Recent advances in the synthesis of antibody-drug conjugates

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Abstract

Antibody-drug conjugates (ADCs) are derived from antibodies covalently attached to highly potent drugs using a variety of conjugation and linker technologies. This class of therapeutic conceptually combines the exquisite specificity of antibodies (*i.e.* enabling discrimination between healthy and diseased tissue) with the cell-killing ability of potent cytotoxic drugs. This powerful and exciting class of targeted therapy has shown considerable promise in the treatment of various cancers with two US Food and Drug Administration (FDA) approved ADCs currently on the market (*i.e.* Adcetris™ and Kadcyla™) and *ca.* 40 ADCs currently undergoing clinical evaluation. However, in order for ADCs to deliver their full potential, sophisticated site-specific conjugation technologies to connect the drug to the antibody are vital. This perspective discusses the strategies currently used for the site-specific construction of ADCs and appraises their merits and disadvantages.

i. Introduction

Antibodies are macromolecular Y-shaped proteins of *ca.* 150 kDa and above (see Figure 1), which are produced by B-cells in the blood upon activation of the immune system. Antibodies can be generated to selectivity target a specific antigen binding partner that is unique to, or over-expressed, on cancer cells and have therefore received much attention in cancer treatment in recent years as they greatly reduce off target toxicity. These targeted antibodies can be used as therapeutics alone, *via* antibody-dependent cell-mediated cytotoxicity, or as vehicles for drug delivery and imaging.

Antibody-drug conjugates (ADCs) are a type of targeted therapy. They consist of an antibody armed with potent cytotoxic drugs using various conjugation and linker technologies. The antibody's binding region allows selective targeting of certain cell types (*i.e.* allowing for discrimination between healthy and diseased tissue) and the potent cytotoxic drug element effects cell-killing independent of antibody-dependent cell-mediated cytotoxicity. Hence, ADCs offer the prospect for delivery of a toxic payload directly to a target, with minimisation of "off-target" toxicity. This in turn allows for the use of particularly toxic drug molecules that are conventionally excluded from use in chemotherapy.

The concept of delivering a toxic payload to cancer cells using a targeting agent dates back to 1913 when Paul Ehrlich described a "heptophore" that can deliver a "toxophore" selectively to a tumor.[1] However, it took 45 years for such a species to be constructed in the form of an ADC.[2] In the 1970's, ADCs were first tested on animals[3, 4] and less than a decade later the first tests on humans showed promising results.[5] The first ADCs based on chimeric and humanised monoclonal antibodies (mAbs) were reported in the 1990s,[6] and throughout this decade, ever increasing payload potency and improved target selection were achieved.[7] These advances finally led, in 2000, to the first US Food and Drug Administration (FDA) approved ADC (Mylotarg, gemtuzumab ozogamicin).[8]

Despite promising preliminary results, Mylotarg was voluntarily withdrawn from the market in June 2010 as post-approval clinical trials for patients with acute myeloid leukemia showed that the ADC offered no clinical benefit over standard chemotherapy. Nevertheless, this class of targeted therapy showed considerable promise in the following years with two further ADCs gaining FDA approval (*i.e.* Adcetris, brentuximab vedotin in 2011[9, 10] and Kadcyla, trastuzumab emtansine in 2013[11, 12]). It is

estimated that there are *ca.* 40 ADCs currently in the clinic and it is predicted that the market for ADCs will grow rapidly in coming years.[13]

Both Kadcyla and Mylotarg were generated by modifying accessible lysine residues on the surface of the relevant antibody.[11, 12] In fact, many ADCs are conjugated through lysine modification. However, with *ca.* 90 accessible lysines, any chemical modification strategy that is non-selective has the potential to generate complex mixtures, with up to 10^6 distinct species statistically possible when targeting drug-to-antibody ratios of 2–4. Such heterogeneous mixtures of ADCs may differ in both drug loading and conjugation site and this can result in a narrow therapeutic window with major pharmacokinetic implications. Although this non-specific conjugation technique has been used in FDA-approved ADCs, the use of non-selective approaches is now considered suboptimal in developing next generation ADCs.

In order for ADCs to deliver their full potential, sophisticated conjugation technologies to connect the cytotoxic drug to the antibody are needed. In a promising early development, Adcetris was obtained by reacting some of the eight free cysteines obtained by reduction of the four interchain disulfides of an antibody (see Figure 1). Whilst this approach still generates *ca.* 15 different species when targeting typical average drug-to-antibody ratios of 2–4, it offers a significant improvement over lysine modification strategies in terms of reduced heterogeneity.[14] There is a growing appreciation of the importance of developing site-specific methods, with several reports highlighting the advantages for generating near homogenous conjugates due to a better defined and improved pharmacokinetic profile.[14-16] This perspective will give an overview of the different methods used for the site-specific attachment of cytotoxic agents to antibodies.

ii. Current methods for site-selective attachment of drugs to antibodies

a. Engineered antibody modification

With the rapid advances in protein engineering, methods to site-specifically functionalise antibodies have become more accessible in recent years. There are three main strategies to generate site-selectively modified ADCs with re-engineered mAbs (see Figure 2): (i) insertion of cysteine residues

in the antibody sequence by mutagenesis;[17, 18] (ii) enzymatic conjugation;[19] and (iii) insertion of an unnatural amino acid with a functional group that can be chemoselectively reacted.[20-22]

i. Engineered cysteines

The thiol moiety of the cysteine side chain has the highest nucleophilicity of all proteinogenic amino acid functional groups under physiological conditions. This makes it a useful target for the selective and site-specific modification of antibodies. Moreover, with the use of site-directed mutagenesis, cysteine residues can readily be inserted at a specific position on a protein.

In 2008, Junutula *et al.* (Genentech) described a method for the introduction of additional cysteine residues on a mAb using site-directed mutagenesis.[23] This procedure is non-trivial for an antibody as the engineered cysteine residues can pair with other free cysteines (*e.g.* to form protein dimers or to scramble disulfides), which could reduce or remove activity.[24, 25] However, by screening conjugation sites on an antibody against the ovarian cancer antigen MUC16, an engineered thio-antibody (THIOMAB) containing two new cysteine sites for attachment was generated successfully.

However, although the engineered cysteines were introduced successfully, they were mostly found as mixed disulfides with glutathione. As no reliable method to target reduction of these mixed disulfides over native disulfides was achieved, an alternative strategy was needed for engineered thiol unmasking. To do this, both the mixed and native interchain disulfides were reduced initially. This was followed by mild re-oxidation of the interchain disulfides with a gentle oxidant (*e.g.* $CuSO_4$) to afford an antibody with all of its native disulfide bonds intact, and two reduced engineered cysteines available for conjugation.

Using this strategy, ADCs comprised of monomethyl auristatin E (MMAE), a potent tubulin inhibitor, and an anti-MUC16 mAb were generated. They were judged to have an average drug-to-antibody ratio of 1.9 with more than 90% homogeneity and were active *in vitro* and *in vivo*.[18] The THIOMAB-drug conjugates were also shown to be significantly less toxic in rat and cynomolgus monkey models when compared with analogous ADCs that were obtained by lysine conjugation with an average drug-

to-antibody ratio of 4. Moreover, as described by Hamblett *et al.*, despite having half the average drug load, the THIOMAB-drug conjugates were as efficacious *in vivo*.[14, 18]

ii. Enzyme-directed modification

Another approach to achieving site-selective modification is *via* enzymes that react with a particular amino acid in a specific amino acid sequence. This has been used to site-selectively attach drugs to antibodies.

Transglutaminases

Transglutaminases (TGs) play an important role in biological processes which depend on the covalent crosslinking of proteins,[26] where they catalyse the formation of amide bonds between the primary amine of a lysine and the amide group of a glutamine. However, bacterial TG isolated from *Streptoverticillium mobaraense* has an atypical catalytic site compared to other TGs.[27, 28] It does not catalyse a reaction with any of the naturally occurring glutamine residues in the constant regions of an antibody, but will recognise a "glutamine tag" (*i.e.* LL**Q**G).[29] By appraising the merits of incorporation of this sequence into 12 surface accessible regions of an anti-M1S1 IgG1, Strop *et al.* (Rinat-Pfizer) found two optimal locations for the "Q-tag". Two ADCs based on monomethyl dolastatin 10 (MMAD, a potent tubulin inhibitor) were then generated. These conjugates were found to have good biophysical properties and a drug-to-antibody ratio of *ca.* 1.9.[16] Furthermore, when compared with an analogous ADC with a higher drug loading (average drug-to-antibody ratio of 3.6) based on cysteine alkylation post-native interchain disulfide reduction, the glutamine-tagged ADCs showed similar activity *in vitro* and *in vivo* and were better tolerated *in vivo*. This differential in toxicity profile suggests that either the selected position of the drug and/or the lower loading offers the possibility for improvement of the therapeutic index of ADCs.

Formylglycine-generating enzymes

Formylglycine-generating enzymes (FGE) catalyse the selective conversion of a cysteine to an aldehyde when in a **C**XPXR sequence (where X is usually serine, threonine, alanine or glycine). The incorporated aldehyde can then be readily functionalised with aminooxy- or hydrazine-functionalised

molecules.[30] Using this approach, Drake *et al.* (Redwood Bioscience) recently produced and characterised a series of functionalised antibodies bearing the aldehyde tag at different sites on trastuzumab.[31] They then used hydrozino-*iso*-Pictet-Spengler (HIPS) chemistry to conjugate a cytotoxic maitansine derivative (a potent microtubule-targeted agent) at three different positions. As observed with other methods, the site of conjugation had a significant impact on *in vivo* efficacy and pharmacokinetic profile in rats. Unfortunately, this method suffers from the hydration of the aldehyde from formyglycine in water to form an unreactive gem-diol, which lowers the yield of the process.[21]

iii. Unnatural amino acid incorporation

Recent advances in development of methods for the incorporation of unnatural amino acids into proteins have presented opportunities for the site-selective modification of antibodies.[22, 32] Two unnatural amino acids, *p*-acetylphenylalanine and *p*-azidophenylalanine, have been particularly useful in generating bioconjugates through oxime ligation and classical click chemistry, respectively.

Axup *et al.* described the incorporation of *p*-acetylphenylalanine into several residues in the constant region of trastuzumab to construct several modified antibodies ready for site-specific ADC synthesis.[22] Using this unique functional group, MMAD was coupled through a stable oxime-ligation process to yield several near-homogenous ADCs with a drug-to-antibody ratio of *ca.* 2.0. The resulting conjugates demonstrated good pharmacokinetic properties, potent *in vitro* cytotoxic activity against HER2+ cancer cells, and complete tumor regression in rodents. When compared with ADCs prepared by cysteine alkylation post-native interchain disulfide reduction, site specific unnatural amino acids-based ADCs were shown to have increased *in vitro* cytotoxicity and *in vivo* efficacy. They also had superior *in vitro* serum stability and an improved toxicology profile in rats.[33, 34]

Zimmerman *et al.* (SutroBiopharma) recently observed a similar trend *in vitro* by incorporating *p*-azidomethyl-phenylalanine into trastuzumab and using strain-promoted azide-alkyne cycloaddition click chemistry to conjugate monomethyl auristatin F (a potent tubulin inhibitor).[32]

Even though these unnatural amino acids methods offer the possibility of generating homogenous conjugates by attachment of a drug at virtually any accessible site on a mAb, the potential

immunogenicity of unnatural amino acids is not yet fully understood and more studies are needed to ensure the safety of these engineered ADCs.

b. Native antibody modification

Although the aforementioned methods offer the possibility to synthesise near homogeneous ADCs they require site-directed mutagenesis and optimisation of cell culture conditions, which can increase the overall manufacturing cost of an ADC. The alternative is to develop methods which avoid the requirement for re-engineering by direct site-selective manipulation of native mAbs (see Figure 3).

i. Reduced inter-chain disulfides as targets for site selective modification

Whilst antibodies typically have approximately 90 accessible lysine residues, reduction of the 4 accessible interchain disulfides yields only 8 nucleophilic cysteine residues. Hence, conjugation to antibodies by reaction with cysteine thiols liberated from reduced interchain disulfides will inherently generate a smaller subpopulation of immunoconjugates compared with modification *via* lysine. Moreover, reduction of native interchain disulfide bonds is thought to have a limited effect on antibody structure and stability since assembly of light and heavy chains does not depend primarily on covalent disulfide linkages, but rather on non-covalent interactions.[14, 35]

1. Conjugation of cysteines liberated from reduced interchain disulfides

In 1990, Braslawsky *et al.* described the use of conjugation to cysteines generated by reduction of the native interchain disulfides of a mAb as an ADC construction strategy. Through employing thiol-bearing doxorubicin as a toxin, a disulfide linker-based ADC was generated. However, whilst the resulting ADC was active *in vitro*, poor potency was observed *in vivo*.[36] This limited potency was attributed to the instability of the disulfide linker used.

A decade later, Doronina *et al.* (Seattle Genetics) used a similar approach of initially reducing the 4 interchain disulfide bonds of a chimeric anti-CD30 mAb (*i.e.* cAC10) but conjugating the liberated cysteines to MMAE using a maleimide linker. This provided an ADC that was potent and selective for CD30-positive hematologic malignancies.[37] Originally, the 8 sulfhydryl groups generated by reduction of the interchain disulfides (see Figure 1) were alkylated using maleimide-MMAE, yielding

a near homogenous conjugate with a drug-to-antibody ratio of 8. However, further analysis by Hamblett *et al.* and Beckley *et al.* showed that the high drug loading had a significant impact on conjugate phramacokinetics (*i.e.* poor tolerability, high plasma clearance rate and decreased efficacy *in vivo*) and propensity to aggregate,[14, 38] and that a lower drug-to-antibody ratio resulted in a larger therapeutic window.[14] In particular, they showed that an ADC with a drug-to-antibody ratio of 4 was significantly less toxic to mice than higher loaded conjugates. These observations, along with a screening of various reduction/re-oxidation strategies, resulted in a novel site-selectively modified antibody, Adcetris, with an average drug-to-antibody ratio of 4 with isomeric homogeneities up to 75%.[35] However, one of the main drawbacks of this strategy comes from the use of maleimide chemistry in that the resulting bioconjugate is known to undergo retro-addition reactions with blood thiols, resulting in the transfer of drug to thiol-bearing proteins and thus leading to off-site toxicity.[39] Recently, however, several strategies to overcome this issue and to obtain thiol-stable bioconjugates have been developed.[40]

2. Re-bridging of cysteines liberated from reduced interchain disulfides

To target a drug-to-antibody ratios of 4 and increase the overall homogeneity of their conjugates, Badescu *et al.* (PolyTherics) explored the possibility that reduction of the interchain disulfide bridges could be followed by functional re-bridging of the disulfide (see Figure 3). This would allow insertion of a single cytotoxic payload per disulfide and retain a covalent link between the protein chains. Their method is based on bis-cysteine-selective sulfone reagents that allow for an addition-elimination-addition sequence onto the reduced disulfide bonds of either Fabs or mAbs. Using MMAE as the toxic payload on both trastuzumab and its Fab, they successfully demonstrated increased efficacy over drug alone whilst retaining binding and antigen-selective cytotoxicity *in vitro*, along with efficacy *in vivo*.[41] Other groups (*i.e.* Chudasama, Baker and Caddick) have also developed reagents that allow for the efficient functional re-bridging of interchain disulfides (*i.e.* dibromopyridazinediones and dithiomaleimides).[42, 43] Using these reagents on both trastuzumab and its Fab, Maruani *et al.* and Nunes *et al.* have successfully generated near homogeneous ADCs with *in vitro* efficacy.[42, 43]

ii. Glycan targeting for synthesis of antibody-drug conjugates

All antibodies are glycosylated at conserved positions in their constant regions; they possess an *N*-glycosylation site at the conserved Asn-297 residue of the Fc region (see Figure 1). Thus, this serves as a potentially interesting (and generic) site for antibody modification.

In this context, the use of hydrazone chemistry to attach a cytotoxic agent onto the glycans of an unmodified native antibody has found considerable utility.[44, 45] Sodium periodate at high concentration has been used to oxidise carbohydrate residues in the native glycans to provide aldehydes which are used for ligation to afford relatively homogenous ADCs.[45] The main drawback of this approach arises from the harsh oxidation conditions required to generate the aldehydes, which can result in oxidation of methionine residues located close to FcRn binding site. This over-oxidation is known to affect FcRn binding and to generally reduce serum half-life.[46] To alleviate this issue, milder enzymatic methods have been developed, which are discussed below.

Neuraminidases

Through sequentially using neuraminidases to cleave the glycosidic linkages of neuraminic acids and Gal oxidase to oxidise the galactose residues of an anti-CEA mAb, Stan *et al.* site-selectively attached doxorubicin *via* reductive amination on the generated aldehydes.[15] The resulting ADC had a drug-to-antibody ratio of 3.7 and was four times more toxic *in vitro* than its counterpart generated by lysine conjugation with a drug-to-antibody ratio of 7.8. This demonstrates once again the importance of, and therapeutic opportunities for, site specificity in ADC construction.

Glycosyltransferases

Recently, Zhou *et al.* (Sanofi-Genzyme) described another approach using post-translational modification of trastuzumab to introduce sialic acid moieties onto the native glycans on Asn-297.[47] To achieve site-specific modification, a mixture of galactosyltransferase and sialyltransferase was used to transfer galactose and sialic acid residues onto the native glycans. These sialic acid residues were then oxidised under mild condition to yield aldehyde-functionalised trastuzumab. Even though these conditions led to partial oxidation of the methionines proximal to the FcRn binding site, which compromised FcRn binding by *ca.* 25%, it had a negligible effect on serum half-life.[47] The

enzymatically modified mAb was then conjugated by oxime ligation with aminooxy drug linkers to generate site-selectively modified ADCs. Using MMAE and MMAD, an average drug-to-antibody ratio of 1.6 was obtained and these glyco-conjugated ADCs exhibited antigen-dependent activity *in vitro* and were efficacious *in vivo*.

A similar strategy that avoids the oxidation step by utilising azido-modified sialic acid and strain-promoted azide-alkyne cycloaddition chemistry with a suitable doxorubicin derivative has recently been applied to an anti-CD22 antibody. This resulted in a near homogenous ADC which has been shown to selectively target and kill lymphoma cells *in vitro*.[48]

The main limitation of these glycan-based modifications stems from the complex and heterogeneous population of glycans in mAbs, as well as their reliance on the presence of galactose on an IgG1. These characteristics of antibodies may reduce the homogeneity of the final ADC construct and/or mean that additional steps are required to homogenise the glycan population.[49]

iii. Conclusion and future outlook

In conclusion, a variety of methods have been developed for the site-selective modification of monoclonal antibodies to produce antibody-drug conjugates, each with their own advantages and limitations (Table 1). Although the use of engineered mAbs for ADC construction offers controlled homogeneity, this is offset by their construction and modification often being technically challenging. Modification of native mAbs, on the other hand, involves simple reaction protocols but with the caveat of difficulties in achieving homogeneous modification. Thus there is no leading technology at present and although recent methods for the construction of ADCs have gone some way to addressing the challenging issues of making homogeneous antibody-drug conjugates, significant hurdles still remain. Despite there being a link between a number of parameters that one needs to consider when constructing an ADC (*e.g.* location of cytotoxic agent, drug-to-antibody ratio, homogeneity) and the efficacy and pharmacokinetic profile of the ADC (*e.g.* required dosage, biodistribution, clearance rate, toxicity, accumulation at tumour), the detailed understanding of the interdependencies of these links and combinations of parameters will need a good deal more work before they are fully understood.

Even though there appears to be general recognition that site-specific ADCs with increased homogeneity are likely to have superior therapeutic properties, there is not as yet a single technology that can be generally applied for their preparation. Instead, each ADC is being constructed in a tailor-made fashion for the specific antibody and drug combination in question. At this time, it is unclear what site-specific strategies will be ideal for what drug types or drug-to-antibody ratios, or even which ones will meet low manufacturing cost, safety and tolerability requirements. However, with a better understanding of the influence and consequences of each site-specific modification strategy in the coming years, the next generation of antibody-based targeted therapy will be based on a more rational design of bioconjugates to connect the "A" and the "D". Perhaps a leading single technology will emerge, or particular technologies will be more suited to certain drug types, drug loadings or specific antibodies, however, what is for certain is that ADCs have an important and longstanding role to play in the future of targeted therapeutics.

References

1. Ehrlich, P. Address in Pathology, ON CHEMIOTHERAPY: Delivered before the Seventeenth International Congress of Medicine. *The British Medical Journal* **2**, 353-359 (1913).

2. Mathe, G., Loc, T. B. & Bernard, J. Effet Sur La Leucemie 1210 De La Souris Dune Combinaison Par Diazotation Da-Methopterine Et De Gamma-Globulines De Hamsters Porteurs De Cette Leucemie Par Heterogreffe. *C. r. hebd. séances Acad. sci.* **246**, 1626-1628 (1958).

3. Ghose, T. & Nigam, S. P. Antibody as Carrier of Chlorambucil. *Cancer* **29**, 1398-& (1972).

4. Rowland, G. F., Oneill, G. J. & Davies, D. A. L. Suppression of Tumor-Growth in Mice by a Drug-Antibody Conjugate Using a Novel Approach to Linkage. *Nature* **255**, 487-488 (1975).

5. Ford, C. H. J. *et al.* Localization and Toxicity Study of a Vindesine-Anti-Cea Conjugate in Patients with Advanced Cancer. *Br. J. Cancer* **47**, 35-42 (1983).

6. Trail, P. A. *et al.* Cure of Xenografted Human Carcinomas by Br96-Doxorubicin Immunoconjugates. *Science* **261**, 212-215 (1993).

7. Pietersz, G. A. & Krauer, K. Antibody-Targeted Drugs for the Therapy of Cancer. *J. Drug Target.* **2**, 183-215 (1994).

8. Linenberger, M. L. *et al.* Multidrug-resistance phenotype and clinical responses to gemtuzumab ozogamicin. *Blood* **98**, 988-994 (2001).

9. Younes, A. *et al.* Brentuximab Vedotin (SGN-35) for Relapsed CD30-Positive Lymphomas. *N. Engl. J. Med.* **363**, 1812-1821 (2010).

10. Senter, P. D. & Sievers, E. L. The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. *Nat. Biotechnol.* **30**, 631-637 (2012).

11. Verma, S. *et al.* Trastuzumab Emtansine for HER2-Positive Advanced Breast Cancer. *N. Engl. J. Med.* **367**, 1783-1791 (2012).

12. LoRusso, P. M., Weiss, D., Guardino, E., Girish, S. & Sliwkowski, M. X. Trastuzumab Emtansine: A Unique Antibody-Drug Conjugate in Development for Human Epidermal Growth Factor Receptor 2-Positive Cancer. *Clin. Cancer Res.* **17**, 6437-6447 (2011).

13. Mullard, A. Maturing antibody-drug conjugate pipeline hits 30. *Nat. Rev. Drug Discov.* **12**, 329–332 (2013).

14. Hamblett, K. J. *et al.* Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin. Cancer Res.* **10**, 7063-7070 (2004).

15. Stan, A. C., Radu, D. L., Casares, S., Bona, C. A. & Brumeanu, T. D. Antineoplastic efficacy of doxorubicin enzymatically assembled on galactose residues of a monoclonal antibody specific for the carcinoembryonic antigen. *Cancer Res.* **59**, 115-121 (1999).

16. Strop, P. *et al.* Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. *Chem. Biol.* **20**, 161-167 (2013).

17. Lyons, A. *et al.* Site-Specific Attachment to Recombinant Antibodies Via Introduced Surface Cysteine Residues. *Protein Eng.* **3**, 703-708 (1990).

18. Junutula, J. R. *et al.* Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat. Biotechnol.* **26**, 925–932 (2008).

19. Sunbul, M. & Yin, J. Site specific protein labeling by enzymatic posttranslational modification. *Org. Biomol. Chem.* **7**, 3361-3371 (2009).

20. Young, T. S., Ahmad, I., Yin, J. A. & Schultz, P. G. An Enhanced System for Unnatural Amino Acid Mutagenesis in E. coli. *J. Mol. Biol.* **395**, 361-374 (2010).

21. Rabuka, D., Rush, J. S., deHart, G. W., Wu, P. & Bertozzi, C. R. Site-specific chemical protein conjugation using genetically encoded aldehyde tags. *Nat. Protoc.* **7**, 1052-1067 (2012).

22. Axup, J. Y. *et al.* Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 16101-16106 (2012).

23. Junutula, J. R. *et al.* Rapid identification of reactive cysteine residues for site-specific labeling of antibody-Fabs. *J. Immunol. Methods* **332**, 41–52 (2008).

24. Woo, H. J., Lotz, M. M., Jung, J. U. & Mercurio, A. M. Carbohydrate-binding protein 35 (Mac-2), a laminin-binding lectin, forms functional dimers using cysteine 186. *J. Biol. Chem.* **266**, 18419-18422 (1991).

25. Wootton, S. K. & Yoo, D. Homo-oligomerization of the porcine reproductive and respiratory syndrome virus nucleocapsid protein and the role of disulfide linkages. *J. Virol.* **77**, 4546-4557 (2003).

26. Greenberg, C. S., Birckbichler, P. J. & Rice, R. H. Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB J.* **5**, 3071-3077 (1991).

27. Kanaji, T. *et al.* Primary structure of microbial transglutaminase from Streptoverticillium sp. strain s-8112. *J. Biol. Chem.* **268**, 11565-11572 (1993).

28. Kashiwagi, T. *et al.* Crystal structure of microbial transglutaminase from Streptoverticillium mobaraense. *J. Biol. Chem.* **277**, 44252-44260 (2002).

29. Jeger, S. *et al.* Site-specific and stoichiometric modification of antibodies by bacterial transglutaminase. *Angew. Chem. Int. Ed. Engl.* **49**, 9995-9997 (2010).

30. Wu, P. *et al.* 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**, 3000-3005 (2009).

31. Drake, P. M. *et al.* Aldehyde tag coupled with HIPS chemistry enables the production of ADCs conjugated site-specifically to different antibody regions with distinct in vivo efficacy and PK outcomes. *Bioconjugate Chem.* **25**, 1331-1341 (2014).

32. Zimmerman, E. S. *et al.* Production of site-specific antibody-drug conjugates using optimized non-natural amino acids in a cell-free expression system. *Bioconjugate Chem.* **25**, 351–361 (2014).

33. Sapra, P. *et al.* Long-term tumor regression induced by an antibody-drug conjugate that targets 5T4, an oncofetal antigen expressed on tumor-initiating cells. *Mol. Cancer Ther.* **12**, 38-47 (2013).

34. Tian, F. *et al.* A general approach to site-specific antibody drug conjugates. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 1766-1771 (2014).

35. Sun, M. M. *et al.* Reduction-alkylation strategies for the modification of specific monoclonal antibody disulfides. *Bioconjugate Chem.* **16**, 1282–1290 (2005).

36. Braslawsky, G. R., Edson, M. A., Pearce, W., Kaneko, T. & Greenfield, R. S. Antitumor activity of adriamycin (hydrazone-linked) immunoconjugates compared with free adriamycin and specificity of tumor cell killing. *Cancer Res.* **50**, 6608-6614 (1990).

37. Doronina, S. O. *et al.* Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol.* **21**, 778-784 (2003).

38. Beckley, N. S., Lazzareschi, K. P., Chih, H.-W., Sharma, V. K. & Flores, H. L. Investigation into Temperature-Induced Aggregation of an Antibody Drug Conjugate. *Bioconjugate Chem.* **24**, 1674-1683 (2013).

39. Shen, B. Q. *et al.* Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat. Biotechnol.* **30**, 184-189 (2012).

40. Lyon, R. P. *et al.* Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates. *Nat. Biotechnol.* **32**, 1059–1062 (2014).

41. Badescu, G. *et al.* Bridging disulfides for stable and defined antibody drug conjugates. *Bioconjugate Chem.* **25**, 1124–1136 (2014).

42. Neves, J. P. M. *et al.* Functional native disulfide bridging enables delivery of a potent, stable and targeted antibody-drug conjugate (ADC). *Chem. Commun.*, doi: 10.1039/C5CC03557K (2015).

43. Maruani, A. *et al.* A plug and play approach to antibody-based therapeutics via a chemoselective dual click strategy. *Nat. Commun.* **6**, 6645 (2015).

44. Hinman, L. M. *et al.* Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. *Cancer Res.* **53**, 3336-3342 (1993).

45. Hamann, P. R. *et al.* An anti-MUC1 antibody-calicheamicin conjugate for treatment of solid tumors. Choice of linker and overcoming drug resistance. *Bioconjugate Chem.* **16**, 346–353 (2005).

46. Wang, W. *et al.* Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies. *Mol. Immunol.* **48**, 860-866 (2011).

47. Zhou, Q. *et al.* Site-specific antibody-drug conjugation through glycoengineering. *Bioconjugate Chem.* **25**, 510–520 (2014).

48. Zhou, Q. *et al.* Site-specific antibody-drug conjugation through glycoengineering. *Bioconjugate Chem.* **25**, 510-520 (2014).

49. Jefferis, R. Glycosylation of recombinant antibody therapeutics. *Biotechnol. Prog.* **21**, 11-16 (2005).

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VC and SC are co-founders and directors of the Company ThioLogics™.

Legends

Figure 1: General structure of an IgG1 antibody highlighting key components. Fab fragments comprise the antigen-binding region and consist of the whole light chain (V_L and C_L) and of part of the heavy chain (V_H and C_H1). Fab fragments have a molecular weight of *ca.* 50 kDa. The Fc fragment carries some of the biological properties of the IgG1, in particular its ability to be recognised by effectors of immunity. It consists of identical C_H2 and C_H3 fragments, derived from the heavy chains' constant domains of the antibody. Fc fragments do not recognise the corresponding antigen, they bind to various cell receptors and complement proteins and have a molecular weight of *ca.* 50 kDa. All antibodies are glycosylated at conserved positions in their constant regions ($_C$); they possess an *N*-glycosylation site at the conserved Asn-297 residue of the Fc region.

Figure 2: General scheme highlighting typical methods for the construction of ADCs based on the engineering of antibodies. The engineering methods highlighted consist of: THIOMAB cysteine engineering followed be alkylation; unnatural amino acid incorporation followed by click ligation; Glutamine-tag (Q-tag) insertion followed by transglutaminase functionalisation; and use of formylglycine-generating enzyme to generate an aldehyde followed by hydrozino-*iso*-Pictet-Spengler functionalisation.

Figure 3: General scheme highlighting typical methods for the construction of ADCs based on "off-the-shelf" antibodies. The methods highlighted consist of: native glycan targeting to yield aldehyde-modified mAb by oxidation followed by reductive amination or *O*-substituted oxime functionalisation; and disulfide reduction followed by either cysteine alkylation using maleimide or functional disulfide re-bridging.

Table 1: A summary of the advantages and limitations of the site-selective methods described.

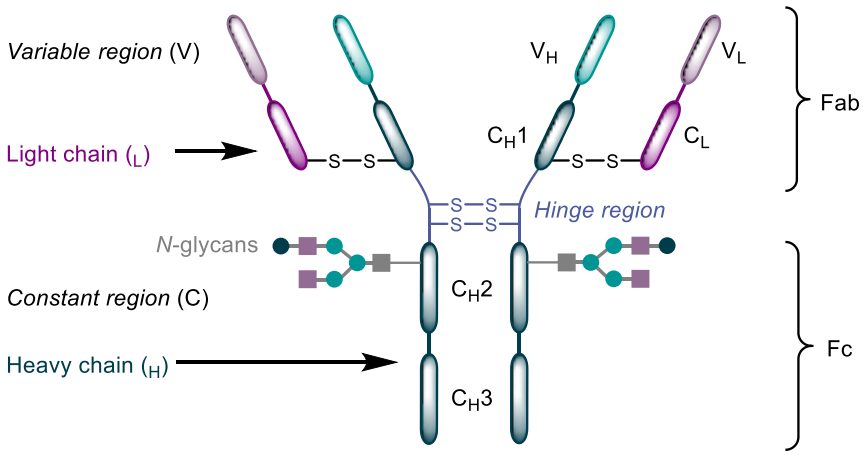

Figure 1

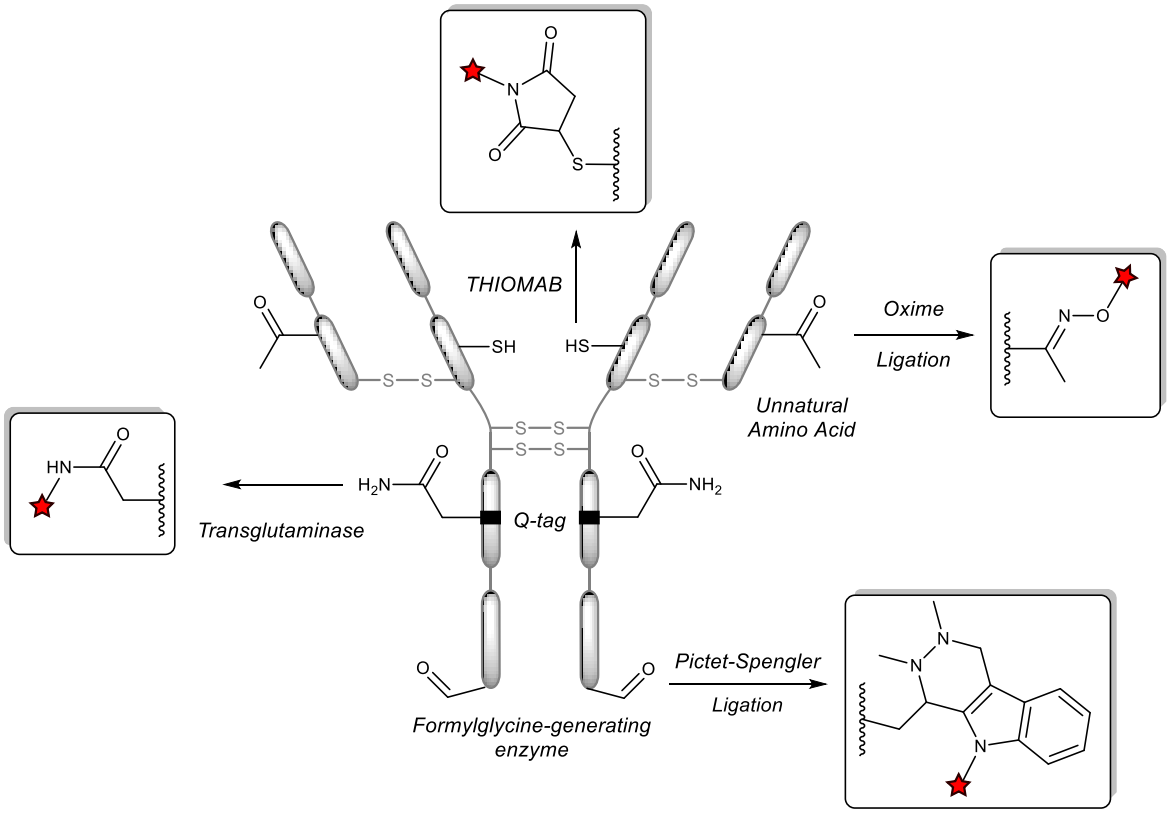

Figure 2

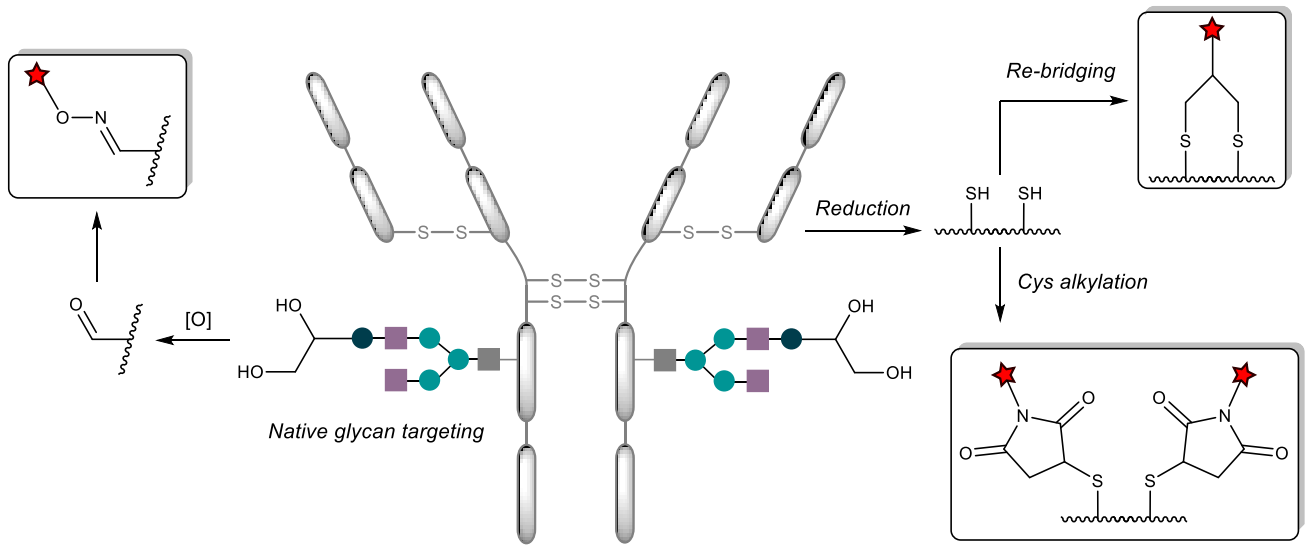

Figure 3

		Advantages	Limitations	Drug-to-Antibody Ratio
Engineered mAbs	THIOMAB (engineered thio-antibody)	Homogeneity, ease of screening	Incorporation of non-orthogonal group leads to issues of ADC production	2
	Enzyme directed	Ease of incorporation, homogeneity	Efficiency is site and antibody-dependent	2
	Unnatural Amino Acid (UAA)	Chemoselectivity, homogeneity	Technically challenging, potential immunogenicity issues	2
Native mAbs	Conjugation of cysteines obtained from native disulfide reduction	Ease of preparation, tuneable average drug-to-antibody ratio	Statistical mixture of products	0, 2, 4, 6, 8
	Functional re-bridging of native disulfides	Homogeneity, no loss of covalent linkage between protein chains	Potential disulfide scrambling	4
	Glycan modification	Ease of preparation, enzymatically controlled selectivity	Heterogeneous glycan population, reduced FcRn binding due to undesired oxidation	2, 4

Table 1