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

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Antibody-drug conjugates (ADC) have considerable promise as anticancer agents by selectively targeting 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 a CD30 (Brentuximab) and a Her2 (ERBB2) specific ADC for treatment of Hodgkin's lymphoma and metastatic breast cancer, respectively.^[3]

Typically, cytotoxic drugs are linked to antibodies by electrophilic modification of lysine or cysteine residues using *N*-hydroxysuccinimide ester or maleimide-activated drugs, respectively.^[2] These conjugation methods lack selectivity and give heterogeneous mixture of products that differ in the sites and stoichiometry of modification. These parameters significantly impact the pharmacokinetic properties of ADCs and therefore there is an urgent need for the development of site-specific conjugation methodologies. Homogeneous ADCs have been obtained by genetic engineering of antibodies to incorporate additional cysteines,^[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]

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Each heavy chain of an 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 glycosyl transferases often tolerate chemical modifications in their sugar nucleotide substrates, allowing the installation of reactive functionalities such as ketones, alkynes or azides.^[9] The incorporation of an azide was expected to be particularly attractive because it is virtually absent in biological systems,^[10] and can be reacted by Staudinger ligation,^[11] copper(I)-catalyzed cycloaddition with terminal alkynes,^[12] or by strain-promoted alkyne-azide cycloaddition (SPAAC).^[13] These conjugation methods should be 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 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 using 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 using trypsin and the generated glycopeptides treated with PNG-F to release the oligosaccharide, which was 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 sialyl transferase, the antibody was treated with galactosyl transferase (GalT) and UPD-Gal in the presence of calf intestine alkaline phosphatase (CIAP), which gave almost exclusively the G2 glycoform (Figure 1b). Next, azido-modified sialic acid was incorporated by treatment with CMP-sialic acid derivative **1** in the presence of recombinant ST6Gal I and CIAP. After 24 h, glycan analysis showed mainly the formation of a mono-sialylated structure, which is in agreement with a previous study^[17] that demonstrated that the α 1,3Man- β 1,2-GlcNAc β 1,4-Gal arm of the glycan of IgG antibody is more assessable for enzymatic remodeling than the other arm. Prolong exposure of the antibody to compound **1** and ST6Gal I resulted, however, in near quantitative bis-sialylation (Figure 1c, Figure S1). 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 acids per antibody (Table S1). The use of α (2,3)-sialyltransferase of *Pasteurella multocida*^[18] or ST3Gal IV resulted in partial modification even after extended incubations times highlighting the favorable properties of ST6Gal I (Figure S2).

DIBO-derivative **2** containing biotin was reacted with antibody **7** to establish conjugation efficiencies. After a reaction time of 2 h, SDS-PAGE using an anti-biotin antibody conjugated to HRP (Figure 2) showed two bands at 37 and 60 kDa, corresponding to labeling of the light and heavy chain, respectively. A similar labeling protocol using DIBO-

FITC (**3**) followed by fluorescence intensity measurements demonstrated the presence of 4.5 fluorophores per antibody molecule. Native gel electrophoresis gave a major band at 150 KDa, which exhibited fluorescence only after remodeling with ST6Gal I and compound **1** and expose 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 explaining the larger 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 antibody 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 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) demonstrated the presence of 4.1 and 3.5 Dox molecules per antibody molecule, respectively.

Effector functions of ADCs may contribute to anticancer properties.^[8] Therefore, the influence of glycan remodeling and attachment of the cytotoxic drug on the binding of Fcγ Receptor IIIA (FcγRIIIa) was analyzed by surface plasmon resonance (SPR).^[19] Thus, 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 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 the antibodies **5**, **6**, **7** and **10**, respectively (Figure S5). Surprisingly, these results demonstrate that the modified of 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 therefore is a suitable target for the development of ADCs. Daudi Burkett lymphoma cells, which express CD22, were incubated with varying concentrations of the anti-CD22 and control antibody with or without Dox modification. After 48 h, cell viability was measured by the MTT assay. The unmodified antibody and control antibody modified by Dox did not possess 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 (inlet), linkered Dox was slightly less active than the unmodified drug highlighting that the hydrazine linkage is cleaved in the acidic environment of endosomes releasing the active drug.

We have demonstrated that ST6Gal I is uniquely suited to install azido-containing sialic acids into the glycans of IgG antibodies to give homogeneous glycoforms having approximately four azido functions. 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 the expectation that in addition to an azide, 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 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 to install reactive functional groups in glycans of antibodies. For example, mild periodate oxidation of 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 oxidation of sensitive amino acids such as methionine. Aldehydes or ketones can also be installed in oligosaccharides of glycoproteins by other means but these methods suffer either from low incorporation of the reactive functionality, the need of complex reagents, less desirable conjugation methods or the formation of heterogeneous products.^[19,25] The new method for homogenous ADC production is attractive because it does not require genetic engineering and therefore is applicable to any type of therapeutic antibody.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. a) Iyer U, Kadambi VJ. *J Pharmacol Toxicol Methods*. 2011; 64:207–212. [PubMed: 21843648] b) Adair JR, Howard PW, Hartley JA, Williams DG, Chester KA. *Expert Opin Biol Ther*. 2012; 12:1191–1206. [PubMed: 22650648]
2. Ducry L, Stump B. *Bioconjug Chem*. 2010; 21:5–13. [PubMed: 19769391]
3. Zolot RS, Basu S, Million RP. *Nat Rev Drug Discov*. 2013; 12:259–260. [PubMed: 23535930]
4. Junutula JR, Raab H, Clark S, Bhakta S, Leipold DD, Weir S, Chen Y, Simpson M, Tsai SP, Dennis MS, Lu Y, Meng YG, Ng C, Yang J, Lee CC, Duenas E, Gorrell J, Katta V, Kim A, McDorman K, Flagella K, Venook RS, Spencer SD, Lee Wong W, Lowman HB, Vandlen R, Sliwkowski MX, Scheller RH, Polakis P, Mallet W. *Nat Biotechnol*. 2008; 26:925–932. [PubMed: 18641636]
5. a) Hutchins BM, Kazane SA, Staflin K, Forsyth JS, Felding-Habermann B, Schultz PG, Smider VV. *J Mol Biol*. 2011; 406:595–603. [PubMed: 21237172] b) Axup JY, Bajjuri KM, Ritland M, Hutchins BM, Kim CH, Kazane SA, Halder R, Forsyth JS, Santidrian AF, Stafin K, Lu Y, Tran H, Seller AJ, Biroc SL, Szydluk A, Pinkstaff JK, Tian F, Sinha SC, Felding-Habermann B, Smider VV, Schultz PG. *Proc Natl Acad Sci U S A*. 2012; 109:16101–16106. [PubMed: 22988081]
6. Strop P, Liu SH, Dorywalska M, Delaria K, Dushin RG, Tran TT, Ho WH, Farias S, Casas MG, Abdiche Y, Zhou D, Chandrasekaran R, Samain C, Loo C, Rossi A, Rickert M, Krimm S, Wong T, Chin SM, Yu J, Dilley J, Chaparro-Riggers J, Filzen GF, O'Donnell CJ, Wang F, Myers JS, Pons J, Shelton DL, Rajpal A. *Chem Biol*. 2013; 20:161–167. [PubMed: 23438745]
7. Panowski S, Bhakta S, Raab H, Polakis P, Junutula JR. *mAbs*. 2014; 6:1–12. [PubMed: 24284905]
8. Jefferis R. *Nat Rev Drug Discov*. 2009; 8:226–234. [PubMed: 19247305]
9. a) Keppler OT, Horstkorte R, Pawlita M, Schmidt C, Reutter W. *Glycobiology*. 2001; 11:11R–18R. [PubMed: 11181557] b) Schmaltz RM, Hanson SR, Wong CH. *Chem Rev*. 2011; 111:4259–4307. [PubMed: 21749134]
10. Debets MF, van der Doelen CWJ, Rutjes FPJT, van Delft FL. *ChemBioChem*. 2010; 11:1168–1184. [PubMed: 20455238]
11. a) Saxon E, Bertozzi CR. *Science*. 2000; 287:2007–2010. [PubMed: 10720325] b) Schilling CI, Jung N, Biskup M, Schepers U, Brase S. *Chem Soc Rev*. 2011; 40:4840–4871. [PubMed: 21687844]

12. a) Meldal M, Tornøe CW. *Chem Rev.* 2008; 108:2952–3015. [PubMed: 18698735] b) Soriano Del Amo D, Wang W, Jiang H, Besanceney C, Yan AC, Levy M, Liu Y, Marlow FL, Wu P. *J Am Chem Soc.* 2010; 132:16893–16899. [PubMed: 21062072] c) Kennedy DC, McKay CS, Legault MC, Danielson DC, Blake JA, Pegoraro AF, Stollow A, Mester Z, Pezacki JP. *J Am Chem Soc.* 2011; 133:17993–18001. [PubMed: 21970470]
13. a) Jewett JC, Bertozzi CR. *Chem Soc Rev.* 2010; 39:1272–1279. [PubMed: 20349533] b) Debets MF, van Berkel SS, Dommerholt J, Dirks AT, Rutjes FP, van Delft FL. *Acc Chem Res.* 2011; 44:805–815. [PubMed: 21766804]
14. Molina A. *Annu Rev Med.* 2008; 59:237–250. [PubMed: 18186705]
15. a) Kajihara Y, Kamitani T, Sato R, Kamei N, Miyazaki T, Okamoto R, Sakakibara T, Tsuji T, Yamamoto T. *Carbohydr Res.* 2007; 342:1680–1688. [PubMed: 17572399] b) Mbua NE, Li X, Flanagan-Steet HR, Meng L, Aoki K, Moremen KW, Wolfert MA, Steet R, Boons GJ. *Angew Chem Int Ed.* 2013; 52:13012–13015. *Angew Chem.* 2013; 125:13250–13253.
16. Ning XH, Guo J, Wolfert MA, Boons GJ. *Angew Chem Int Ed.* 2008; 47:2253–2255. *Angew Chem.* 2008; 120:2285–2287.
17. Barb AW, Meng L, Gao Z, Johnson RW, Moremen KW, Prestegard JH. *Biochemistry.* 2012; 51:4618–4626. [PubMed: 22574931]
18. Yu H, Chokhawala H, Karpel R, Yu H, Wu B, Zhang J, Zhang Y, Jia Q, Chen X. *J Am Chem Soc.* 2005; 127:17618–17619. [PubMed: 16351087]
19. Huang W, Giddens J, Fan SQ, Toonstra C, Wang LX. *J Am Chem Soc.* 2012; 134:12308–12318. [PubMed: 22747414]
20. Ning X, Temming RP, Dommerholt J, Guo J, Ania DB, Debets MF, Wolfert MA, Boons GJ, van Delft FL. *Angew Chem Int Ed.* 2010; 49:3065–3068. *Angew Chem.* 2010; 122:3129–3132.
21. Sanders BC, Friscourt F, Ledin PA, Mbua NE, Arumugam S, Guo J, Boltje TJ, Popik VV, Boons GJ. *J Am Chem Soc.* 2011; 133:949–957. [PubMed: 21182329]
22. Selvaraj R, Fox JM. *Curr Opin Chem Biol.* 2013; 17:753–760. [PubMed: 23978373]
23. Okeley NM, Toki BE, Zhang X, Jeffrey SC, Burke PJ, Alley SC, Senter PD. *Bioconjug Chem.* 2013; 24:1650–1655. [PubMed: 24050213]
24. a) Rodwell JD, Alvarez VL, Lee C, Lopes AD, Goers JW, King HD, Powsner HJ, McKearn TJ. *Proc Natl Acad Sci U S A.* 1986; 83:2632–2636. [PubMed: 3458222] b) Van Lenten L, Ashwell G. *J Biol Chem.* 1971; 246:1889–1894. [PubMed: 4323238] c) Zuberbuhler K, Casi G, Bernardes GJL, Neri D. *Chem Commun.* 2012; 48:7100–7102.
25. a) Morell AG, Van den Hamer CJ, Scheinberg IH, Ashwell G. *J Biol Chem.* 1966; 241:3745–3749. [PubMed: 5916390] b) Boeggeman E, Ramakrishnan B, Pasek M, Manzoni M, Puri A, Loomis KH, Waybright TJ, Qasba PK. *Bioconjug Chem.* 2009; 20:1228–1236. [PubMed: 19425533]

Figure 1.

Mass spectrometry-based determination of glycan structures of IgG control antibody. (A) *N*-Glycans isolated from the immunoglobulin G. (B) Galactosylation of the IgG antibody resulted in primarily a di-galactosylated glycan. (C) Galactosylation followed by sialylation of the IgG antibody using ST6Gal I resulted primarily in a bis-sialylated glycan.

Galactosyltransferase/UDP-Gal:	-	-	+	+	-	-	+	+
ST6Gal I/CMP-Neu5Ac9N ₃ :	-	-	-	-	+	+	+	+
DIBO-biotin:	-	+	-	+	-	+	+	-

Figure 2. Confirmation of IgG labeling of CMP-Neu5Ac9N₃ by ST6Gal I before and after remodeling with galactosyltransferase. (A) Western blotting using an anti-biotin antibody conjugated to HRP. (B) Total protein staining by Coomassie blue.

Figure 3.

Cytotoxicity of anti-CD22 antibody conjugated to Dox. Daudi B cells were incubated with various concentrations of Dox, Dox-DIBO, IgG-Dox-DIBO, and CD22-Dox-DIBO for 48 h at 37 °C. Cell viability was assessed using the MTT assay. Data were fitted using Prism nonlinear regression software. EC₅₀ values for Dox, Dox-DIBO, and CD22-Dox-DIBO were found to be 0.1, 1.5, and 1.4 μM, respectively.

Scheme 1.

Glycan remodeled of IgG antibodies to produce a homogenous glycoform that have azido moieties for strain promoted cycloadditions with compound **2-4**. Reagents and conditions: (i) UDP-Gal, galactosyltransferase, MOPS buffer, pH 7.2; (ii) Compound **1**, sialyltransferase, cacodylate buffer pH 7.6; (iii) Compound **2**, **3**, or **4** in cacodylate buffer pH 7.6.