Bioconjugate Chemistry

Communication

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Metabolic Engineering of Monoclonal Antibody Carbohydrates for Antibody–Drug Conjugation

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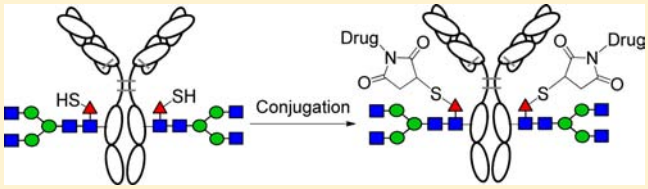
Supporting Information

ABSTRACT: The role that carbohydrates play in antibody function and pharmacokinetics has made them important targets for modification. The terminal fucose of the N-linked glycan structure, which has been shown to be involved in modulation of antibody-directed cellular cytotoxicity, is a particularly interesting location for potential modification through incorporation of alternative sugar structures. A library of fucose analogues was evaluated for their ability to incorporate into antibody carbohydrates in place of the native fucose. A number of efficiently incorporated molecules were identified, demonstrating the ability of fucosyltransferase VIII to utilize a variety of non-natural sugars as substrates. Among these structures was a thiolated analogue, 6-thiofucose, which was incorporated into the antibody carbohydrate with good efficiency. This unnatural thio-sugar could then be used for conjugation using maleimide chemistry to produce antibody–drug conjugates with pronounced cytotoxic activities and improved homogeneity compared to drug attachment through hinge disulfides.



Metabolic modification of protein glycans can be used to generate proteins possessing novel carbohydrate structures without the need to carry out post-translational modification of the proteins using enzymatic or chemical processes. Examples include the incorporation of sialic acid analogues using either direct sialic acid analogues (e.g., diazirine analogue 5-siaDAz[1] and other functionalities[2]) or precursor analogues (the thio-sugar analogue *N*-thioglycolyl-D-mannosamine pentacetate,[3] the azido analogue ManNAz, diazirine-containing sugar ManNDAz,[1] ketone functionalized ManLev,[4] or acyl modified *N*-propanoylglucosamine,[5] *N*-propanoylmannosamine,[6,7] or *N*-glycolylmannosamine pentaacetate[8]), and alkynyl-sugars or azido-sugars such as 5-alkynyl- or 5-azidofucose,[9] GalNAz, and GlcNAz.[10,11] The ability to incorporate unnatural sugars into glycoproteins enables studies to assess the importance of individual sugars on biological activity,[2,5,6,8] and in cases where the modified glycan is labeled, can allow for the visualization of glycosylated proteins in cells.[1,3,4,7,9,10]

Antibodies are important examples of glycoproteins whose functions can be affected by alteration of the carbohydrate structures, ranging from binding to Fcγ receptors that lead to effector function activities to recycling of the antibody through FcRn which impacts antibody pharmacokinetics.[12−15] Carbohydrates on antibodies have also long been used for drug and label attachment, through periodate oxidation of vicinal diols which are exclusively present within the glycan structure.[16−18] However, the reaction is inherently heterogeneous, difficult to control, and can lead to oxidation of sensitive amino acids such as methionine. These shortcomings might be overcome by metabolically incorporating unnatural sugars into the antibody carbohydrate for the purposes of introducing novel structures that modulate activity or offer the potential to introduce new sites for drug conjugation.

Previously, we have shown that the terminal fucose on the antibody heavy chain N-linked carbohydrate (Figure 1) could be almost entirely deleted when antibody producing cells were exposed to biochemical inhibitors of the fucosylation pathway.[19] In an extension of this work, we observed that selected fucose analogues could be incorporated in place of fucose, allowing for the generation of novel glycan structures. There have been literature reports that synthetic fucose analogues can be incorporated into carbohydrates by fucosyltransferases both in vitro and in cells.[9,20−22] Here we describe several such molecules that are incorporated in place of fucose with high efficiency, and show that 6-thiofucose peracetate (**3**, Figure 1) provides a convenient handle for drug conjugation. The resulting new antibody–drug conjugates (ADCs) are active, immunologically specific, and have favorable composition and stability profiles.

IDENTIFICATION OF INCORPORATORS

Approximately 200 synthetic fucose-related molecules were generated for screening. The library contained hydroxyl group modifications (esterification for cellular penetration, ketal formation, or alkylation) or heteroatom substitutions, replacements of the 5-methyl group by alkyl groups, and alterations of the stereochemical configurations about the ring system.[23]

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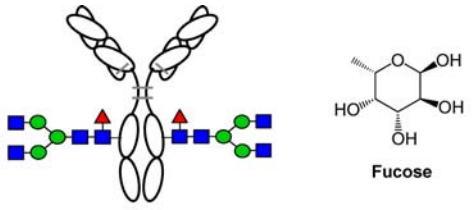
A



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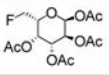
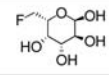
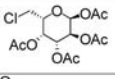
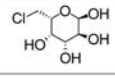
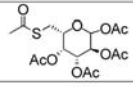
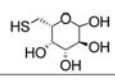
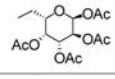
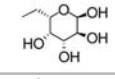
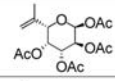
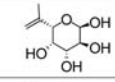
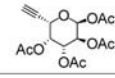
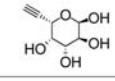
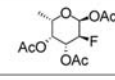
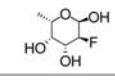
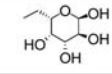
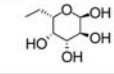
Compound		Incorporated structure		% Incorporated analogue (1 mM treatment)
	1		8	>90%
	2		9	80%
	3		10	70%
	4		11	>90%
	5		12	80%
	6*		13*	3%
	7*		14*	0.2%
	11		11	>90%

Figure 1. (A) Schematic representation of an antibody with its attached carbohydrate and the structure of fucose. Monosaccharide symbols used are as follows: triangle, Fuc; square, GlcNAc; circle, Man. (B) Structures of fucose analogues, the actual structures incorporated into antibodies place of fucose (triangle), and the incorporation efficiency as determined by LC-MS. *Ref 19.

Incorporation was assayed by evaluating the glycosylated heavy chain molecular weights using liquid chromatography–mass spectrometry (LC-MS) of antibodies produced by CHO cells cultured in the presence of each test article at 1 mM. Humanized 1F6 (h1F6, anti-CD70 IgG_1)[24] was used as the reporter antibody and was isolated from CHO cell culture supernatants. Molecules with varying ability to incorporate were identified, with the most efficiently incorporated molecules from the screen being compounds **1** through **5** (Figure 1). These molecules, according to MS (SI Figure S1), provided between 70 and >90% incorporation into the antibody glycan in place of fucose. Some compounds with similar structures failed to incorporate, such as compounds **6** and **7**, which were previously reported to be inhibitors of antibody and cellular fucosylation.[19] The hypothesized mechanism by which these molecules are incorporated begins with their deacetylation within CHO cells to generate the free alcohols, with generation of compounds **8** through **12** from **1** through **5**, respectively. Indeed, compounds **4** and **11** displayed identical properties on cells (Figure 1). In addition, we have previously reported that **13** and **14** were as active at inhibiting antibody fucosylation as their acetylated counterparts, **6** and **7**.[19] The deprotected sugars are converted to their GDP-sugar analogues through the fucose salvage pathway in CHO cells through fucokinase-catalyzed phosphorylation, followed by conversion to GDP-derivatives by GDP-fucose pyrophosphorylase.[25−27] The conversion of fucose analogues to their GDP-analogues has been previously reported[19,28] and is a common mechanism by which unnatural sugars have been incorporated into protein carbohydrates.[29] These activated sugars must then be shuttled into the Golgi apparatus by the GDP-fucose transporter[30,31] where fucosyltransferases transfer the fucose moiety of the GDP-fucose analogue to glycoprotein substrates.

DRUG CONJUGATION TO ANTIBODY CARBOHYDRATES THROUGH INCORPORATED 6-THIOFUCOSE (10)

Anti-CD70 (h1F6) and anti-CD30 (cAC10) antibodies were used to generate ADCs through incorporated 6-thiofucose. For both antibodies, treatment of CHO cells with 1 mM compound **3** resulted in incorporation of compound **10** into the antibody glycan with 60−70% efficiency, so that there were 1.2−1.4 equiv of the unnatural sugar per antibody. Antibody yield and CHO cell viability were not noticeably affected by the addition of compound **3**. Thiols are useful handles for protein conjugation, since they can be site-selectively conjugated under mild conditions.[32] LC-MS of native antibody cAC10 revealed that the thiol of incorporated compound **10** was present as a cysteine disulfide, which could be liberated upon reduction with dithiothreitol. Efficient reduction required that the antibody interchain disulfides also be reduced (Figure 2). Generation of an ADC using the drug linker vc-mc-PAB-MMAE[33] (Figure 3A) with completely reduced **10**-modified IgG (cAC10) led to the expected 9.3 drugs per antibody over the typical 8 drug loading observed with normally fucosylated antibodies (Figure 3B). This is in good agreement with the 1.4 additional thiol sites per antibody (cAC10) suggested by MS incorporation data. Reoxidation of the **10**-modified antibody prior to conjugation resulted in reformation of the interchain disulfides, leaving the glycan thiols in the reduced state (Figure 2). The incorporated sugar thiols were reacted with mc-vc-PAB-MMAE, generating an ADC with 1.3 drugs per antibody (Figure 3C). Comparison of the PLRP traces of the thio-sugar ADC with an ADC of the same drug loading generated by partial reduction of the interchain disulfides reveals that the

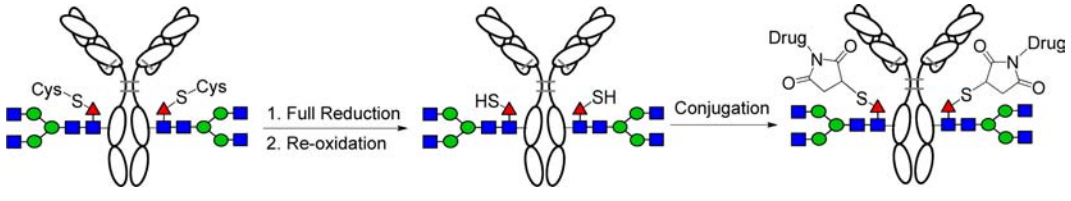


Figure 2. Procedure for conjugation of **10**-modified antibody. Monosaccharide symbols used are as follows: triangle-S, compound **10**; square, GlcNAc; circle, Man.

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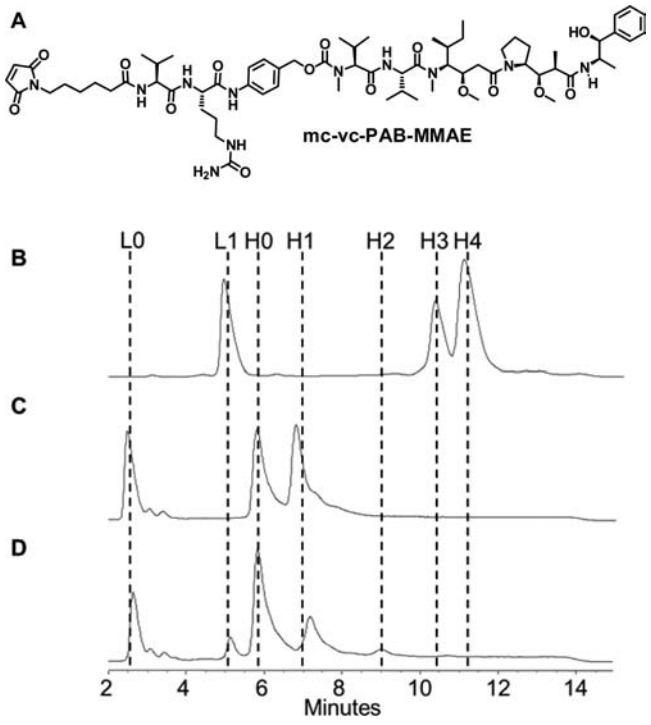

Figure 3. Structure of mc-vc-PAB-MMAE (A) and PLRP characterization of conjugates made with this drug linker (B-D). L0/1 = light chain with zero or one drug, H0/1/2/3/4 = heavy chain with zero to four drugs. (B) Fully reduced and conjugated **10**-modified antibody (cAC10, 9.3 drugs/mAb). (C) Reduced, reoxidized, and conjugated **10**-modified cAC10 (1.3 drugs/mAb), and (D) partially reduced and conjugated native cAC10 (1.3 drugs/mAb). Similar data was obtained with h1F6-**10**-modified antibody.

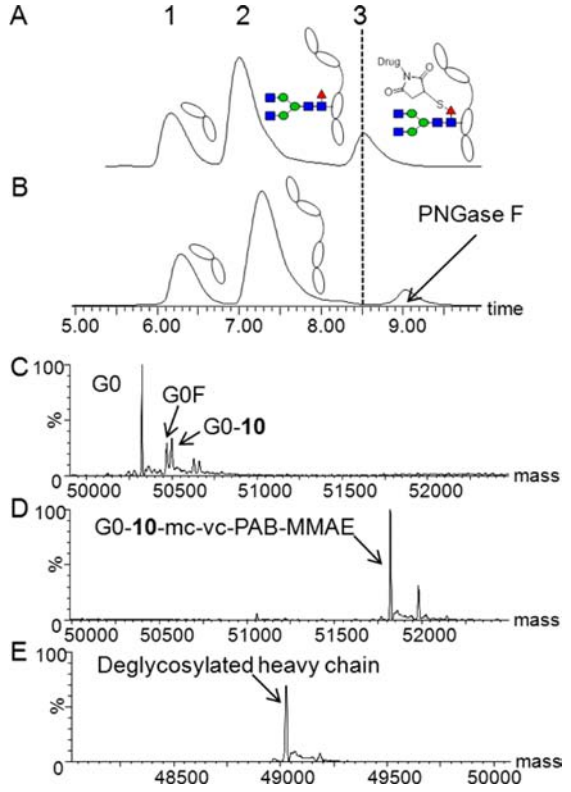

Figure 4. Determination of the drug attachment site in a thio-sugar ADC. (A) PLRP of h1F6-**10**-mc-vc-PAB-MMAE (0.4 drugs/mAb). (B) PLRP of PNGase F-treated h1F6-**10**-mc-vc-PAB-MMAE showing the loss of peak 3 after treatment. C, D, and E display the deconvoluted mass spectra of PLRP peaks 2 and 3. Mass spectrum of (C) peak 2 of h1F6-**10**-mc-vc-PAB-MMAE, (D) peak 3 of h1F6-**10**-mc-vc-PAB-MMAE, and (E) peak 2 of PNGase-F-treated h1F6-**10**-mc-vc-PAB-MMAE.

thio-sugar ADC had decreased heterogeneity with drug attached at only one site while the partial reduction resulted in drug distributed on light chain as well as on the heavy chain (Figure 3C-D). Specifically, in the thio-sugar ADC ~60% of the heavy chains had a single drug attached, and there were no light chain-associated drugs. In contrast, generation of ADCs with 1.3 drugs/mAb via partial reduction of mAb interchain disulfides resulted in considerably more heterogeneity. Such conjugates had 30% of the heavy chains and 20% of light chains with one drug attached, and 10% of the heavy chains with two drugs. Exclusive attachment of the drug to the 6-thiofucose of **10**-modified heavy chain was established by releasing carbohydrate-bound drug from a lower loaded version of the h1F6 thio-sugar ADC using the glycosidase PNGase F. This resulted in the loss of the both the drug linker and the carbohydrate from the ADC, as determined by MS analysis (Figure 4).

CYTOTOXICITY ASSAY

Anti-CD30 (cAC10) and anti-CD70 (h1F6) thio-sugar ADCs were evaluated for cytotoxicity on CD30 positive cell lines and compared to ADCs of similar drug loadings made by partial reduction (Figure 5A-B). The anti-CD30 thio-sugar ADCs were as potent as corresponding ADCs with hinge-linked drug on antigen positive cells. Nonbinding anti-CD70 ADCs showed no activity against these cell lines and the anti-CD30 thio-sugar ADC displayed no activity on an antigen negative cell line (SI Figure S2), demonstrating strong immunologic specificity. Thus, thio-sugar based ADCs can be as active as their more heterogeneous hinge-linked counterparts.

PLASMA STABILITY

We and others have demonstrated some degree of reversibility of thioether formation when maleimides are reacted with antibody cysteine residues. This is the result of a retro-Michael-type reaction.[34,35] However, there is now evidence that the level of maleimide conjugate stability can vary with respect to the position of substitution on the mAb.[35,36] The novel attachment site and identity of the thiol for the thio-sugar ADC prompted us to evaluate stability in plasma. We found that over the course of four days in rat plasma, the thio-sugar conjugate maintained at least 85% of its conjugated drug while a 4-loaded conjugate made through interchain disulfides lost 40% over the same time period (Figure 5C). This suggests that conjugation through unnatural thio-fucose analogue **10** resulted in adducts that are highly resistant to retro-Michael elimination.

CONCLUSIONS

Conjugation technology can be a key determinant in bioconjugate composition, activity, and stability. Because of this, considerable interest has surrounded technologies that provide much greater degree of homogeneity than that achieved through random modification of amino acid side chains. While this has been accomplished through chemical reactions using natural or unnatural amino acids,[36−40] both

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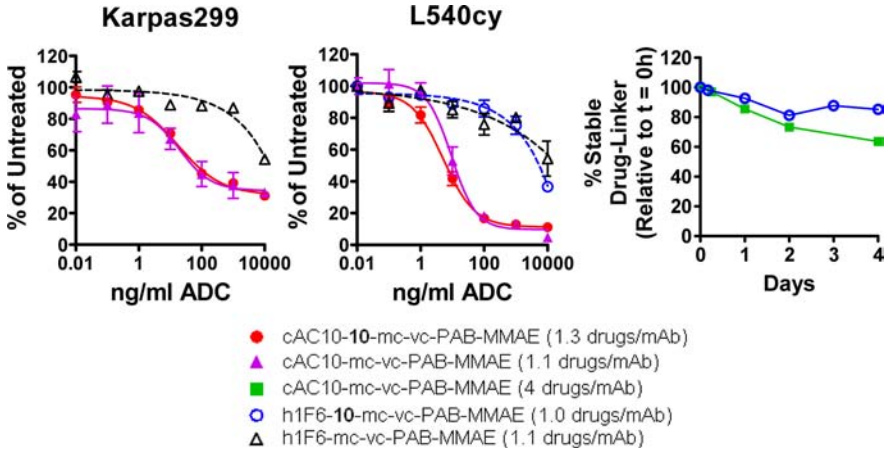

Figure 5. (A-B) Cytotoxicity comparison of anti-CD30 ADCs made with mc-vc-PAB-MMAE attached through interchain disulfides or through antibody carbohydrate-incorporated **10** (cAC10, anti-CD30 mAb) with nonbinding control ADCs (h1F6, anti-CD70 mAb). (A) Karpas299 and (B) L540cy cells express CD30. (C) Ex vivo plasma stability of the h1F6-**10**-mc-vc-PAB-MMAE conjugate compared to an ADC made by conjugation through interchain disulfides (h1F6-mc-vc-PAB-MMAE, data from a single measurement).

approaches can suffer limitations and complications. For example, modification of mutated natural amino acids is complicated by the presence of multiple copies of the same residue elsewhere in the protein structure, and the use of unnatural amino acids requires bioengineered cell lines and novel expression systems. Metabolic incorporation of unnatural carbohydrate units is an alternative to existing technologies, and has become increasingly utilized in studies for the specific labeling of proteins.[3,9,10,29] The work described here is the first example of antibody carbohydrate engineering for the purposes of making site-selectively modified ADCs.

We demonstrate that the fucose moiety of antibody carbohydrates can be substituted through metabolic incorporation of unnatural sugars such as **8**−**12**, likely by hijacking the fucose salvage pathway to generate the activated unnatural sugar for presentation to fucosyltransferase VIII, the enzyme responsible for antibody fucosylation. One of the incorporators identified, compound **3**, generated antibodies with thiolated structure **10** present in place of fucose, presenting a commonly used chemical handle for site-specific conjugation. Antibodies were generated with this thiol handle by simply culturing the CHO production cell line in the presence of the incorporator, alleviating the need to perform complex bioengineering to generate novel conjugation sites. Unfortunately, the most efficient incorporators (compounds **1** and **4**) did not provide a handle that would allow for conjugation. The only exploitable molecule from the library of fucose analogues was compound **3**, which gave 70% incorporation per heavy chain glycan, for a total of approximately 1.4 available new thiol residues/mAb. Approximately 90% of the introduced thiols could be modified with drugs, leading to ADCs with 1.3 drugs/mAb. Not only was the level of heterogeneity decreased compared to conjugates generated from hinge disulfides, but stability was measurably increased. Since the degree of stability of maleimide-based ADCs is dependent on the position of substitution, it is not surprising that glycan attached drugs differ in stability compared to other conjugation methodologies. The influence of the conjugation of incorporated **10** on effector functions of these ADCs was not evaluated in this study; however, this will be an interesting focus of future work.

The technology described here complements the increasing number of methods designed to achieve site-specific conjugation.[36−40] The advantage that such methods have over random or semirandom conjugation strategies is that the resulting product has greater uniformity and the pharmacokinetics and pharmacodynamics are more predictable. We are currently engaged in assessing the properties of site-specifically conjugated ADCs in vivo with the intent of applying the advancements toward next generation targeted therapeutics.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental methods for compound screening, thio-sugar conjugation, cytotoxicity, and drug linker stability as well as two additional figures described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare the following competing financial interest(s): All authors are employees and shareholders of Seattle Genetics, Inc.

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