10 mg/kg dose except for vehicle group and the numbers inside parentheses represent the data from animals treated with Thiol Dol10 at 3 mg/kg dose.

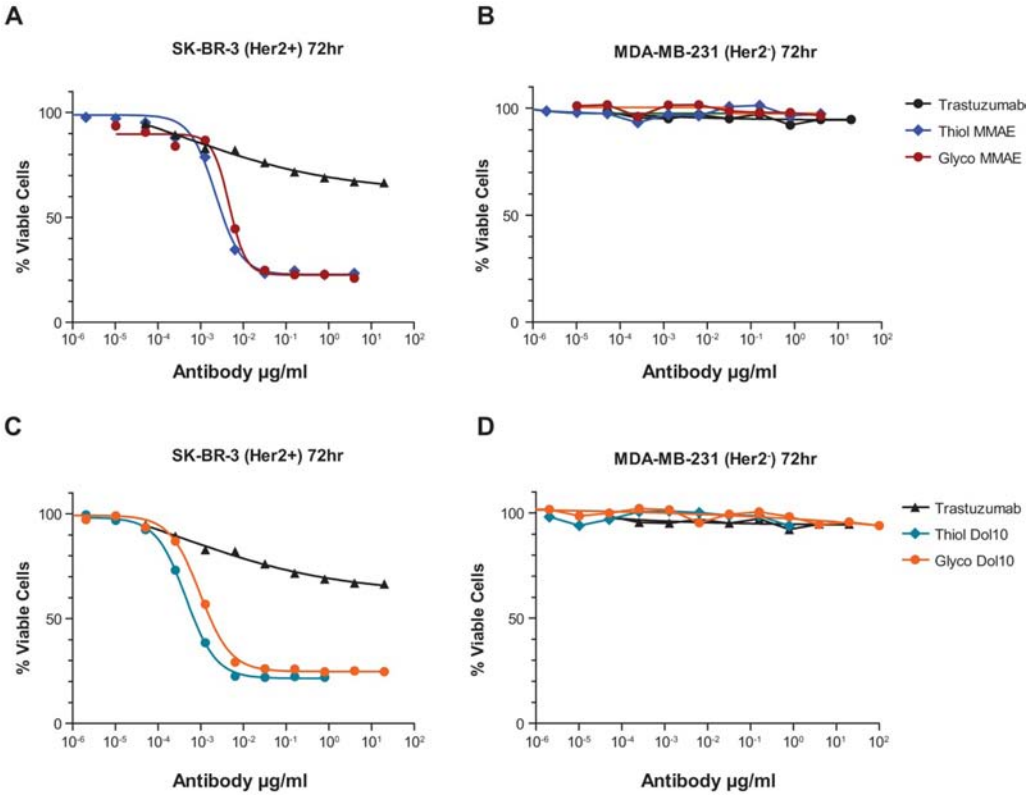


Figure 4. Relative potency of trastuzumab glyco- and thiol conjugates *in vitro*. Cell viability was determined following 72 h exposure to ADCs containing either MMAE (A and B) or PEG8-Dol10 (C and D) linked to the glycans ("Glyco") or by conventional chemistry to hinge region cysteines ("Thiol"). Trastuzumab was used as control. The treated cells include the Her2 antigen expressing cells, SK-BR-3 (A and C) or nonexpressing cells, MDA-MB-231 (B and D).

(SEC) analysis of all ADCs showed the presence of very low levels of aggregation (1–7%).

***In Vitro* Cytotoxicity of Glyco ADCs.** Both trastuzumab derived Glyco ADCs (Glyco MMAE and Glyco Dol10) were tested for their cytotoxicities in a cell proliferation assay using Her2-positive SK-BR-3 and Her2-negative MDA-MB-231 cell lines. Corresponding ADCs prepared using thiol-maleimide chemistry (Thiol MMAE or Thiol Dol10) by conjugating the drugs to the antibody through hinge region disulfides were used as positive controls. Cells were treated with the ADCs for 72 h prior to viability assay. As shown in Table 1 and Figure 4A and C, conjugates prepared by either method were very potent. As might be expected from the DAR values, approximately 2-fold lower EC_{50} values were observed for the thiol conjugates compared to their corresponding Glyco ADCs versions. This suggests a similar efficiency for intracellular delivery of cytotoxin. The Glyco ADCs, similar to Thiol ADCs, also showed undetectable antiproliferative effect on the Her2-negative cell line (Glyco MMAE, Thiol MMAE, and Thiol Dol10 at up to 4 μg/mL; Glyco Dol10 at up to 100 μg/mL), indicating the preservation of target specificity of the antibody after modification (Figure 4B and D). Unmodified trastuzumab

dx.doi.org/10.1021/bc400505q | *Bioconjugate Chem.* 2014, 25, 510–520

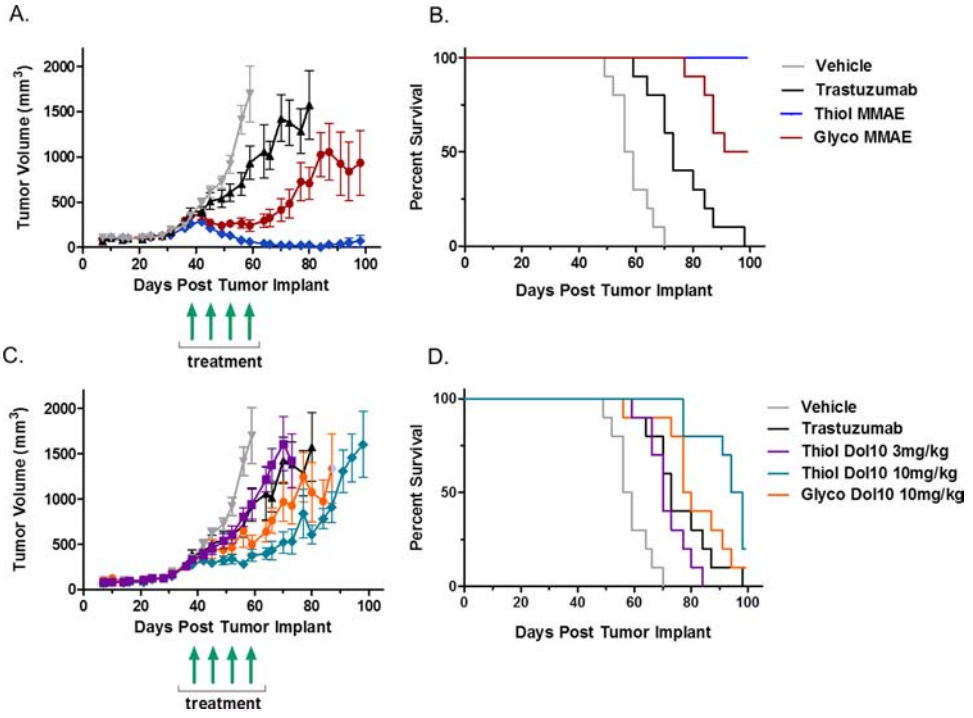


Figure 5. Comparison of *in vivo* efficacy of trastuzumab glyco- and thiol conjugates in a Her2- positive tumor cell xenograft model. SCID mice implanted with Her2-positive SK-OV-3 tumor cells were dosed with MMAE (A and B) and PEG8-Dol10 (C and D) containing Glyco ADCs or thiol conjugate comparators with about 2-fold higher DAR. Trastuzumab was also included as a control. The effect of MMAE Glyco ADC ("Glyco MMAE") and thiol conjugate ("Thiol MMAE") on tumor regression (A) and animal survival (B) was illustrated. The effect of aminooxy- PEG8-Dol10 Glyco ADC ("Glyco Dol10") and thiol conjugate ("Thiol Dol10") on tumor growth (C) and animal survival (D) was also shown. The tumor volumes were not shown once >60% of each cohort was removed from the study.

demonstrated a slight inhibition of proliferation of SK-BR-3 cells broadly over several orders of magnitude higher concentrations compared to the ADCs. Similarly, it did not show any inhibition of the Her2-negative MDA-MB-231 cells at up to 20 μg/mL.

Similar trends in cytotoxicity were also observed in the cell proliferation assay with ADCs prepared using antibodies against the tumor antigen FAP, which is highly expressed by stromal fibroblasts in epithelial cancers including colon, pancreatic, and breast cancers.[29] As with the trastuzumab conjugates, these ADCs were prepared by conjugating either aminooxy MMAE or maleimide MMAE drug-linkers to glycans or thiol groups, respectively. By cell proliferation assay, the thiol-maleimide based ADC was highly target-selective, showing ~100-fold greater potency on CHO cells expressing human FAP than the same cells lacking FAP (Figure S2). Similar to the results with trastuzumab, the Glyco ADC of anti-FAP (B11) with a DAR of 1.5 showed about 2-fold higher EC_{50} than the corresponding Thiol ADC (DAR 3.3) (Table 1).

***In Vivo* Efficacy of Glyco ADCs in a Her2-Positive Tumor Cell Xenograft Model.** Beige/SCID mice were implanted with Her2-positive SK-OV-3 tumor cells and tumors were allowed to grow until they reached an average size of >200 mm^3. At this stage, the animals were intravenously administered with trastuzumab derived MMAE Glyco ADC or the corresponding Thiol ADC comparator (about 2-fold higher DAR). The effect of the MMAE ADC treatment on tumor growth is shown in Figure 5A. The Glyco ADC showed significantly greater tumor growth repression than the trastuzumab ($p < 0.0001$) and vehicle groups but less than the Thiol ADC comparator ($p < 0.0001$). For the MMAE derived Glyco ADC treated animals, there was significant increase in animal survival ($p < 0.01$ as compared to trastuzumab) with a ~20 day delay in tumor growth and ~20 day (1.3-fold) increase in median survival time compared to those treated with the naked trastuzumab (Figure 5B and Table 1). While no response was observed in any animal in the trastuzumab group, nine out of ten animals in the Glyco ADC treated group showed at least a partial response with their tumors decreased in size for three consecutive measurements following the initiation of treatment (Table 1). The Thiol ADC treatment led to near-complete tumor repression at the same dose (10 mg/kg) with four and six out of ten animals showing partial and complete response, respectively ($p < 0.0001$ as compared to trastuzumab). Neither of these ADCs showed any effect on body weight (Figure S3A).

The *in vivo* efficacy of the PEG8-Dol10 containing Glyco trastuzumab ADC (Glyco Dol10) and its corresponding Thiol ADC comparator (Thiol Dol10) was also investigated in the same experiment. The PEG8-Dol10 drug-linker conjugated either by the thiol or by the Glyco showed similar tumor growth repression although with a somewhat lower efficacy than the MMAE-derived ADCs (Figure 5C and D). At 10 mg/kg dose, the efficacy of the glycoconjugate in suppressing tumor growth was intermediate between that of 3 and 10 mg/kg of the thiol conjugate having at least a 2-fold higher DAR ($p < 0.05$, < 0.0001, and >0.05 for Glyc Dol10 at 10 mg/kg, Thiol Dol10 at 10 mg/kg, and Thiol Dol10 at 3 mg/kg, respectively, compared to trastuzumab). There was no significant effect on animal survival for both Glyco Dol10 at 10 mg/kg and Thiol Dol10 at 3 mg/kg ($p > 0.05$), although significant increase in survival was observed with Thiol Dol10 at 10 mg/kg ($p < 0.01$). Like

dx.doi.org/10.1021/bc400505q | *Bioconjugate Chem.* 2014, 25, 510−520

MMAE conjugates, there was no effect of either of the Dol10 conjugates on animal body weight (Figure S3B).

DISCUSSION

Site-specific conjugation of recombinant proteins and antibodies offers a significant advantage over nonspecific conjugation targeting multiple lysine or cysteine residues in IgGs. These include more homogeneous product profile and well-defined conjugate structures. Other approaches that have been explored for site-specific conjugation of drugs and other agents to proteins and antibodies include the introduction of free cysteine, unnatural amino acids, thiolfucose or specific amino acid tags through site-directed mutagenesis or engineering a new biosynthetic pathway inside expressing cells.[9−13,30−32] Junutula et al. showed that unique unpaired cysteine can be introduced into antibody heavy chain and used as a site for maleimide drug−linker conjugation, leading to conjugates with an improved preclinical safety over conventional ADCs.[9,10] The *in vivo* instability of the thioether linkage connecting the drug to the antibody has been found to be highly dependent on the site of conjugation, leading to substantial variations in efficacy.[33] Site-specific drug conjugation has also been achieved for antibodies with a tag sequence containing a glutamine residue that can be coupled to an amine group of a drug-linker by the use of a microbial transglutaminase. However, even in this case, the site of conjugation can also influence the *in vivo* stability and efficacy.[11] Recently, it was reported that thiolfucose can be incorporated into the antibody *N*-glycans for conjugation using maleimide chemistry to produce ADC with high cytotoxicity *in vitro*.[13] The introduction of unnatural amino acids as sites for conjugation has also been described.[12] All these approaches resulted in potent *in vitro* cytotoxicity and in some cases *in vivo* efficacy. However, they potentially lengthen development time due to the requirement to either re-engineer the protein sequence, which can affect protein expression and stability, or change cell culture conditions in the bioreactor. Furthermore, the use of unnatural amino acids introduces additional safety considerations and the potential for immune recognition of the ADCs. Use of the present approach would potentially shorten the development time and can be applied to existing therapeutic antibodies.

Conjugation of drug-linker onto unmodified native antibody glycans has previously been employed using hydrazone chemistry.[34,35] An ADC containing calicheamicin attached to an anti-CD33 antibody through a hydrazone linkage (gemtuzumab ozogamicin) has been used in the clinic, but was withdrawn due to concerns over linkage stability and associated off-target toxicity.[36] The arguably aggressive condition with greater than a 10-fold higher concentration (12.5 mM) of periodate than current approach was required to oxidize fucose, galactose, mannose, or GlcNAc in the native glycans to provide aldehydes necessary for ligation, then likely leading to linkages of undefined structure with increased ADC heterogeneity. This exposure of the antibody to such high concentrations of periodate will also result in undesired oxidation of amino acid residues, potentially affecting antibody integrity, functionality, and efficacy. We have observed that higher concentration of periodate (7.5 mM) produces modified antibodies that are highly prone to aggregation (data not shown). By incorporating periodate-sensitive sialic acid residues using sialyltransferase, the present approach enables us to introduce aldehyde groups by using very low concentrations of periodate, which has minimal effect on antibody integrity, functionality, and aggregation. Oxidation of methionine residues, including Met-252 and Met-428 (located in Fc CH3 region close to FcRn binding site), is known to affect FcRn binding.[27,28] In particular, oxidation of 80% of Met-252 in trastuzumab by hydrogen peroxide has been shown to result in a ∼60% decrease in FcRn binding and 80% reduction in serum half-life.[28] However, partial oxidation (∼40%) of Met-252, while showing a somewhat lower effect (∼25%) on FcRn binding, was found to have a minimal effect on the serum half-life in human FcRn transgenic mouse. This diminishing effect at lower extents of oxidation suggests a single intact FcRn site on each antibody may be sufficient to preserve the long circulating half-life attributed to FcRn binding.[28] The present approach using sialic acids as the linkage site for oxime ligation provides the ability to employ milder conditions allowing flexibility in ADC synthesis and leading to improved safety profile without affecting serum half-life. Interestingly, we have also observed that low concentrations of periodate have a lower effect on the FcRn binding of the sialylated antibody than unsialylated antibody. This might be due to fast depletion of periodate by reaction with sialic acid, thus limiting its ability to react with amino acid residues and providing a basis for at least partial selectivity.

Another approach to introduce sugar residues to antibodies utilizes mutant glycosyltransferases to transfer 2-acetonyl-2-deoxy-galactose or 2-*N*-acetyl azide GalNAc at nonreducing termini of the native glycans. These galactose or GalNAc derivatives can subsequently be conjugated to bioactive agents carrying orthogonally reactive functional groups, such as aminooxy or alkyne groups.[37,38] However, the efficiency of the process is unclear since the reactivity of the introduced sugars has been demonstrated only with aminooxy biotin and fluorescent probes.

Although the native glycans are somewhat heterogeneous, remodeling through galactosyl and sialyltransferases leads to near homogeneous glycans, in the form of monosialylated biantennary structures (A1F). The introduction of only a single sialic acid on the two galactose acceptors on each biantennary glycan of the antibody may be due to a limited accessibility of one of the terminal galactoses, which might be partially buried in the antibody structure or in noncovalent interaction with the protein surface.[39] The observed number of drugs per antibody was also slightly lower than the sialic acid content. This might be due to either incomplete accessibility of the sialic acids for coupling or steric hindrance for drug conjugation by presence of the first drug-linker on a biantennary structure. This issue could be addressed through changes in drug-linker design, which merits further investigation.

Our studies demonstrate that site-specific conjugation of cytotoxic agents using sialic acids as the linkage sites yields ADCs with significant anti-tumor activity both *in vitro* and *in vivo*. The relatively lower *in vitro* potency as compared to thiol ADC controls is consistent with lower DAR, which results in fewer cytotoxic payloads carried into the tumor cells, thus explaining the somewhat lower efficacy *in vivo*. However, unlike hydrazone linkages, oximes are significantly more stable at physiological pH,[40,41] and resistant to exchange even at high concentrations of hydroxylamine, the lowest molecular weight aminooxy analog.[42] Moreover, glycoconjugation may offer additional advantages as a consequence of its carbohydrate attachment site. Many highly potent cytotoxic agents are very hydrophobic, which facilitates their translocation into the cytosol after lysosomal release from the ADC, as well as

bystander effects, due to killing of host-derived antigen-negative tumor supporting stromal cells. However, the hydrophobicity of those drugs also contributes to undesirable properties such as aggregation of the ADCs. Introduction of a hydrophilic linker element such as a short PEG chain has improved solubility of ADCs.[43] The hydrophilic nature of oligosaccharides and the sialic acid conjugation site may itself afford a similar improvement in solubility as we demonstrated previously in a conjugation of glycan through sialic acid, which showed much lower protein aggregation than that through lysine residues.[16] The glycoengineering may allow the use of more hydrophobic and potent drugs. Taken together, the present strategy represents a promising approach for the generation of site-specific antibody−drug conjugates with potentially superior pharmacological and pharmaceutical properties that may further expand the scope of antibody−drug conjugates as an important class of therapies for the treatment of cancer.

■ CONCLUSIONS

By using an enzymatic remodeling process, we have successfully incorporated α2,6-linked sialic acids, which are present in nature as minor species in polyclonal antibodies, to the nonreducing terminus of *N*-glycans at Asn-297 of IgG. These sialic acid functionalized antibodies have been demonstrated to be used for the preparation of ADCs by site-specific conjugation of anti-cancer drugs via oxime ligation. The generality of this method was demonstrated by comparable results obtained with three different antibodies and two different cytotoxic agents. The glycoconjugates tested showed high target selectivity in *in vitro* antiproliferation and effective *in vivo* anti-tumor activities. Thus, a novel approach of site-specific antibody−drug conjugation is presented for existing antibody without any change in its amino acid sequence or cell culture condition.

■ EXPERIMENTAL PROCEDURES

Materials. Trastuzumab was obtained from Genentech (South San Francisco, CA) and monoclonal IgG1 against fibroblast activation protein (FAP) was produced in our laboratories. Unless stated otherwise, all chemicals were obtained from Sigma (St. Louis, MO) and were used as received. The drug linkers, Maleimidocaproyl-valine-citrulline-((*p*-aminobenzyl)oxy)carbonyl-monomethylauristatine (MC-VC-PABC-MMAE) and Maleimidocaproyl-valine-citrulline-((*p*-aminobenzyl)oxy)carbonyl octaethyleneglycol-dolostatin-10 (MC-VC-PABC-PEG8-Dol 10) were synthesized according to published procedures with >95% purity.[14,15]

Synthesis of 2-(2-(Aminooxy) acetamido)-3-((succinamido)thio)propanamidocaproyl-valine-citrulline-((*p*-aminobenzyl)oxy)carbonyl-MMAE (AO-Cys-MC-VC-PABC-MMAE) (1). *(a). Synthesis of 2-(2-(Aminooxy)-acetamido)-3-mercaptopropanamide.* *S*-Trityl-L-cysteinamide (362 mg, 1 mmol) was added to 3 mL of dimethylformamide (DMF) solution of *t*-BOC-aminooxyacetic acid *N*-hydroxysuccinimide ester (289 mg, 1 mmol). The reaction was completed after 3 h as evident from HPLC analysis. The reaction mixture was diluted with 30 mL of dichloromethane and was washed with 0.1 M sodium bicarbonate solution (2 × 20 mL), deionized water (2 × 20 mL), and brine (2 × 20 mL). The organic phase was separated and was dried over anhydrous sodium sulfate, filtered, and concentrated to dryness. The residue was treated with 3 mL of trifluoroacetic acid (TFA) followed by 150 μL of triethylsilane. The resulting solution was precipitated from *t*-butyl methyl ether. The process of dissolution in TFA and precipitation from *t*-butyl methyl ether was repeated three times. After filtration, the residue was dried under reduced pressure yielding an off-white solid (205 mg, 67% yield). The compound was used in the next step without further purification.

(b). Synthesis of AO-Cys-MC-VC-PABC-MMAE. 2-(2-(Aminooxy)acetamido)-3-mercaptopropanamide (30.1 mg, 0.098 mmol) and MC-VC-PABC-MMAE (64.9 mg. 0.049 mmol), were dissolved in 3 mL of DMF. To this solution was added 100 μL of triethylamine. The resulting reaction mixture was stirred at room temperature for 15 min, by which time reaction was deemed complete by HPLC analysis. The compound was purified by preparative HPLC yielding the desired product as an off-white solid (45 mg, 62%). Reversed-phase HPLC analysis suggested the purity of the compound to be >96%. Mass spectrum (ESI) calcd. for $C_{73}H_{116}N_{14}O_{18}S$ $(MH)^+$ 1509.8501; found, *m*/*z* 1509.8469.

Synthesis of 2-(2-(Aminooxy)acetamido)-3-((succinamido)thio)propanamidocaproyl-valine-citrulline-((*p*-aminobenzyl)oxy)carbonyloctaethyleneglycol-Dolostatin10 (AO-Cys-MC-VC-PABC-PEG8-Dol10) (2). In 3 mL of DMF were taken 2-(2-(aminooxy)acetamido)-3-mercaptopropanamide (7.4 mg, 0.024 mmol,), MC-VC-PABC-PEG8-Dol10 (12 mg, 0.008 mmol), and triethylamine (30 μL). The reaction mixture was stirred at room temperature and was found to be completed within 15 min according to HPLC analysis. Preparative HPLC purification offered the desired product as an off-white solid (6.2 mg, 46%). Reversed-phase HPLC analysis indicated the purity of the compound to be >96%. Mass spectrum (ESI) calcd. for $C_{80}H_{124}N_{16}O_{19}S_2$ $(MH)^+$ 1678.0664; found, *m*/*z* 1678.0613.

***In Vitro* Sialylation of Antibodies.** Galactosylation and sialylation of the antibodies were carried out in one-pot by using β1,4-galactosyltransferase (50 mU/mg, Sigma) and α2,6-sialyltranaferase (5 μg/mg, R&D system) with donor sugar nucleotide substrates, UDP-galactose (10 mM) and CMP-sialic acid (10 mM) in 50 mM MES buffer (pH 6.5) containing 5 mM $MnCl_2$. The reaction mixture containing 5 mg/mL antibody was incubated at 37 °C for 48 h. Incorporation of sialic acids to the antibodies was verified by glycan analysis. The sialylated antibody was further purified using protein A sepharose column. The antibody was eluted with 25 mM citrate phosphate buffer (pH 2.9) and was immediately neutralized with dibasic phosphate buffer before buffer-exchanged using Amicon ultra 50 kDa centrifugal filters (Millipore, Billerica, MA).

Sialic Acid-Mediated (SAM) Conjugation. Drug conjugation to sialic acids on the antibody was performed according to a method previously described for sialic acid-mediated glycan conjugation of enzymes with some modification necessary for ADC.[16,17] Briefly, the sialylated antibody (1 to 30 mg) was oxidized with 1 mM sodium periodate (Sigma), in 100 mM sodium acetate buffer (pH 5.6) on an ice bath for 30 min. The reaction mixture was protected from light during the course of the reaction and the reaction was quenched by adding cold aqueous glycerol (3% v/v) for 15 min. The product was desalted and buffer-exchanged into 100 mM sodium acetate (pH 5.6) by five rounds of ultrafiltration over Amicon ultra 50 kDa centrifugal filters. Subsequently, 25 mM of drug-linkers (0.167 v/v) dissolved in 75% aqueous DMSO was added to the antibody solution at 0 °C. The

dx.doi.org/10.1021/bc400505q | *Bioconjugate Chem.* 2014, 25, 510−520

reaction mixture has drug-linker to antibody ratio at 24:1 (mol/mol) and a final antibody concentration at 5 mg/mL. The reaction mixture was incubated 16 h at room temperature. The unincorporated drug-linkers and any free drugs were scavenged using BioBead SM2 resin. After filtering off the Biobead resins, the solution was buffer-exchanged into 20 mM histidine and 0.005% Polysorbate 80 using PD-10 columns and sterile filtered. The endotoxin levels were determined and were found to be less than 0.1 EU/mg ADC.

Synthesis of Hinge Disulfide Directed ADC Using Thiol-Maleimide Chemistry. Hinge disulfide targeted ADC synthesis was performed following a reported procedure.[18] Briefly, buffer-exchanged antibody in borate buffer (pH 8.0) was treated with Tris (2-carboxyethyl) phosphine at an appropriate molar ratio that results in reduction of approximately two hinge disulfides. After allowing the reaction to proceed for 2 h, at 37 °C, 10−12 molar excess of the maleimide drug-linkers were added to the reaction mixture. The resulting reaction mixture was kept at 10−15 °C for an hour. Excess drug linker was scavenged by incubating with BioBead SM2 resin for 2 h at room temperature. The conjugate was desalted and buffer-exchanged on PD-10 columns using 20 mM histidine and 0.005% Polysorbate 80. The final products contained less than 0.1 EU/mg of endotoxin.

Characterization of Oxidized Antibodies and ADCs. Analysis of permethylated glycans released from antibody with PNGase F was performed by MALDI-TOF mass spectrometry.[19,20] Extent of protein aggregation was determined by size exclusion chromatography.[21] Identification of oxidized methionine residues was performed as described previously.[22] The sialic acid content was measured using HPAEC-PAD after the antibody was hydrolyzed with 0.5 M formic acid.[23] The lectin blotting with SNA, a lectin specific for α2,6-linked sialic acid, was performed following a published procedure.[24] FcRn binding was carried out using surface plasmon resonance on Biacore (Biacore 3000 system). Samples were diluted to 100 nM in PBS (pH 6.0) containing 0.005% Polysorbate 20 and passed over a CM5 chip coupled with ~1000 RU mouse FcRn using amine chemistry on flowcell 2. The flowcell 1 was used as a control surface for reference subtraction. The percent binding was calculated by dividing the relative binding (RU) of oxidized antibody by that from unmodified control at the maximal binding in the sensorgram (~160 s). Hydrophobic interaction chromatography was performed following the published procedure.[18] After the proteins were injected onto a TSK gel Butyl-NPR column (2.5 μm, 4.6 mm × 3.5 cm, Tosoh Bioscience LLC, King of Prussia, PA), the gradient was held at 100% mobile phase A (1.5 M ammonium sulfate in 50 mM potassium phosphate, pH 7.0) for one minute followed by 14 min gradient of 0−100% mobile phase B (20% isopropanol and 80% 50 mM potassium phosphate, pH 7.0). The protein absorbance was measured at 248 and 280 nm. Integration was done using Chemstation software to quantify ratios of different peaks. LC-MS analysis of conjugates to determine DAR was performed as described,[18,25] except that the Glyco ADCs were partially reduced with 20 mM dithiothreitol without deglycosylation.

Cell Culture. SK-BR-3, SK-OV-3, and MDA-MB-231 cells were obtained from ATCC (Manassas, VA). SK-OV-3 and MDA-MB-231 cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). SK-BR-3 cells were cultured in McCoy's 5A media supplemented with 10% heat-inactivated FBS. All cells were cultured in a 37 °C humidified incubator. For *in vivo* studies, SK-OV-3 cells were expanded in Hyper-Flasks (Corning, Corning, NY). All cells were tested and confirmed to be free from endotoxin and virus using LAL assay (Charles River Laboratories, Wilmington, MA).

***In Vitro* Cell Viability Assay.** Exponentially growing cells were trypsinized, collected by centrifugation (1200 rpm, room temperature), and resuspended in growth media. Cell numbers and viability were assessed using a Vi-CELL Cell Viability Analyzer (Beckman Coulter, Brea, CA). Cells were adjusted to 10×10^5 cells/mL in the culture media. Cells were subsequently plated in white-sided, clear bottom 96-well plates to yield 5000 cells per well (50 μL) and brought into the incubator and incubated for 24 h. Antibodies and antibody−drug conjugates were prepared as 2X stock solutions in growth media prior to addition to the microtiter plates. A 5-fold serial dilution series was set up with naked trastuzumab and individual ADCs starting with the initial concentration of 200 μg/mL. Trastuzumab was reconstituted in sterile water according to the manufacturer's instructions. The 2X stock solutions and dilutions were added to the wells containing the cells. For each sample, the measurements were taken in triplicate. Following addition of the antibodies and ADCs, the plates were kept in the incubator for three days. Viability was assessed using the CellTiter-Glo (Promega, San Luis Obispo, CA) assay following the manufacturer's instructions. Luminescence was detected using a Tecan plate reader (1 s/well). Viability curves were generated in GraphPad Prism using nonlinear regression, and EC_{50} values were calculated from these curves using the equation: Y = Bottom + (Top−Bottom)/(1 + 10((Log(EC_{50}) − X).

Animal Studies. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, NIH Publication No. 86−23) and were approved by Genzyme's Institutional Animal Care and Use Committee. Beige/SCID mice (Charles River Laboratories, Wilmington, MA) were injected with 1×10^7 cells mixed in a 2:1 ratio with Matrigel (BD Biosciences, San Jose, CA) in a total volume of 300 μL. The cell preparations were given subcutaneously on the right flank of anesthetized animals. Once established, the tumors were allowed to grow until the average volume of tumor reached a volume of 150 mm^3 at which point the animals were randomized and regrouped (10 animals per group) prior to treatment initiation. Tumor volumes were calculated as: tumor volume (mm^3) = (tumor shorter diameter)^2 × (tumor long diameter) × 0.5. The native trastuzumab, drug conjugates of trastuzumab, or vehicle were administered via tail vein injection at either 3 or 10 mg/kg of the body weight. Tumor-bearing mice received a total of four doses at day 38, 45, 52, and 59. Tumors were measured twice weekly using calipers until they reached a volume of 2000 mm^3 at which point mice were euthanized. The partial response is defined as those tumors that decreased in size for three consecutive measurements following the initiation of treatment. For complete response, the tumors from mice were below the limit of detection for at least three consecutive measurements in a row. The Kaplan−Meier survival curve was plotted using GraphPad Prism. Two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical analysis of the tumor growth data, while Log-rank (Mantel-Cox) and/or Gehan-Breslow-Wilcoxon test was used

for comparing two survival curves. A probability value of $p < 0.05$ was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

Additional supplementary figures and tables described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 508-270-2599. Fax: 508-872-9080. E-mail: qun.zhou@genzyme.com.

Notes

The authors declare the following competing financial interest(s): All authors are employees of Genzyme Corporation, A Sanofi Company.

■ ACKNOWLEDGMENTS

The authors would like to thank John McPherson, Robert Mattaliano, Tim Edmunds, Huawei Qiu, John Harrahy, Peng Pan, Joanne Cotton, Karin Jensen, and Alison Schroeer for suggestions and help in preparing the manuscript.

■ REFERENCES

(1) Alley, S. C., Okeley, N. M., and Senter, P. D. (2010) Antibody-drug conjugates: targeted drug delivery for cancer. *Curr. Opin. Chem. Biol. 14*, 529−37.

(2) Carter, P. J., and Senter, P. D. (2008) Antibody-drug conjugates for cancer therapy. *Cancer J. 14*, 154−69.

(3) Gualberto, A. (2012) Brentuximab Vedotin (SGN-35), an antibody-drug conjugate for the treatment of CD30-positive malignancies. *Expert Opin. Invest. Drugs 21*, 205−16.

(4) Haddley, K. (2012) Brentuximab vedotin: its role in the treatment of anaplastic large cell and Hodgkin's lymphoma. *Drugs Today (Barcelona) 48*, 259−70.

(5) LoRusso, P. M., Weiss, D., Guardino, E., Girish, S., and Sliwkowski, M. X. (2011) Trastuzumab emtansine: a unique antibody-drug conjugate in development for human epidermal growth factor receptor 2-positive cancer. *Clin. Cancer Res. 17*, 6437−47.

(6) Niculescu-Duvaz, I. (2010) Trastuzumab emtansine, an antibody-drug conjugate for the treatment of HER2+ metastatic breast cancer. *Curr. Opin. Mol. Ther. 12*, 350−60.

(7) Wang, L., Amphlett, G., Blattler, W. A., Lambert, J. M., and Zhang, W. (2005) Structural characterization of the maytansinoid-monoclonal antibody immunoconjugate, huN901-DM1, by mass spectrometry. *Protein Sci. 14*, 2436−46.

(8) Hamblett, K. J., Senter, P. D., Chace, D. F., Sun, M. M., Lenox, J., Cerveny, C. G., Kissler, K. M., Bernhardt, S. X., Kopcha, A. K., Zabinski, R. F., Meyer, D. L., and Francisco, J. A. (2004) Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin. Cancer Res. 10*, 7063−70.

(9) Junutula, J. R., Raab, H., Clark, S., Bhakta, S., Leipold, D. D., Weir, S., Chen, Y., Simpson, M., Tsai, S. P., Dennis, M. S., Lu, Y., Meng, Y. G., Ng, C., Yang, J., Lee, C. C., Duenas, E., Gorrell, J., Katta, V., Kim, A., McDorman, K., Flagella, K., Venook, R., Ross, S., Spencer, S. D., Lee Wong, W., Lowman, H. B., Vandlen, R., Sliwkowski, M. X., Scheller, R. H., Polakis, P., and Mallet, W. (2008) Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat. Biotechnol. 26*, 925−32.

(10) Junutula, J. R., Flagella, K. M., Graham, R. A., Parsons, K. L., Ha, E., Raab, H., Bhakta, S., Nguyen, T., Dugger, D. L., Li, G., Mai, E., Lewis Phillips, G. D., Hiraragi, H., Fuji, R. N., Tibbitts, J., Vandlen, R., Spencer, S. D., Scheller, R. H., Polakis, P., and Sliwkowski, M. X. (2010) Engineered thio-trastuzumab-DM1 conjugate with an improved therapeutic index to target human epidermal growth factor receptor 2-positive breast cancer. *Clin. Cancer Res. 16*, 4769−78.

(11) Strop, P., Liu, S. H., Dorywalska, M., Delaria, K., Dushin, R. G., Tran, T. T., Ho, W. H., Farias, S., Casas, M. G., Abdiche, Y., Zhou, D., Chandrasekaran, R., Samain, C., Loo, C., Rossi, A., Rickert, M., Krimm, S., Wong, T., Chin, S. M., Yu, J., Dilley, J., Chaparro-Riggers, J., Filzen, G. F., O'Donnell, C. J., Wang, F., Myers, J. S., Pons, J., Shelton, D. L., and Rajpal, A. (2013) Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. *Chem. Biol. 20*, 161−7.

(12) Axup, J. Y., Bajjuri, K. M., Ritland, M., Hutchins, B. M., Kim, C. H., Kazane, S. A., Halder, R., Forsyth, J. S., Santidrian, A. F., Stafin, K., Lu, Y., Tran, H., Seller, A. J., Biroc, S. L., Szydlik, A., Pinkstaff, J. K., Tian, F., Sinha, S. C., Felding-Habermann, B., Smider, V. V., and Schultz, P. G. (2012) Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proc. Natl. Acad. Sci. U. S. A. 109*, 16101−6.

(13) Okeley, N. M., Toki, B. E., Zhang, X., Jeffrey, S. C., Burke, P. J., Alley, S. C., and Senter, P. D. (2013) Metabolic engineering of monoclonal antibody carbohydrates for antibody-drug conjugation. *Bioconjugate Chem. 24*, 1650−55.

(14) Doronina, S. O., Toki, B. E., Torgov, M. Y., Mendelsohn, B. A., Cerveny, C. G., Chace, D. F., DeBlanc, R. L., Gearing, R. P., Bovee, T. D., Siegall, C. B., Francisco, J. A., Wahl, A. F., Meyer, D. L., and Senter, P. D. (2003) Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol. 21*, 778−84.

(15) Pettit, G., Singh, S., Srirangam, J., Hogan-Pierson, F., and Williams, M. (1994) The dolastatins. 19. synthesis of dolaisoleuine. *J. Org. Chem. 59*, 1796−1800.

(16) Zhou, Q., Stefano, J. E., Harrahy, J., Finn, P., Avila, L., Kyazike, J., Wei, R., Van Patten, S. M., Gotschall, R., Zheng, X., Zhu, Y., Edmunds, T., and Pan, C. Q. (2011) Strategies for Neoglycan conjugation to human acid alpha-glucosidase. *Bioconjugate Chem. 22*, 741−51.

(17) Zhu, Y., Jiang, J. L., Gumlaw, N. K., Zhang, J., Bercury, S. D., Ziegler, R. J., Lee, K., Kudo, M., Canfield, W. M., Edmunds, T., Jiang, C., Mattaliano, R. J., and Cheng, S. H. (2009) Glycoengineered acid alpha-glucosidase with improved efficacy at correcting the metabolic aberrations and motor function deficits in a mouse model of Pompe disease. *Mol. Ther. 17*, 954−63.

(18) Stefano, J. E., Busch, M., Hou, L., Park, A., and Gianolio, D. A. (2013) Micro- and mid-scale maleimide-based conjugation of cytotoxic drugs to antibody hinge region thiols for tumor targeting. *Methods Mol. Biol. 1045*, 145−71.

(19) Wada, Y., Azadi, P., Costello, C. E., Dell, A., Dwek, R. A., Geyer, H., Geyer, R., Kakehi, K., Karlsson, N. G., Kato, K., Kawasaki, N., Khoo, K. H., Kim, S., Kondo, A., Lattova, E., Mechref, Y., Miyoshi, E., Nakamura, K., Narimatsu, H., Novotny, M. V., Packer, N. H., Perreault, H., Peter-Katalinic, J., Pohlentz, G., Reinhold, V. N., Rudd, P. M., Suzuki, A., and Taniguchi, N. (2007) Comparison of the methods for profiling glycoprotein glycans–HUPO Human Disease Glycomics/Proteome Initiative multi-institutional study. *Glycobiology 17*, 411−22.

(20) Zhou, Q., Park, S. H., Boucher, S., Higgins, E., Lee, K., and Edmunds, T. (2004) N-linked oligosaccharide analysis of glycoprotein bands from isoelectric focusing gels. *Anal. Biochem. 335*, 10−6.

(21) Van Patten, S. M., Hughes, H., Huff, M. R., Piepenhagen, P. A., Waire, J., Qiu, H., Ganesa, C., Reczek, D., Ward, P. V., Kutzko, J. P., and Edmunds, T. (2007) Effect of mannose chain length on targeting of glucocerebrosidase for enzyme replacement therapy of Gaucher disease. *Glycobiology 17*, 467−78.

(22) Sendak, R. A., Ganesa, C., Lee, K. L., Harrahy, J. J., Theberge, R., Morgan, C. J., Cole, E. S., Kohn, L. D., and Mattaliano, R. J. (2003) The effect of posttranslational modifications on the in vitro activity of recombinant human thyroid-stimulating hormone. *Thyroid 13*, 1091−101.

(23) Lee, K., Jin, X., Zhang, K., Copertino, L., Andrews, L., Baker-Malcolm, J., Geagan, L., Qiu, H., Seiger, K., Barngrover, D., McPherson, J. M., and Edmunds, T. (2003) A biochemical and

pharmacological comparison of enzyme replacement therapies for the glycolipid storage disorder Fabry disease. *Glycobiology 13*, 305−13.
(24) Kaneko, Y., Nimmerjahn, F., and Ravetch, J. V. (2006) Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science 313*, 670−3.
(25) Gianolio, D. A., Rouleau, C., Bauta, W. E., Lovett, D., Cantrell, W. R., Jr., Recio, A., 3rd, Wolstenholme-Hogg, P., Busch, M., Pan, P., Stefano, J. E., Kramer, H. M., Goebel, J., Krumbholz, R. D., Roth, S., Schmid, S. M., and Teicher, B. A. (2012) Targeting HER2-positive cancer with dolastatin 15 derivatives conjugated to trastuzumab, novel antibody-drug conjugates. *Cancer Chemother Pharmacol. 70*, 439−49.
(26) Anthony, R. M., Wermeling, F., Karlsson, M. C., and Ravetch, J. V. (2008) Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc. Natl. Acad. Sci. U. S. A. 105*, 19571−8.
(27) Vaccaro, C., Zhou, J., Ober, R. J., and Ward, E. S. (2005) Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels. *Nat. Biotechnol. 23*, 1283−8.
(28) Wang, W., Vlasak, J., Li, Y., Pristatsky, P., Fang, Y., Pittman, T., Roman, J., Wang, Y., Prueksaritanont, T., and Ionescu, R. (2011) Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies. *Mol. Immunol. 48*, 860−6.
(29) Teicher, B. A. (2009) Antibody-drug conjugate targets. *Curr. Cancer Drug Targets 9*, 982−1004.
(30) Wu, P., Shui, W., Carlson, B. L., Hu, N., Rabuka, D., Lee, J., and Bertozzi, C. R. (2009) Site-specific chemical modification of recombinant proteins produced in mammalian cells by using the genetically encoded aldehyde tag. *Proc. Natl. Acad. Sci. U. S. A. 106*, 3000−5.
(31) Gao, W., Liu, W., Christensen, T., Zalutsky, M. R., and Chilkoti, A. (2010) In situ growth of a PEG-like polymer from the C terminus of an intein fusion protein improves pharmacokinetics and tumor accumulation. *Proc. Natl. Acad. Sci. U. S. A. 107*, 16432−7.
(32) Qiu, H., Boudanova, E., Park, A., Bird, J. J., Honey, D. M., Zarazinski, C., Greene, B., Kingsbury, J. S., Boucher, S., Pollock, J., McPherson, J. M., and Pan, C. Q. (2013) Site-Specific PEGylation of human thyroid stimulating hormone to prolong duration of action. *Bioconjugate Chem. 24*, 408−18.
(33) Shen, B. Q., Xu, K., Liu, L., Raab, H., Bhakta, S., Kenrick, M., Parsons-Reponte, K. L., Tien, J., Yu, S. F., Mai, E., Li, D., Tibbitts, J., Baudys, J., Saad, O. M., Scales, S. J., McDonald, P. J., Hass, P. E., Eigenbrot, C., Nguyen, T., Solis, W. A., Fuji, R. N., Flagella, K. M., Patel, D., Spencer, S. D., Khawli, L. A., Ebens, A., Wong, W. L., Vandlen, R., Kaur, S., Sliwkowski, M. X., Scheller, R. H., Polakis, P., and Junutula, J. R. (2012) Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat. Biotechnol. 30*, 184−9.
(34) Hamann, P. R., Hinman, L. M., Beyer, C. F., Greenberger, L. M., Lin, C., Lindh, D., Menendez, A. T., Wallace, R., Durr, F. E., and Upeslacis, J. (2005) An anti-MUC1 antibody-calicheamicin conjugate for treatment of solid tumors. Choice of linker and overcoming drug resistance. *Bioconjugate Chem. 16*, 346−53.
(35) Hinman, L. M., Hamann, P. R., Wallace, R., Menendez, A. T., Durr, F. E., and Upeslacis, J. (1993) Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. *Cancer Res. 53*, 3336−42.
(36) Reichert, J. M. (2012) Marketed therapeutic antibodies compendium. *MAbs 4*, 413−5.
(37) Boeggeman, E., Ramakrishnan, B., Pasek, M., Manzoni, M., Puri, A., Loomis, K. H., Waybright, T. J., and Qasba, P. K. (2009) Site specific conjugation of fluoroprobes to the remodeled Fc N-glycans of monoclonal antibodies using mutant glycosyltransferases: application for cell surface antigen detection. *Bioconjugate Chem. 20*, 1228−36.
(38) Ramakrishnan, B., Boeggeman, E., Pasek, M., and Qasba, P. K. (2011) Bioconjugation using mutant glycosyltransferases for the site-specific labeling of biomolecules with sugars carrying chemical handles. *Methods Mol. Biol. 751*, 281−96.
(39) Jefferis, R. (2009) Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. *Trends Pharmacol. Sci. 30*, 356−62.
(40) Kalia, J., and Raines, R. T. (2008) Hydrolytic stability of hydrazones and oximes. *Angew. Chem., Int. Ed. Engl. 47*, 7523−6.
(41) Mueller, B. M., Wrasidlo, W. A., and Reisfeld, R. A. (1990) Antibody conjugates with morpholinodoxorubicin and acid-cleavable linkers. *Bioconjugate Chem. 1*, 325−30.
(42) Park, A., Honey, D. M., Hou, L., Bird, J. J., Zarazinski, C., Searles, M., Braithwaite, C., Kingsbury, J. S., Kyazike, J., Culm-Merdek, K., Greene, B., Stefano, J. E., Qiu, H., McPherson, J. M., and Pan, C. Q. (2013) Carbohydrate-mediated polyethylene glycol conjugation of TSH improves its pharmacological properties. *Endocrinology 154*, 1373−83.
(43) Zhao, R. Y., Wilhelm, S. D., Audette, C., Jones, G., Leece, B. A., Lazar, A. C., Goldmacher, V. S., Singh, R., Kovtun, Y., Widdison, W. C., Lambert, J. M., and Chari, R. V. (2011) Synthesis and evaluation of hydrophilic linkers for antibody-maytansinoid conjugates. *J. Med. Chem. 54*, 3606−23.

NOTE ADDED AFTER ASAP PUBLICATION

There was an error in Figure 1, the TOC graphic, and the abstract graphic in the version published on 2/28/2014. This was corrected in the version published on 3/19/2014.