Drug Design

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Preparation of Well-Defined Antibody–Drug Conjugates through Glycan Remodeling and Strain-Promoted Azide–Alkyne Cycloadditions**

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Abstract: *Antibody–drug conjugates hold considerable promise as anticancer agents, however, producing them remains a challenge and there is a need for mild, broadly applicable, site-specific conjugation methods that yield homogenous products. It was envisaged that enzymatic remodeling of the oligosaccharides of an antibody would enable the introduction of reactive groups that can be exploited for the site-specific attachment of cytotoxic drugs. This is based on the observation that glycosyltransferases often tolerate chemical modifications in their sugar nucleotide substrates, thus allowing the installation of reactive functionalities. An azide was incorporated because this functional group is virtually absent in biological systems and can be reacted by strain-promoted alkyne–azide cycloaddition. This method, which does not require genetic engineering, was used to produce an anti-CD22 antibody modified with doxorubicin to selectively target and kill lymphoma cells.*

Antibody–drug conjugates (ADCs) have considerable promise as anticancer agents owing to their ability to selectively target cytotoxic drugs to cells expressing tumor-associated cell surface proteins.[1] It has been proposed that ADCs are endocytosed after binding a cell-surface protein and degraded in the lysosome to release the cytotoxic drug. Alternatively, a drug can be attached to an antibody through a linker that is selectively cleaved after cellular uptake.[2] The promise of ADC technology has been demonstrated by the approval of CD30 (Brentuximab) and Her2 (ERBB2) specific ADCs for the treatment of Hodgkin's lymphoma and metastatic breast cancer, respectively.[3]

Typically, cytotoxic drugs are linked to antibodies through the electrophilic modification of lysine or cysteine residues by using *N*-hydroxysuccinimide ester or maleimide activated drugs, respectively.[2] These conjugation methods lack selectivity and give heterogeneous mixtures of products that differ in the sites and stoichiometry of modification. These parameters significantly impact the pharmacokinetic properties of ADCs and there is therefore an urgent need for the development of site-specific conjugation methodologies. Homogeneous ADCs have been obtained by genetically engineering antibodies for the incorporation of additional cysteine residues,[4] unnatural amino acids,[5] or tags for transamination reactions.[6] These approaches have provided ADCs that have improved therapeutic and pharmacokinetic properties in animal models.[7]

Each heavy chain of an immunoglobulin G (IgG) antibody is modified at Asn297 with a complex biantennary N-linked oligosaccharide, which does not affect antigen binding but influences effector functions.[8] We envisaged that enzymatic remodeling of the oligosaccharide of an antibody would make it possible to introduce reactive groups that can be exploited for the site-specific attachment of cytotoxic drugs. The premise of such an approach is based on the observation that glycosyltransferases often tolerate chemical modifications in their sugar nucleotide substrates, thus allowing the installation of reactive functionalities such as ketones, alkynes, or azides.[9] The incorporation of an azide is particularly attractive because this functional group is virtually absent in biological systems[10] and can be reacted by Staudinger ligation,[11] copper(I)-catalyzed cycloaddition with terminal alkynes,[12] or strain-promoted alkyne–azide cycloaddition (SPAAC).[13] These conjugation methods are more attractive than the conventionally used electrophilic conjugation methods for ADC preparation.

We set out to remodel the oligosaccharides of an anti-CD22 monoclonal antibody[14] and a control polyclonal antibody by using CMP sialic acid derivative **1**, which has an azide at C-9 of the sialic acid moiety (Scheme 1).[15] The azido moieties of the glycans of the resulting antibodies can then be reacted by SPAAC with dibenzylcyclooctynol (DIBO)[16] modified by, for example, biotin (**2**), FITC (**3**), or a cytotoxic drug such as doxorubicin (**4**).

Efficient remodeling of the glycans of antibodies with azido-containing sialic acid requires a detailed knowledge of their compositions. Therefore, the control antibody was proteolyzed with trypsin and the generated glycopeptides treated with PNG-F to release the oligosaccharide, followed by permethylation and analysis of the resulting compounds by mass spectrometry. Mainly core fucosylated G0, G1, and G2 glycoforms were present, with only a trace amount of a sialylated structure (Figure 1a). To create the maximum number of acceptor sites for a sialyltransferase, the antibody was treated with galactosyltransferase (GalT) and UPD-Gal

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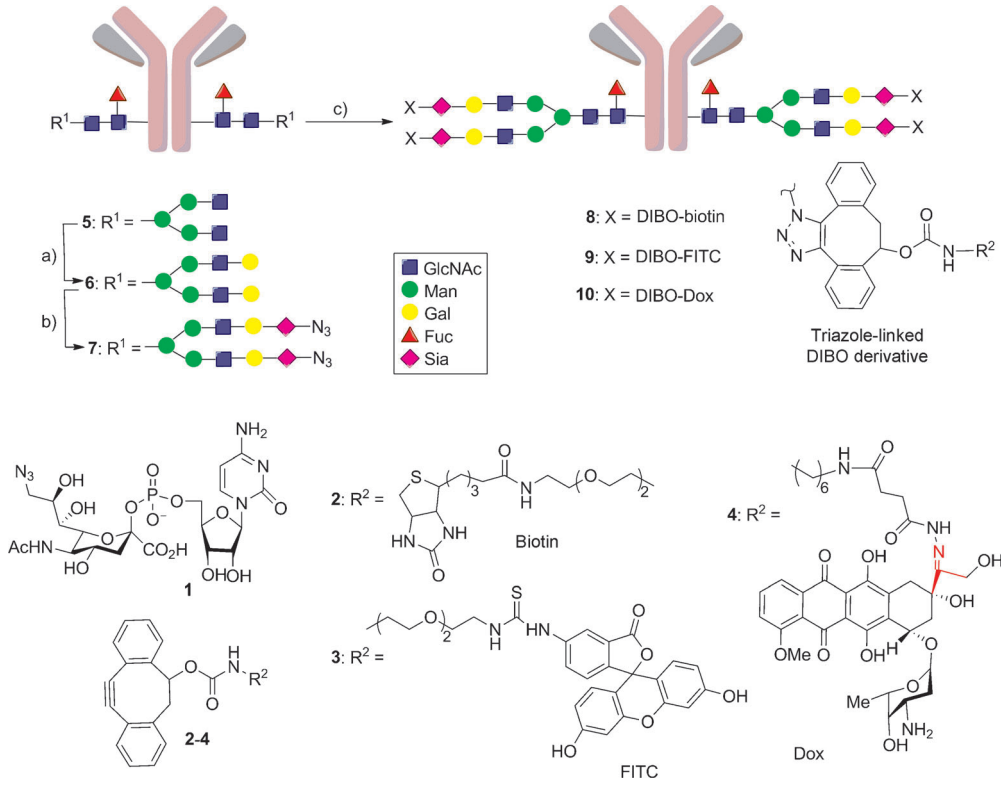

Scheme 1. Glycan remodeling of IgG antibodies to produce a homogenous glycoform with azido moieties for strain-promoted cycloadditions with compound **2**–**4**. Reagents and conditions: a) UDP-Gal, galactosyltransferase, MOPS buffer, pH 7.2; b) CMP sialic acid derivative **1**, sialyltransferase, cacodylate buffer pH 7.6; c) Compound **2**, **3**, or **4** in cacodylate buffer pH 7.6. UDP = uridine diphosphate, GlcNAc = *N*-acetylglucosamine, Man = mannose, Gal = galactose, Fuc = fucose, Sia = sialic acid, CMP = cytidine monophosphate.

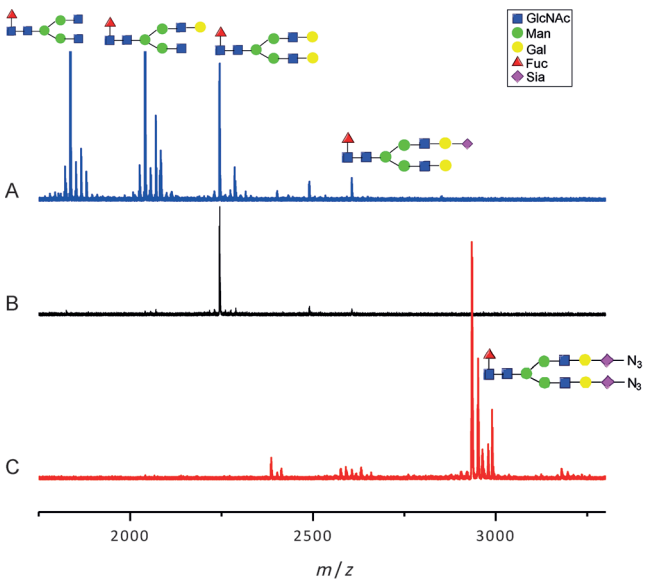

Figure 1. Mass spectrometry based determination of the glycan structures of the IgG control antibody. A) N-Glycans isolated from the IgG. B) Galactosylation of the IgG antibody resulted primarily in a digalactosylated glycan. C) Galactosylation followed by sialylation of the IgG antibody by using ST6Gal I resulted primarily in a bis-sialylated glycan.

in the presence of calf intestine alkaline phosphatase (CIAP). This process gave almost exclusively the G2 glycoform (Figure 1 b). Next, azido-modified sialic acid was incorporated by treatment with CMP sialic acid derivative **1** in the presence of recombinant sialyltransferase ST6Gal I and CIAP. After 24 h, glycan analysis showed the formation mainly of a monosialylated structure, which is in agreement with a previous study[17] that demonstrated that the α1,3-Man-β1,2-GlcNAc-β1,4-Gal arm of the glycan of IgG antibodies is more accessible for enzymatic remodeling than the other arm. Prolonged exposure of the antibody to compound **1** and ST6Gal I, however, resulted in near quantitative bis-sialylation (Figure 1 c and Figure S1 in the Supporting Information). Treatment of the remodeled antibody with aqueous acetic acid (2 M) at 80 °C followed by analysis with high-pH anionic exchange chromatography (HPAEC) showed the presence of 4.3 azido-containing sialic acid residues per antibody (Table S1 in the Supporting Information). The use of the α(2,3)-sialyltransferase of *Pasteurella multocida*[18] or ST3Gal IV resulted in partial modification even after extended incubations times, thus highlighting the favorable properties of ST6Gal I (Figure S2).

DIBO derivative **2**, which contains biotin, was reacted with antibody **7** to establish conjugation efficiencies. After a reaction time of 2 h, SDS-PAGE with an anti-biotin antibody conjugated to HRP (Figure 2) showed bands at 37 and 60 kDa, which correspond to labeling of the light and heavy chain, respectively. A similar labeling protocol with DIBO-FITC (**3**) followed by fluorescence intensity measurements demonstrated the presence of 4.5 fluorophore molecules per antibody. Native gel electrophoresis gave a major band at 150 kDa, which exhibited fluorescence only after remodeling with ST6Gal I and compound **1** and exposure to FITC-DIBO (**3**) (Figure S4). These results demonstrate that in addition to an N-glycan at Asn297, the light chain of the control antibody is partially modified by a glycan, thus explaining the fact that there are more than four glycans per antibody molecule. The various experiments also demonstrate that the labeling procedure is highly efficient and selective for azido-modified antibodies. The anti-CD22 anti-

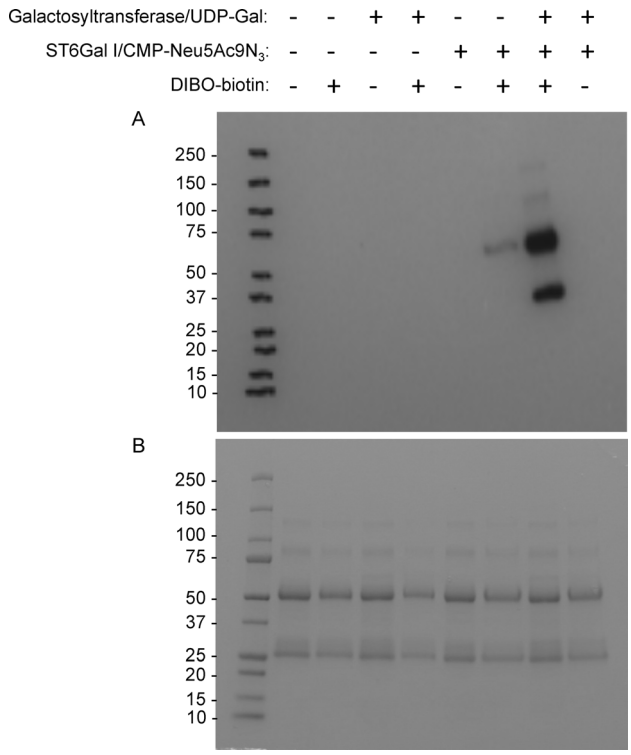

Figure 2. Confirmation of IgG labeling with CMP-Neu5Ac9N$_3$ by ST6Gal I before and after remodeling with galactosyltransferase. A) Western blot with an anti-biotin antibody conjugated to HRP. B) Total protein staining by Coomassie blue. HRP = horseradish peroxidase.

body was remodeled in a similar fashion and, in this case, no glycosylation of the light chain was observed (Figure S3).

Compound **4** (Scheme 1), which is composed of DIBO attached to Dox through an acid-sensitive hydrazine linker, was synthesized by condensation of DIBO modified by a hydrazine linker with the ketone moiety of Dox (Scheme S1). The remodeled control and anti-CD22 antibody (**7**) were exposed to **4** and subsequent fluorescent intensity measurements (Table S1 in the Supporting Information) demonstrated the presence of 4.1 and 3.5 Dox molecules per antibody molecule, respectively.

The effector functions of ADCs may contribute to their anticancer properties.[8] Therefore, the influence of glycan remodeling and the attachment of the cytotoxic drug on binding to the Fcγ Receptor IIIA (FcγRIIIa) was analyzed by surface plasmon resonance (SPR).[19] The various glycoforms of the antibodies were immobilized on a sensor chip modified by protein A and different concentrations of recombinant FcγRIIIa were employed as the analyte. The resulting data were fitted to a 1:1 Langmuir binding model to give equilibrium constants (K_D) of 110, 131, 110, and 119 nM for antibodies **5**, **6**, **7**, and **10**, respectively (Figure S5). Surprisingly, these results demonstrate that the modified sialic acids do not influence FcγRIIIa binding.

The cell surface receptor CD22 is a clinically validated target for B-cell lymphoma that undergoes constitutive endocytosis,[14] and it is therefore a suitable target for the development of ADCs. Daudi Burkett lymphoma cells, which express CD22, were incubated with varying concentrations of the anti-CD22 (CD22) and control (IgG) antibodies with or without Dox modification. After 48 h, cell viability was measured by MTT assay. The unmodified antibody and control antibody modified by Dox did not exhibit cytotoxicity, whereas the anti-CD22 antibody linked to Dox exhibited dose-dependent cytotoxicity. The influence of modifying Dox with an acid-sensitive hydrazine linker, which in turn is attached to DIBO, was also investigated. As can be seen in Figure 3 (inset), DIBO-linked Dox was slightly less active than the unmodified drug, thus highlighting the importance of the fact that the hydrazine linkage is cleaved in the acidic environment of the endosome to release the active drug.

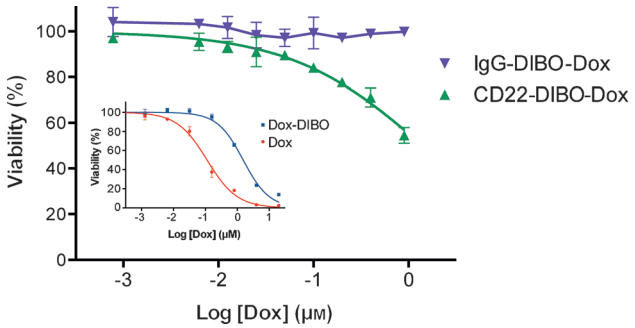

Figure 3. Cytotoxicity of the anti-CD22 antibody conjugated to Dox. Daudi B cells were incubated with various concentrations of IgG-DIBO-Dox or CD22-DIBO-Dox (or Dox or Dox-DIBO (**4**); see inset) for 48 h at 37 °C. Cell viability was assessed by MTT assay. Data were fitted with Prism nonlinear regression software. The EC_{50} values for Dox, Dox-DIBO (**4**), and CD22-DIBO-Dox were found to be 0.1, 1.5, and 1.4 μM, respectively.

We have demonstrated that ST6Gal I is uniquely suited to installing azido-containing sialic acid residues into the glycans of IgG antibodies to give homogeneous glycoforms with approximately four azido moieties. A cytotoxic drug can be attached to such an antibody by strain-promoted azide–alkyne cycloaddition, which is attractive for ADC preparation because it does not require a toxic metal catalyst and proceeds very efficiently at ambient temperature. Furthermore, ST6Gal I is a very stable protein that can be expressed in large quantities, and it is expected that, in addition to an azide moiety, it can tolerate other and even more reactive functional groups in its sugar donor, such as nitrones,[20] nitrile oxides,[21] or *trans*-cyclooctene.[22] Previously, site-specific attachment of a drug to the glycan of antibodies has been accomplished by metabolic labeling with 6-thiofucose.[23] This approach resulted in a relatively low level of drug incorporation, depends on a less attractive conjugation approach, and relies on the presence of a core fucose moiety that inhibits antibody effector functions.[8] Several other methods have been reported for installing reactive functional groups in the glycans of antibodies. For example, mild periodate oxidation of the sialic acids of glycoproteins makes it possible to introduce aldehyde functions, which can be used for coupling purposes.[24] Such reactions are difficult to control and can lead to the oxidation of sensitive amino acids such as

methionine. Aldehydes or ketones can also be installed in the oligosaccharides of glycoproteins by other means but these methods suffer either from low incorporation of the reactive functionality, the need for complex reagents, less desirable conjugation methods, or the formation of heterogeneous products.[19,25] The method for homogenous ADC production reported herein is attractive because it does not require genetic engineering and is therefore applicable to any type of therapeutic antibody.

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