Chapter 3
Stable and Homogeneous Drug Conjugation by Sequential Bis-Alkylation at Disulphide Bonds Using Bis-Sulphone Reagents

Martin Pabst, Matthew Bird, Mark Frigerio, and Antony Godwin

Abstract Antibody drug conjugates (ADCs) have begun to have a tremendous impact on the treatment of cancer and other pathological conditions. A current limitation in ADC development is that much effort and time is needed to fully optimise the combination of antibody, linker and drug. New linker strategies are required to ensure that more homogeneous and stable ADCs can be produced with more predictable in vivo behaviour without the need for extensive re-optimisation, especially if one component of the ADC is changed. In order to improve both the homogeneity and the stability of ADCs, we have developed linkers that allow site-specific drug conjugation based on bis-sulphones that covalently re-bridge reduced disulphide bonds. The bis-sulphone reagents comprise a drug, a linker and a bis-reactive conjugating moiety that is capable of undergoing reaction with both sulphur atoms derived from a reduced disulphide bond in antibodies and antibody fragments. We have demonstrated that the bis-sulphone-derived conjugates retain antigen-binding, are stable in serum and exhibit potent and antigen-selective cell killing in both in vitro and in vivo cancer models. Disulphide re-bridging conjugation is a general approach to prepare stable ADCs, which does not require the antibody to be recombinantly re-engineered for site-specific conjugation. The bis-sulphone linker-platform is being developed by Abzena plc under the trade name ThioBridge™.

Keywords Antibody drug conjugates • Disulphide re-bridging • Bis-alkylation • Monoclonal antibodies • Antibody fragments • PET imaging • In vivo efficacy

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3.1 Introduction

Structural heterogeneity has been a common feature of the antibody drug conjugates (ADCs) that have progressed to clinical evaluation and of the few ADCs that have so far obtained marketing authorisation (Sochaj et al. 2015; Panowski et al. 2014). While ADCs are beginning to have a tremendous impact on the treatment of cancer in particular, their therapeutic window remains persistently narrow. Developers of new ADCs face a complex array of interrelated considerations to produce an optimal medicine. Arguably, many of the issues are still to be fully elucidated and understood and in vivo behaviour remains hard to predict from in vitro studies. Considerable effort in the field by pharma and biotech companies is leading to increased understanding of how the drug conjugation site, drug loading and conjugate stability impact both efficacy and tolerability (Strop et al. 2013, 2015; Junutula et al. 2008; Shen et al. 2012; Senter 2009). One way this has been achieved is through a focus on site-specific conjugation methods to prepare ADCs that are sufficiently homogeneous to elucidate structure–activity relationships. ADCs with more defined drug loadings are also now reaching the clinic, which may ultimately lead to a stricter regulatory environment for the homogeneity of future ADCs (Mack et al. 2014) as the state-of-the-art evolves. Site-specific conjugation methods are also generally efficient in the use of reagents and generate high yields of desirable product(s). Manufacturability may also be enhanced by choosing a site-specific conjugation method, as reagent requirements are lower and reaction conditions may be more robust, thus limiting batch-to-batch variability.

A considerable number of new approaches are currently being developed in attempts to achieve site-specific and homogeneous conjugation, including re-engineered antibodies to introduce natural or non-natural amino acids specifically for conjugation (Tian et al. 2014; Hallam et al. 2015; Hallam and Smider 2014; Zimmerman et al. 2014; Axup et al. 2012; Behrens and Liu 2014; Sochaj et al. 2015; Panowksi et al. 2014). Chemo-enzymatic conjugation is another promising approach, for example using glycotransferases or transglutaminases (Dennler et al. 2014; Jeger et al. 2010). The reader is referred to several recent reviews on linker chemistries describing the many approaches in detail (Sievers and Senter 2013; Beck 2014; Chari et al. 2014; Behrens and Liu 2014; Flemming 2014; Panowksi et al. 2014; Agarwal and Bertozzi 2015).

The overwhelming majority of ADCs currently in clinical development are based on IgG1 isotype whole antibodies, with few ADCs being of IgG2 and IgG4 isotypes (Trail 2013; www.ClinicalTrials.gov). Seattle Genetics pioneered the use of maleimide linker-based reagents to prepare ADCs via reaction at the cysteine thiols generated from reduced inter-chain disulphide bonds of IgG1 antibodies (Wahl et al. 2001; Francisco et al. 2003; Willner et al. 1993). There are many examples in the clinic of this mono-alkylation approach, and Adcetris® (brentuximab vedotin) has been on the market since 2011 for the treatment of relapsed or refractory Hodgkin's lymphoma and anaplastic large cell lymphoma (Younes et al. 2010).

In humans, there are four subclasses of IgG known to exist; IgG1, IgG2, IgG3 and IgG4 where each of these subclasses can show further variations according to its allotype (Vidarsson et al. 2014; Liu and May 2012). All IgG molecules are made up of two heavy chains of approximately 50 kDa and two light chains of approximately 25 kDa. The intra-chain disulphide bonding in IgG molecules is fixed between the four subclasses at 12 per molecule (four per heavy chain and two per light chain). Intra-chain disulphide bonds are buried between two layers of β-sheets, and consequently they are not solvent exposed and are unavailable for conjugation. In IgG antibodies, the two heavy and two light chains are covalently linked via inter-chain disulphide bonds. Each light chain is linked to a separate heavy chain through a single disulphide bridge. Disulphide bridges also link the heavy chains in the hinge region with the total number varying between the four subclasses or allotypes, respectively. IgG1 and IgG4 have only two inter-heavy chain disulphide bonds, whereas IgG2 has four and IgG3 eleven (Liu and May 2012). The positioning of the inter-heavy chain disulphide bonds in the hinge region and the inter-light–heavy chain disulphides near the C-terminus of the light chain means that these disulphides are highly solvent exposed and therefore are available for chemical modification.

Thiol-selective conjugation is generally extremely efficient, typically requiring stoichiometric or slight excesses of reagents, and is also very fast under conditions (i.e. pH and temperature) that do not denature the antibody. The cysteine thiol, with a pK_a of ca. 8.3, is the most nucleophilic amino acid side chain, thus affording site-selectivity in conjugation with appropriate reagents (Wong 1991).

The susceptibility of the inter-chain disulphide bonds in IgG1 antibodies to chemical reduction is well-established, and some studies have indicated that this may vary depending upon bond location. In the study of Liu et al. (2010), for example, inter-light chain and heavy chain disulphide bonds were the most susceptible to reduction followed by the upper inter heavy chain disulphide. The least susceptible was the lower inter-heavy chain disulphide. Reduction of the intra-chain disulphide bonds required the presence of a denaturant (guanidine hydrochloride) and were not reduced under native conditions.

In practise, achieving completely selective reduction is simply not feasible. Partial reduction of the four inter-chain disulphide bonds for maleimide-based conjugation in IgG1 therefore produces a heterogeneous population of antibody species with varying reduction states (Sun et al. 2005) and distributions of antibody molecules with differing numbers of free thiols ranging from 0–8 are typically produced. Conjugation at these reduced cysteines gives rise to varying DAR species which possess different biological and physicochemical properties. Highly drug-loaded species (e.g. DAR 8) have been shown to clear from circulation quickly and display increased toxicity (Hamblett et al. 2004a, b). Species with low drug loads, including unconjugated mAb, may not deliver enough drug to the tumour and may act competitively with higher drug loaded species to reduce efficacy.

Where partial reduction is used, all mono-thiol (mono-alkylation) conjugation linkers, e.g. maleimide, haloacetamide and vinyl sulphone, result in loss of disulphide bonds in the final ADC and therefore may leave the antibody susceptible to fragmentation in vivo. Cysteine disulphides in proteins are formed during an

early stage in post-translational protein modification and stabilise the protein structure by making the folded conformation thermodynamically favourable over the unfolded form (Fass 2012; Narayan 2012; Trivedi et al. 2009; Wedemeyer et al. 2000; Darby 1997; Darby and Creighton 1995; Betz 1993). Furthermore, disulphide bonds afford protection from oxidants and proteolytic enzymes, preventing protein damage and potentially increasing in vivo half-life (Hogg 2003). The integrity of disulphide bonds is therefore important.

A limitation of mono-thiol conjugation is that antibodies do not naturally have single free cysteines available for conjugation. To address the issues of heterogeneity and stability with monoalkylation at reduced disulphides, antibodies with few engineered cysteine residues have been developed, often referred to as THIOMABs. Typically, two cysteine residues are engineered into the antibody's amino acid sequence to enable production of homogenous ADCs with a DAR of 2 (Behrens and Liu 2014; Sochaj et al. 2015; Panowksi et al. 2014).

Our conjugation approach is to utilise the natural cysteines available in IgG antibodies by using a bis-sulphone linker, which is capable of 're-bridging' disulphide bonds without disrupting antibody function and at the same time enhancing disulphide and ADC stability. With full inter-chain disulphide reduction, homogenous DAR 4 ADCs can be produced efficiently from IgG1 antibodies. In addition, lower DAR variants can be produced by partial reduction. As protein re-engineering is not required to optimise the ADC, development time spent on optimising the site of conjugation and producing new antibody variants can be minimised. Our efforts have been directed towards examining bis-sulphone-based alkylation as a site-directed linker-platform for developing the next generation of ADCs.

3.2 Bis-Sulphone Reagents for Disulphide Bridging Conjugation

Bis-alkylation conjugation is the reaction of a single conjugation reagent at two different reactive sites of the same protein or peptide. Bis-alkylation linkers based on bis-sulphone ($R\text{-}S(=O)_2\text{-}R'$) functionality were first demonstrated by Lawton et al. for cross-linking antibodies and conjugating fluorescent and radio labels (del Rosario et al. 1990; Liberatore et al. 1990). Mechanistically, the bis-sulphone reagents require β-elimination of a sulfinate anion to form a reactive α,-β-unsaturated ketone, which acts as a Michael acceptor in reaction with Michael donors, such as the free thiols liberated following disulphide reduction (Fig. 3.1). The β-elimination reaction is base mediated and occurs readily in solution at neutral pH so that activation to the reactive form can occur in situ with a disulphide-reduced protein. Conjugation of the α,β-unsaturated enone is believed to proceed via a sequence of Michael addition and elimination reactions and ultimately results in the formation of a conjugate characterised by two thioether bonds linked via a three-carbon bridge. There is no bis-sulphone functionality in the final conjugate as the bis-sulphone groups are eliminated from the linker, acting as protecting groups for the latent Michael acceptor functionality.

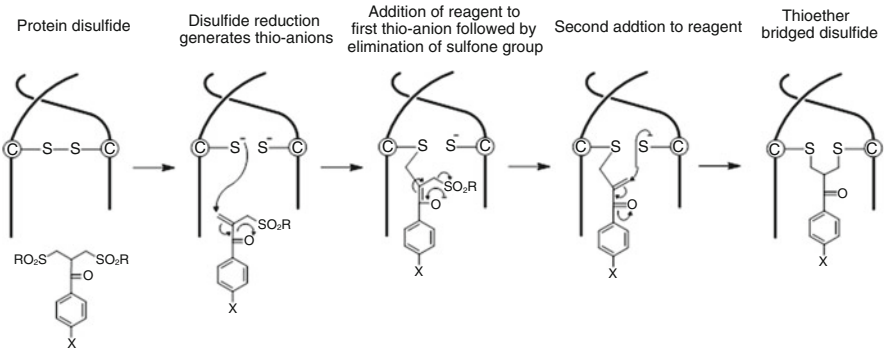

Fig. 3.1 General reaction scheme of the sequential bis-alkylation reaction of a bis-sulphone linker

We further developed the bis-sulphone approach for PEGylation at accessible disulphide bonds to extend in vivo half-lives, demonstrating the wide applicability to all major classes of therapeutic proteins and peptides that possess one to many disulphide bonds (Brocchini et al. 2006, 2008; Shaunak et al. 2006; Balan et al. 2007; Choi et al. 2009; Khalili et al. 2012, 2013). For PEGylation, the PEG component is attached directly to the bis-sulphone linker.

More recently, we have developed bis-alkylation via bis-sulphones for the generation of ADCs that are more homogeneous and stable than mono-alkylation ADCs. Bis-alkylation reagents have been used to produce whole antibody and fragment drug conjugates (Badescu et al. 2014; Bryant et al. 2015). The main features of bis-sulphone-based bis-alkylation for producing ADCs are narrow DAR distributions of DAR 4 that can be produced without re-engineering the antibody for conjugation and that the two thioether to antibody bonds together have increased stability compared to the thioether bonding of maleimide-based conjugation.

With disulphide-bridging conjugation now established, other groups have developed reagents that are capable of undergoing reaction with two thiols to introduce molecular bridges across disulphides. For example, bis-functionalised maleimides have been developed by Baker and co-workers (Maruani et al. 2015; Marculescu et al. 2014; Schumacher et al. 2011, 2013, 2014; Bryden et al. 2014; Castaneda et al. 2013; Jones et al. 2012; Chudasama et al. 2011; Ryan et al. 2011; Smith et al. 2010), while Concortis Biosystems have documented disulphide bridging linkers for the insertion of 2–5 atom bridges (Miao et al. 2013). Unlike bis-sulphone-based reagents, these other strategies do not work by sequential addition reactions. Thiol exchange reactions of bis-functionalised maleimides may be useful for in vivo release of a conjugated protein or peptide (Smith et al. 2010; Ryan et al. 2011; Chudasama et al. 2011).

Disulphide rebridging conjugation requires two steps performed in sequence (1) disulphide reduction within the antibody to liberate free cysteine thiols for conjugation and (2) incubation of the reduced antibody with the drug containing reagent (Fig. 3.2).

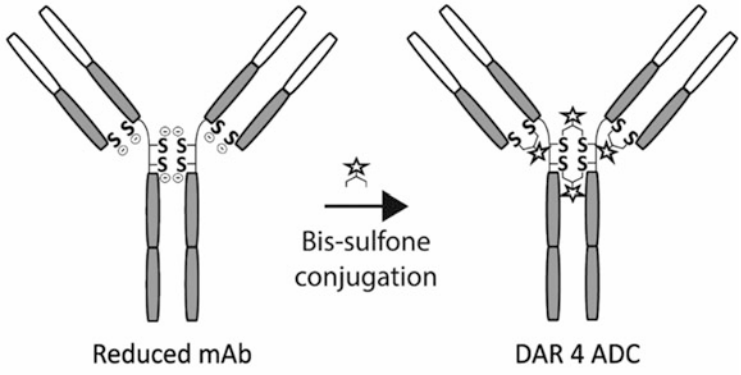

Fig. 3.2 Conjugation of bis-sulphone ADC reagents to an IgG1 type antibody at the four interchain disulphides to give an ADC with a DAR of 4 as the major product

Reagents commonly used to reduce disulphides in antibodies are either thiol- or phosphine-based. Thiol-based reducing agents, such as mercaptoethanol and dithiothreitol (DTT), reduce disulphide bonds via a disulphide exchange mechanism and are typically used in stoichiometric excess to ensure efficient reduction. A buffer exchange step is then required prior to adding any Michael-addition-based conjugation reagent, including maleimide linkers, to avoid quenching of the reagent. Phosphine-based reducing agents such as tris(carboxyethyl)phosphine (TCEP) are more efficient reductants requiring only stoichiometric amounts or a slight excess to be used and may also have catalytic activities under some conditions (Cline et al. 2004). Consequently, reduction can be performed without a subsequent buffer exchange step. Phosphines are also effective reductants over a wide range of pH range (e.g. pH 1.5–8.5) (Han & Han 1994) which may be useful in optimising the conjugation. Where full antibody reduction is required, we have found that 6 equivalents of TCEP to antibody (1.5 to 1 equivalents to disulphide) is effective at 40 °C over 1 h using an antibody concentration of 5 mg/mL at pH 7.5. Partial reduction can be achieved by lowering the stoichiometry of reducing agent to mAb (Sun et al. 2005).

As with other thiol-based conjugation chemistries, bis-sulphone conjugations can be performed under mild conditions of pH (e.g. 7.5) and at ambient temperatures to afford high to full conversions to conjugated product (ADC) within 4–24 h. Reagent usage is also highly efficient with only slight excesses of reagents being required for optimal conversion, e.g. 1.3–1.6 equivalents of reagent per reduced disulphide (Badescu et al. 2014; Bryant et al. 2015).

While the rate of β-elimination and Michael addition increases as reaction buffer basicity increases, a neutral pH is generally suitable for conjugation, without the need for denaturants. These conditions are also typical for maleimide conjugation. The bis-sulphone linkers are very stable and are not readily susceptible to hydrolysis on storage or during conjugation. Under these conditions, the linker is also very tolerant of many buffer additives such as metal ion chelators (e.g. EDTA) and surfactants (e.g. Tween-20 and Triton-X100).

Cytotoxic payloads tend to be hydrophobic and are often insoluble in aqueous solutions. Incorporation of a relatively short poly(ethylene glycol) (PEG) of discrete mass helps to solubilise the reagent and minimise the need for organic solvents in the conjugation. Having PEG in the final ADC also lowers the propensity of ADCs to aggregate, which can be an issue when very hydrophobic payloads are used. When solvent is required, acetonitrile, dimethylsulfoxide (DMSO) and dimethyl formamide (DMF) have all been found to be compatible with bis-suflone conjugation. Typically, the reagent can be dissolved in pure organic solvent before addition to the reduced antibody in aqueous solution affording 5% (v/v) of organic solvent in the reaction mixture.

For ADCs prepared with non-site-specific conjugation chemistries such as amine conjugation or mono-alkylation of reduced native inter-chain disulphides, individual DAR species often account for less than 40% of the total DAR variants present in a reaction mixture (Sun et al. 2005; Behrens and Liu 2014). While separation of individual DAR variants can be achieved by chromatography, such purification steps are usually avoided at production scale, presumably to minimise production costs and maximise yields. Purification is typically limited to a diafiltration process such as tangential flow filtration (TFF) to primarily remove unreacted reagent, but leaving a mixed population of DAR variants (EMA Assessment report: Adcetris 2012; Rohrer 2012) However, for site-specific conjugation methods that afford reaction mixtures rich in desirable DAR variant or variants, chromatography may become attractive as a fractionation step to remove unconjugated antibody, undesirable DAR variants and unreacted reagent that may otherwise negatively impact the toxicological properties of the ADC. For very hydrophobic drugs, removal of high DAR species may also be beneficial for improving aggregation and other physicochemical related properties. Hydrophobic interaction chromatography (HIC) is commonly used to determine DAR distribution and is scalable for purification purpose with very high recoveries possible. Synthon's anti-*her2* ADC, SYD985, is purified by HIC to give a mixture containing >95% DAR 2 and DAR 4 species and has recently entered clinical evaluation (Dokter et al. 2014). Prior to purification, the trastuzumab duocarmycin conjugate which is prepared through maleimide-thiol chemistry has a typically wide DAR profile. HIC purification can be applied to ADCs prepared by bis-alkylation to give single DAR purities of >95% (Badescu et al. 2014; Bryant et al. 2015) and recoveries of >85% have been achieved at laboratory research scale. Overall process yields of >70% for HIC purified DAR 4 ADCs have been achieved in our laboratories at research scale, which is likely to increase with scale as losses during purification and isolation invariably decrease.

3.3 Reagent

The example bis-sulphone reagent shown in Fig. 3.3 consists of the bis-sulphone linker, a semi-telechelic PEG, a dipeptidyl self-immolative, valine-citrulline-*para*-aminobenzoyloxycarbonyl (val-cit-PAB), linker and the well-known tubulin inhibitor auristatin payload, monomethyl auristatin E. The bis-sulphone linker is

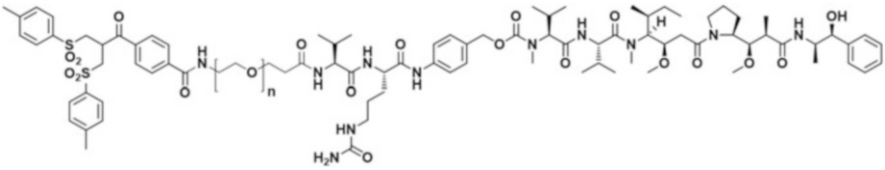

Fig. 3.3 Example bis-sulphone reagent consisting of (*left* to *right*) a bis-sulphone linker, a PEG unit to aid solubility, an enzymatically cleavable self-immolative linker (valine-citrulline-*p*-aminobenzyloxycarbonyl, *PAB*) and a cytotoxic payload (monomethyl auristatin E, *MMAE*). Reprinted with permission from Mol. Pharmaceutics 2015, 12, 1872–1879. Copyright 2015 American Chemical Society

routinely produced in very high purity (>98%) from benzoic acid acetophenone in a two-step reaction that has been successfully performed at kilogram scale. The bis-sulphone groups are protecting groups masking the unsaturated ketone functionality that can be derivatised at the carboxylic acid group, tolerating coupling of cleavable and non-cleavable linker payloads without activation to the mono-sulphone or undergoing hydrolysis. The linker payload (e.g. val-cit-PAB-MMAE in Fig. 3.3) can be added to a bis-sulphone-PEG-COOH precursor using the terminal carboxylic acid group of the PEG with standard amide coupling chemistry. Payloads and linkers with amino or hydroxyl functionality are particularly suited to this approach, allowing to prepare reagents with auristatin, maytansine, duocarmcyin and pyrrolobenzodiazepine (PBD) payloads for example. The final reagents can be purified by reverse phase chromatography using an acidified mobile phase and stored as solids until use.

3.4 ADC Characterisation

The multi-component complexity of ADCs require a range of analytical methods to provide sufficient information on the physicochemical properties and purity of the conjugates. ADC homogeneity can generally be assessed by determining the average drug-to-antibody ratio, the drug-to-antibody ratio distribution and finally conjugation site heterogeneity. Bis-sulphone produced ADCs are amenable to characterisation by HIC combined with UV detection for characterisation of the most important parameters of ADC homogeneity. As few DAR variants are generated, ample resolution can be achieved for both analytical and preparative scale chromatography. Figure 3.4c shows a typical crude conjugation mixture analysed by HIC for a conjugation process using a bis-sulphone reagent possessing a vc-PAB-MMAE cleavable linker/payload applied to an IgG1 monoclonal antibody after reduction of the four inter-chain disulphide bonds. No unconjugated antibody remains as there is full conversion to ADC. A very narrow DAR distribution is seen, with the major ADC product being the DAR 4 variant. DAR 4 loading is arguably a sweet spot for tubulin binding payloads, such as auristatins, offering a balance between a reduced circulation half-life compared to the naked antibody and efficacy (Hamblett 2004b). As well as the DAR 4 variant, there are

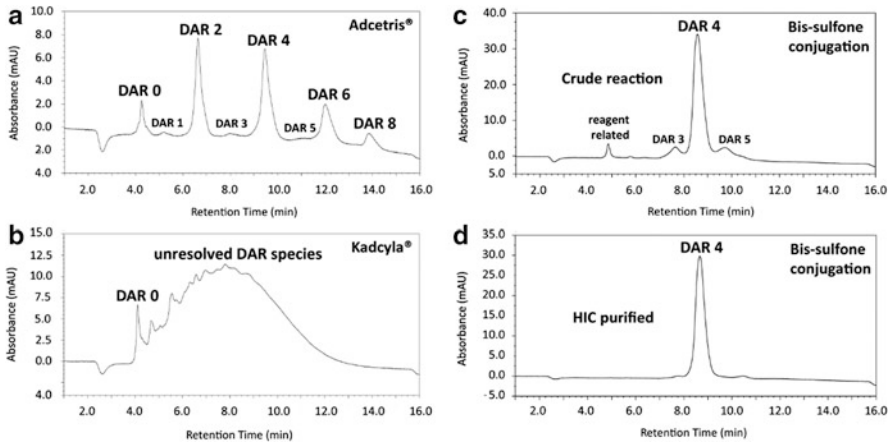

Fig. 3.4 HIC profiles for (**a**) Brentuximab vedotin (Adcetris®), (**b**) Trastuzumab emtansine (Kadcyla®), (**c**) example crude reaction mixture for a bis-sulphone val-cit-PAB-MMAE reagent conjugated to brentuximab to give a DAR 4 as the major product, (**d**) Bis-sulphone conjugated brentuximab-MMAE reaction mixture after purification by HIC

Table 3.1 Important physiochemical properties of ADCs and analytical methods usually used for bis-sulphone-based ADCs to address those parameters.

Analysis	Method(s)[a]
% Unconjugated antibody	HIC-UV
DAR distribution	HIC-UV
Average DAR	HIC-UV
% Unconjugated drug	LC-MS
% Monomeric antibody	SEC-UV
Intact mass	Protein MS (e.g.: TOF, Q-TOF)
Content determination	UV absorbance at 280 nm, Bradford or BCA assay

[a]*HIC-UV* Hydrophobic interaction chromatography with UV detection, *LC-MS* liquid chromatography - mass spectrometry, *SEC-UV* Size exclusion chromatography with UV detection

minor products of DAR 3 and DAR 5. DAR 5 is likely to be the result of a reduced disulphide reacting with two molecules of reagent rather than one, i.e. one reagent with each cysteine thiol before rebridging occurs. Since both DAR 3 and DAR 5 are very minor products and the average DAR is very close to 4 (e.g. 3.9), we have found these DAR variants have minimal impact on in vitro potency and they can be removed during purification using chromatography to give a homogeneous DAR 4 product. This is the approach we have taken to prepare ADCs for in vivo evaluation. Figure 3.4a shows a HIC profile for a typical maleimide mono-alkylation conjugation with an average DAR of about 4 and a broad DAR distribution between DAR 0 to DAR 8. Lysine conjugated antibodies typically do not allow a clear characterisation of the DAR homogeneity by HIC (as demonstrated by the analysis of Kadcyla in Fig. 3.4b) and require more sophisticated methods such as protein mass spectrometry for characterisation (Wakankar et al. 2011; Debaene et al. 2014; Marcoux et al. 2015). Table 3.1 lists the

analytical methods that are required for bis-sulphone-based ADCs to answer the most relevant physicochemical properties. For further reading regarding the bioanalytical considerations for ADCs and current industry practices, we refer the reader to Gorovits et al. (2013).

3.4.1 ADC Stability

ADC linkers must prevent the premature release of a potent cytotoxic drug in circulation to allow maximum delivery to the target cell to maximise efficacy and to limit off-tumour-related toxicity. For an ADC designed to liberate the cytotoxic within target cells, the ability of the linker to be cleaved effectively intracellularly is essential. We have shown both in vitro and in vivo that the thioether bonding to antibodies produced from the bis-sulphone reagents is stable in circulation and does not readily undergo retro-Michael addition reaction unlike the thio-ether bonds produced by maleimide linkers (Fig. 3.5). Furthermore, the serum stability of the bis thio-ether bonding does not appear to be species dependent. This intrinsic stability is a clear differentiator to mono-thiol alkylation where deconjugation and cross-conjugation to albumin are concerns (Alley et al. 2008). The excellent stability is also supportive of site-specificity of the conjugation to thiols, as thioether bonds would be expected to be more stable than bonding generated from other (amine) nucleophiles. As the covalent bonding to antibody is very stable, a second and potentially less stable (cleavable) linker is needed in the reagent where the release of free, underivatised payload is ensured. The majority of our exemplification work has focused on dipeptide-PAB cleavable linkers such as valine-citrulline-PAB and valine-alanine-PAB, which are enzymatically hydrolyzed by

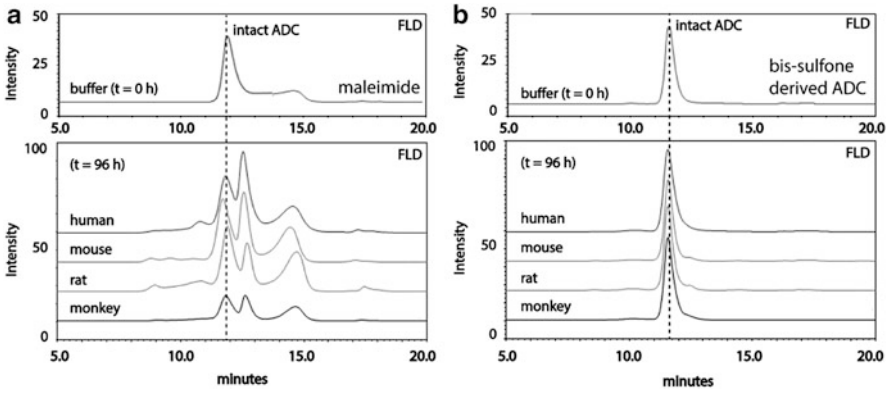

Fig. 3.5 Stability of conventional maleimide ADC (**a**) versus bis-sulphone-derived ADC (**b**) in different sera. ADCs were prepared with a fluorescent dye as a mock payload to allow analysis by size-exclusion chromatography. The bis-sulphone-derived ADC showed excellent stability after 96 h human, mouse, rat and monkey sera. The maleimide ADC showed instability in all sera, with significant cross-conjugation to albumin (c. 12.5 min peak) and breakdown products due to unbridged disulphides (c. 14.5 min) and free payload (c. 17.4 min)

lysosomal proteases such as cathepsin B to release the cytotoxic cleanly in the lysosomes of the target cell. Dipeptidyl linkers are straight-forward to incorporate into bis-sulphone reagents and are compatible with a wide range of payloads. For cytotoxic payloads that retain potency if released from the ADC covalently conjugated with additional moieties, such as monomethyl auristatin F (MMAF), we have shown that direct conjugation to the bis-sulphone linker via a PEG spacer creating a non-cleavable (amide) linker also resulted in active ADCs in vitro and in vivo as described later in this chapter.

3.5 In Vitro Potency

ADCs are required to bind cancer cell antigens, internalise and subsequently show drug release to be effective. The majority of our ADC exemplification work has been completed with trastuzumab and brentuximab as antibodies as there are well-established in vitro and in vivo models, and our ADCs could be benchmarked against Kadcyla® and Adcetris®. Trastuzumab is a monoclonal antibody that binds *Her2* and is internalised. We studied the binding activity of bis-sulphone conjugated trastuzumab-MMAE conjugate by ELISA, following the binding of the ADC to the extracellular domain of *Her2* (Fig. 3.6), and demonstrated antigen-specific binding of the antibody was fully retained after conjugation. Internalisation was assessed using confocal microscopy by incubating a trastuzumab conjugate, prepared with fluorescein as a fluorescent mock payload, with SK-BR-3 cells, a high *Her2* expressing cell line. Visualisation of the cells showed that the prepared ADC had been internalised after incubation at 37 °C for 4 h as efficiently as the unconjugated antibody. No internalisation was observed in a non-*Her2* expressing control cell line (A549), as shown for the tested conjugate (Badescu et al. 2014).

ADC potency is commonly determined in vitro using anti-cell proliferation assays on cultured antigen-positive and antigen-negative cell lines. An example is

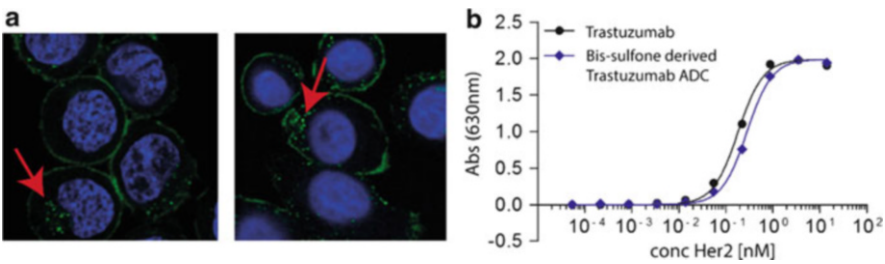

Fig. 3.6 (**a**, *Left image*) Internalisation of a bis-sulphone-derived trastuzumab-FITC conjugate into *Her2* positive SK-BR-3 cells. (**a**, *Right image*) Internalisation of trastuzumab, detection with an anti-human mAb conjugated to Alexa Fluor® 488. (**b**) *Her2* target binding, comparison of native trastuzumab with a bis-sulphone-derived trastuzumab-MMAE conjugate as determined by ELISA. Reprinted with permission from Bioconjug Chem 25 (6):1124–1136. Copyright 2015 American Chemical Society

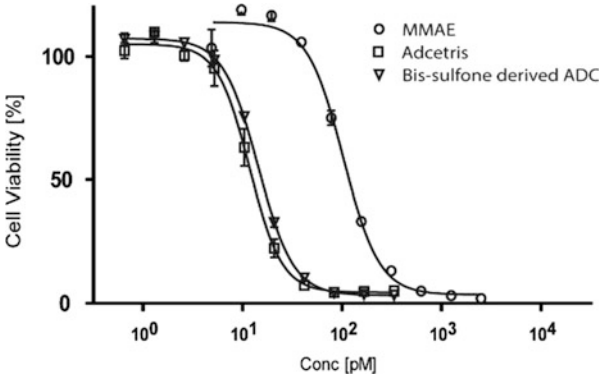

Fig. 3.7 In vitro cell viability assay in an antigen positive cell line (Karpas 299) for a bis-sulphone-derived brentuximab conjugate (DAR 4), a maleimide-derived brentuximab MMAE conjugate (average DAR 4, Adcetris®) and MMAE. Both conjugates showed a comparable in vitro potency, superior to unconjugated MMAE

the CellTiter-Glo® Luminescent Cell Viability Assay which is based on measuring a luminescent response proportional to the amount of ATP present within the cell. MMAE, for example, inhibits cell division by blocking tubulin polymerisation, leading to a decrease in ATP levels. While in vitro potency is a useful readout to confirm antigen-selective cell killing (Senter 2009), ADCs with the same cytotoxic payload and antibody but different linker chemistry or site-of conjugation may exhibit similar activities. In vivo xenograft studies on the other hand can lead to discrimination between ADCs with equivalent drug loading due to factors such as stability, PK and tissue distribution. Figure 3.7 shows an anti-proliferation assay in an antigen positive cell line (Karpas 299) for bis-sulphone- and malemide-derived brentuximab conjugates, all prepared with val-cit-PAB-MMAE. The bis-sulphone conjugated DAR 4 ADC was found to have potent anti-proliferative activity in the antigen positive cell line with an IC50 value comparable to the marketed ADC product, Adcetris® [average DAR of 4 (Debaene et al. 2014)]. In an antigen negative cell line, the conjugates were found to decrease viability, but the potency was about three orders of magnitude lower than that of free drug, supporting antigen-selective delivery of the MMAE (data not shown).

Several ADCs under clinical evaluation employ the auristatin payload, monomethyl auristatin F, MMAF. MMAF unlike MMAE is highly potent as an ADC when used with non-cleavable maleimide linkers that ultimately releases an MMAF adduct after mAb digestion within lysosomes (Doronina et al. 2006; Hamblett et al. 2004a; Hamblett et al. 2004b). There is evidence to suggest that the drug released is cysteine derivatised, e.g. a cysteine-maleimidocaproyl-MMAF adduct. Since non-cleavable linker approaches rely on enzymatic degradation of the antibody for drug release, a concern for bis-alkylation-derived ADCs could be that the improved stability compared to mono-alkylation prevents or slows the rate of antibody breakdown, so that potency is reduced or eliminated. Using a bis-sulphone PEG(6)-amide-MMAF reagent to prepare a trastuzumab ADC, we have observed potency comparable to a maleimide PEG(6)-amide-MMAF ADC in SK-BR-3 cells indicating the antibody of the bis-sulphone-derived ADC was degraded and the released drug entity(ies) could escape the lysosomes and bind tubulin.

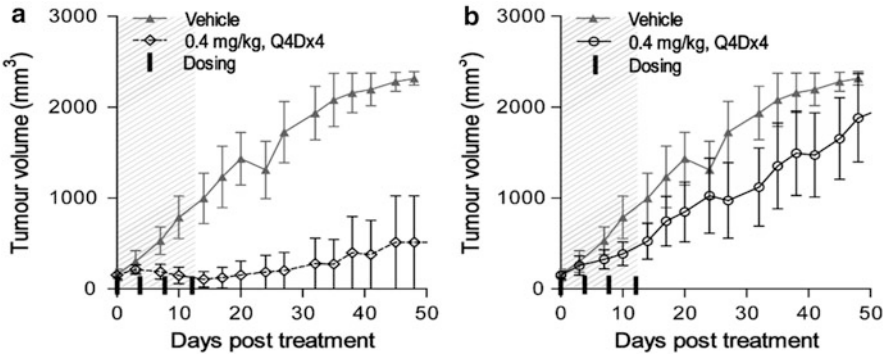

Fig. 3.8 In vivo efficacy in a Karpas 299 SCID mouse xenograft model. The bis-sulphone conjugated brentuximab-MMAE conjugate (*left*) showed better efficacy compared to the maleimide conjugated brentuximab-MMAE conjugate Adcetris® (*right*)

3.6 In Vivo Efficacy

Bis-sulphone-derived ADCs have shown promising results in murine xenograft models. Adcetris® and a DAR 4 bis-sulphone-derived brentuximab ADC were evaluated in a Karpas 299 xenograft model using a multiple dosing regimen of every fourth day for four doses (q4dx4). The brentuximab-MMAE conjugate was highly active against the Karpas 299 xenografts resulting in a significant delay in tumour growth which was superior to the response to the marketed maleimide conjugate (Fig. 3.8). A target non-binding comparator ADC had no effect on tumour volume as compared to the vehicle control group (data not shown). The ADC was also well tolerated with no significant change in body weight.

3.7 Antibody Fragments

The pre-clinical and clinical pipeline of antibody fragments continues to expand with a growing number under evaluation as fragment drug conjugates (FDCs). For all therapeutic indications, the hope is that fragments will offer differentiated performance compared to whole antibodies, primarily due to altered PK and tissue distribution as a consequence of their smaller size (Nelson 2012). For FDCs, there is the potential for faster penetration into solid tumours but also for faster clearance from systemic circulation. With ADCs, which are used in the clinic at, or close to their maximum tolerated dose, off-target toxicities and side effects are seemingly related to systemic exposure to the drug. It is hoped that high tumour-to-blood ratios and reduced systemic toxicity may be possible using FDCs to enhance the therapeutic index, a primary goal of drug conjugation. Additionally, as fragments lack an Fc domain, there is an absence of antibody-dependent cell-mediated cytotoxicty

(ADCC) or complement-dependent cytotoxicity (CDC). Clinical data is now beginning to emerge to support the hypothesis that FDCs can accumulate significantly in tumours (Powers et al. 2012), although it should be noted that is a dearth of data directly comparing whole antibodies and fragments in the same studies.

Fabs are arguably the most explored fragment format in the clinic and ideal for drug conjugation via bis-alkylation conjugation as they possess a single, solvent accessible inter-chain disulphide bond. ScFvs and domain antibodies are less clinically mature fragment formats but are also amenable to bis-alkylation conjugation through engineered disulphides. We have shown that conjugation at the inter-chain disulphide of a Fab is highly selective, leading to homogeneous conjugates and ideally situated distal to the binding domain. Therefore, conjugation would be expected to have a minimal impact on the binding properties of the Fab. Furthermore, the PK properties may be more easily tailored for a Fab or fragment by varying the length of a PEG component incorporated in the reagent to affect the overall size of the drug conjugate and retard clearance from circulation via glomerular filtration in the kidneys.

3.8 Fab Drug Conjugates

Conjugation of Fab fragments via bis-alkylation follows the same general process as conjugation of mAbs. Selective reduction of the Fab inter-chain disulphide bonds to liberate two free thiols can be achieved using DTT or TCEP followed by incubation with the bis-sulphone drug reagent. Under non-reducing conditions, a Fab will migrate as a single band at approximately 50 kDa, when analysed by SDS-PAGE. Under reducing conditions, Fabs generally migrate at what appears to be a single band at approximately 25 kDa. The change in molecular weight is a result of the reduction of the single inter-chain disulphide that covalently links the V_HC_{H1} and V_LC_L and their subsequent dissociation due to SDS present in the analysis. This migration difference between reduced and non-reduced Fab allows insight into the bis-alkylation conjugation, as the bis-sulphone reagents do not allow the heavy and light chains to dissociate in the presence of reductant once covalently conjugated across the inter-chain disulphide (Fig. 3.9). From an SDS-PAGE gel, the extent of bridging can be determined. The stability of the bis-thioether bonding is demonstrated by the fact the conjugates are resistant to thiol exchange reactions.

Several studies have shown the utility of bis-sulphone linkers to efficiently and site-specifically conjugate PEG to Fabs (Khalili et al. 2012, 2013). Surface plasmon resonance (SPR) experiments using Biacore were used to examine the comparative binding of PEGylated Fab constructs derived from clinically used monocloncal antibodes: bevacizumab, ranibizumab and trastuzumab. PEGylated Fabs displayed only a 2-fold reduction in apparent affinity without any change in the dissociation rate. Differences were found to occur in the association rates (ka) rather than the dissociation rates (kd) and were thought to be due to steric shielding effects of PEG.

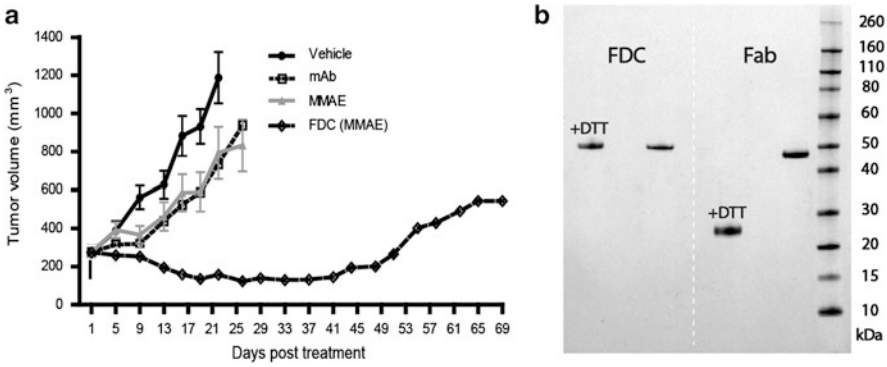

Fig. 3.9 (**a**) Evaluation of in vivo efficacy of a bis-sulphone-derived Fab MMAE conjugate (DAR 1) in a BT-474 SCID mouse xenograft model showing tumour regression and 5/10 tumour free survivors. (**b**) SDS-PAGE analysis of Fab and a bis-sulphone-derived Fab MMAE conjugate under reducing and non-reducing conditions. SDS-PAGE gel was stained with InstantBlue™. Reprinted with permission from Bioconjug Chem 25 (6):1124–1136. Copyright 2015 American Chemical Society

Interestingly, changing the molecular weight of the conjugated PEG did not appear to affect the apparent binding rates and affinities indicating that in an FDC context, PEG could be used to optimise pharmacokinetics (PK) and pharmacodynamics (PD).

We have prepared a vc-PAB-MMAE-based FDC from proteolytically digested trastuzumab. No conjugation was observed unless the Fab was first incubated with DTT. The FDC retained antigen specific binding with no loss of affinity to *Her2* compared to the unconjugated Fab, as measured by ELISA. In addition, the conjugate showed similar potency to unconjugated MMAE in *Her2* positive cell lines (SK-BR-3 and BT-474) and several fold lower potency on *Her2* negative cells (MCF-7 and A549). The FDC was also compared with unconjugated trastuzumab and MMAE in a BT-474 SCID mouse xenograft model, and a clear and promising tumour response was observed resulting in a marked delay in tumour growth (Fig. 3.9). Mice were treated with 20 mg/kg of the FDC with dosing on alternate days over 25 d. Control groups were treated with either 20 mg/kg trastuzumab (every fourth day), 0.3 mg/kg free MMAE (alternate days) or vehicle (alternate days). For the FDC and free MMAE dosed groups, each dose was equivalent with respect to the amount of MMAE administered. A median time to end point (TTE) of 66.3 days was obtained for the FDC, corresponding to a tumour growth delay (TGD) of 48.7 d. In contrast, trastuzumab (TTE 24.6 d) and free drug (TTE 21.8 d) each elicited only marginal TGDs of 7.0 d and 4.2 d, respectively, which did not translate to meaningful activity. The FDC was well tolerated and fluctuations in body weight were similar between groups. These results indicate that bis-sulphone-derived FDCs have a great deal of potential with respect to homogeneity and ease of production and characterisation, as well as flexibility to optimise a new therapeutic.

3.9 Imaging Applications

Another promising application of the disulphide bridging conjugation approach is in the production of antibody conjugates for imaging applications, where a radiolabel is carried by the antibody rather than a cytotoxic drug. We have worked with ImaginAb Inc. to develop desferrioxamine conjugates using their minibody antibody format. Using the bis-sulphone conjugation approach, we were able to prepare conjugates containing one copy of desferrioxamine per minibody using two bis-sulphone reagents with different sizes of PEG (6 and 36 repeat units). The conjugates were then radiolabeled with ^{89}Zr and their immunoreactivity was evaluated in mice after 48 h in comparison with a maleimide conjugate. For all the conjugates, a similar distribution of radioactivity was observed in all tissues except for the kidneys. Kidney uptake was significantly reduced for the bis-sulphone-derived conjugates, indicating that disulphide bridging stabilised the minibody and extended their circulation half-lives, with a slightly longer blood clearance observed with the 36- versus the 6-repeat unit PEG conjugate (Fig. 3.10).

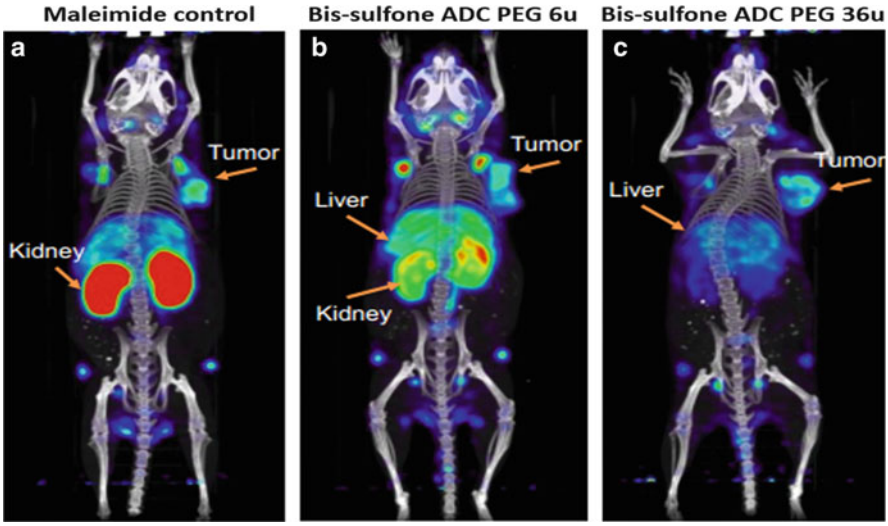

Fig. 3.10 PET images, provided courtesy of ImaginAb, Inc. U.S., showing SCID mice 48 h after administration with ^{89}Zr-labelled minibody conjugates prepared with (**a**) malemide–desferrioxamine reagent (**b**) bis-sulphone–desferrioxamine reagent containing a 6 repeat unit PEG (**c**) bis-sulphone–desferrioxamine reagent containing a 36 repeat unit (36u) PEG. Images show better tumour to kidney for the bis-sulphone conjugates and indicate tumour contrast can be modulated by changes in PEG size. Minibody with desferrioxamine and ^{89}Zr based on (*left*) maleimide; and (*middle*) bis-sulphone with a PEG 6u spacer, (*right*) bis-sulphone with a PEG 36u spacer

3.10 Conclusion

Considerable effort by the ADC community is now focused on developing approaches to control the site and number of drug molecules conjugated to the antibody. Any new conjugation approach ideally needs to be flexible in design to allow the systematic determination and optimisation of biological properties, ultimately producing ADCs with an improved therapeutic index. In our studies with bis-sulphone-based conjugation reagents, we have shown promising preclinical results demonstrating that whole antibodies and antibody fragments are well suited to disulphide re-bridging conjugation, affording well-defined and stable products. It is our hope that the promising qualities already observed for this versatile linker platform will enable bis-sulphone-derived ADCs to become part of the ever-expanding pipeline of ADCs reaching the clinic and ultimately help patients.

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