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Protein Bioconjugation

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Cysteine-Selective Reactions for Antibody Conjugation**

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In memory of Carlos Barbas III

antibodies · bioorganic chemistry · conjugation · drug delivery · maleimides

The unique targeting ability of antibodies has triggered burgeoning interest in the attachment of potent cytotoxic drugs onto these biomolecules to create antibody–drug conjugates (ADCs) which are able to spare healthy tissue by releasing its cargo only upon specific cancer-cell antigen recognition.[1] Despite their conceptual simplicity, the individual components of ADCs must have specific/precise properties to elicit therapeutic benefit. The antibody should have high affinity and specificity for the defined and abundant antigens in tumours and its pharmacokinetic properties should be unaffected upon conjugation with the drug. The cytotoxic molecule should be highly potent, thereby minimizing the number of payload molecules necessary to induce effective cell death. Finally, the conjugation strategy must permit chemical installation of the drug onto the antibody at a pre-determined site(s), and ensure stability of the conjugate whilst in circulation in vivo. Efforts in this field have led to the recent approval of two ADCs as drugs: Adcetris (brentuximab–vedotin), for the treatment of refractory Hodgkin lymphoma and anaplastic large-cell lymphoma, and Kadcyla (trastuzumab–emtansine), for the treatment of metastatic Her2+ breast cancer.[2]

A key factor in the design of therapeutically useful ADCs is the ability to create chemically defined, stable protein–drug conjugates. Early attempts to conjugate antibodies and cytotoxic molecules relied on the reactivity of abundant solvent-accessible lysine residues and cysteine residues reactin rapidly with *N*-hydroxysuccinimide esters (e.g., brentuximab–vedotin) and maleimide reagents (e.g., trastuzumab–emtansine), respectively. However, such modifications often result in heterogeneous drug-to-antibody ratio mixtures and conjugation site occupancy, and potentially, different pharmacokinetic and therapeutic properties.[3] These problems fuelled the development and use of robust chemical site-selective modification strategies to prepare homogeneous conjugates with improved properties. For instance, a chemically defined ADC prepared through oxime formation between ketones on the side chain of a non-native amino acid, and an hydroxylamine functionalized drug which bears a noncleavable linker was shown to display enhanced pharmacokinetic stability and improved in vitro and in vivo efficacy relative to an ADC equipped with a cleavable linker and an ADC prepared by means of conventional conjugation chemistry.[4] The aim of this highlight is to discuss new methods for site-selective bioconjugation at native or engineered cysteines, methods which may be used to build homogeneous and stable ADCs. The field of ADCs has recently been described in great detail in an excellent review.[1]

To address the homogeneity problems of protein conjugates, efforts to chemically target native or engineered cysteines, and explore the low abundance of the residue and the unique nucleophilicity of its thiol side chain have been developed. In recent years, different reactions to modify cysteine residues have been developed.[5] Noteworthy examples of such strategies involve the 1) reaction of the sulfhydryl side chain of cysteine with α-halocarbonyl compounds, perfluoroaromatic molecules (A; Figure 1),[6] and monobromomaleimides (B);[7] 2) reaction with 2-cyanobenzothiazole and Julia–Kocieński-like reagents, such as a phenyloxadiazole sulfone derivative (C);[8] 3) conversion into dehydroalanine followed by reaction to form thiol Michael-addition adducts (D)[9] or free-radical thiol-ene coupled products (E);[10] and 4) reaction with well-known Michael acceptors such as vinyl sulfones or maleimides (F),[5b] and allenamides (G).[11] Of these examples, maleimides have been the most commonly used scaffold to link different payloads to antibodies.[12] This strategy was used to prepare brentuximab–vedotin and to conjugate a number of different molecules (e.g., proteins, radionuclide chelators, fluorescent labels, and others) to antibodies. Mixed disulfides have also been ex-

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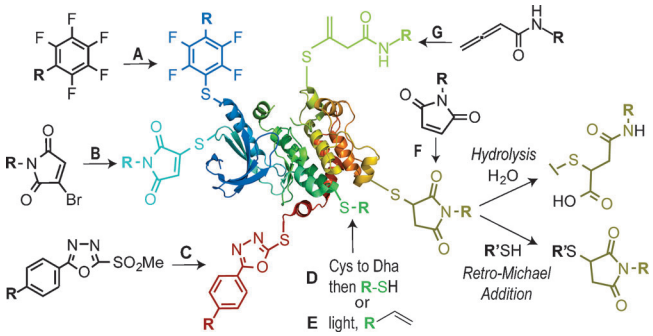

Figure 1. New cysteine-selective bioconjugation reactions and the degradation pathways of maleimide.

plored for building ADCs. For instance, Neri and co-workers modified antibody fragments directly at cysteine with thiol-containing drugs to generate traceless, chemically defined, disulfide-linked conjugates.[13]

The reaction between the sulfhydryl side chain of cysteine and maleimide derivatives proceeds quickly in aqueous media and with high levels of selectivity. However, the resulting succinimide thioethers can undergo either hydrolysis or exchange with other thiols, through a retro-Michael reaction, to yield heterogeneous conjugate mixtures in vivo, and thus alters the therapeutic efficacy and leads to systemic release and potential toxicity.[14] These limitations prompted investigations into the development and use of methods which would yield thioethers at cysteine rapidly and efficiently, and products which would remain stable in human plasma. For instance, a bis(sulfone) reagent which selectively alkylates two free cysteines derived from a native disulfide has been described. This allows the covalent re-bridging of the disulfide bond and the antibody remains structurally intact. Conjugates prepared by using the bis(sulfone) reagent remained largely stable after a 96 hour incubation in both rat and human serum.[15]

One example of a cysteine bioconjugation reaction which leads to a stable thioether conjugate was recently disclosed by Barbas and co-workers.[8] By building on the fact that methyl sulfonyl benzothiazole acts as a selective thiol-blocking reagent,[16] a series of methylsulfone derivatives were synthesized and their reactivity towards a cysteine-protected amino acid studied. Among these, the phenyloxadiazole sulfone **1** (Figure 2) was found to react very rapidly (< 5 min) to yield the desired thioether conjugate in quantitative yield.[8] Importantly, **1** did not react with other nucleophiles present in proteins and the resulting thioether conjugate was shown to be highly stable in both acid and basic conditions, as well as in the presence of glutathione. The sulfone **1** was also shown to be amenable to synthetic manipulation and the modified sulfone **2** allowed the site-selective conjugation of fluorophores and polyethylene glycol (PEG) chains at cysteine-tagged proteins, including recombinant human serum albumin and a maltose-binding protein, MBP-C-HA, which has a cysteine residue linking MBP to an influenza hemagglutinin (HA) peptide tag (Figure 2). Conjugation proceeded with complete conversion at room temperature in aqueous conditions with as few as 10 equivalents of the sulfone reagent. The resulting thioether-linked MBP-C-HA conjugate **4** displayed enhanced stability in human plasma ($t_{1/2}$ of 117 h) relative to the counterpart conjugate prepared by using maleimide chemistry ($t_{1/2}$ of 59.9 h). In fact, and unlike the maleimide conjugate, no fluorescence exchange with plasma proteins was observed with **4**. Although this new class of sulfones, as well as of a number of recently disclosed chemoselective cysteine bioconjugation reactions (Figure 1), have not been applied to antibodies, they hold great promise as an alternative to current maleimide-based technology for the construction of more stable, homogenous, and therapeutically useful conjugates.

Since the discovery of maleimide, iodoacetamide, and Michael-acceptor-based reagents, a number of new reactions have been developed for the selective and controlled modification of proteins at native or engineered cysteines. These new reactions and methods, together with advances in our ability to determine drug loading distribution in vivo[17] and to study the conformation and dynamics of conjugates in solution,[18] have the potential to drive the design and construction of new chemically defined protein conjugates for therapeutic use with, hopefully, improved efficacy and pharmacokinetic properties.

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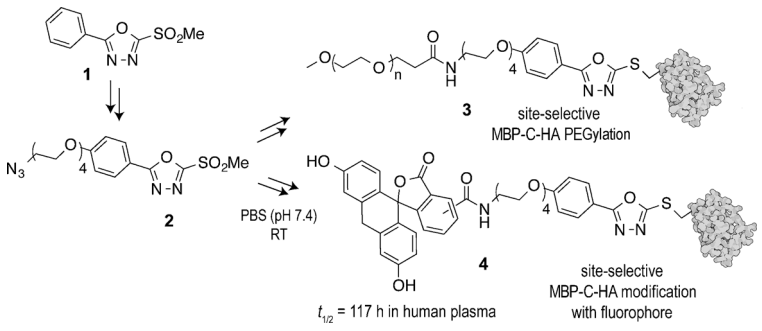

Figure 2. Chemical site-selective modification of MBP-C-HA with phenyloxadiazole derivatives equipped with PEG chains and fluorophores. PBS = phosphate-buffered saline.

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