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# Next Generation Antibody Drug Conjugates (ADCs) and Immunotoxins

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Ulf Grawunder • Stefan Barth
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# Next Generation Antibody Drug Conjugates (ADCs) and Immunotoxins

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# Preface

The specific targeting of toxic substances to disease targets is a historic concept that was already developed by Paul Ehrlich more than 100 years ago, for which he coined the term "magic bullet" (*Zauberkugel*). A "magic bullet" should only destroy the target expressing disease entity, but leave target-negative cells unaffected. While Paul Ehrlich developed the "magic bullet" concept primarily in the area of infectious disease, this principle can also perfectly be applied to the field of oncology.

During the past two decades therapeutic antibodies have led to a revolution of targeted cancer therapy, with more than 30 therapeutic antibodies and antibody fragments, including major blockbusters like trastuzumab (Herceptin®) or rituximab (Mabthera®), being FDA and EMA approved. In the field of oncology, the "magic bullet" concept of Paul Ehrlich is pursued by attaching cellular toxins to cancer cell-specific antibodies or antibody fragments, in order to specifically deliver the toxic payload to the site of the tumor. In the broadest definition, these drugs belong to the class of immuno-conjugates, in which an active pharmaceutical ingredient (API), with a desired pharmacological effect, is attached to a targeting moiety based on an immune-targeting molecule, e.g., an antibody, a soluble receptor, or a ligand for a specific disease target. Immuno-conjugates comprised of an antibody or antibody binding domain coupled to a potent cellular toxin can be grouped into two classes: antibody drug conjugates (ADCs) and immunotoxins (ITs), and both types of molecules are subjects of intense research, both in preclinical and clinical research.

In ADCs, the toxic payload is a small molecular weight compound which is coupled or conjugated to (a) reactive site(s) of the antibody or antibody fragment by means of a linker, either by means of a chemical, or via an enzymatic reaction. In contrast, immunotoxins are comprised of an antibody or an antibody fragment (e.g., $F_{ab}$ or $scF_v$ fragment) coupled to a protein toxin.

While the concept of targeting a toxin to a cancer cell by means of a cancer cell-specific antibody is compelling, research during the last two decades has shown that the practical implementation of Paul Ehrlich's "magic bullet" principle in ADCs

and ITs is anything but trivial. This is due to the fact that both in ADCs and immunotoxins a multitude of functionalities need to be combined in a single, large biological drug molecule—if only a single functional aspect of these complex molecules is not optimal, this will become the limiting factor in determining the therapeutic index of the drug.

## The Complexity of ADCs and ITs

**Finding a Suitable Target** The task of developing an effective ADC or IT with promising therapeutic index already starts with the selection of the cancer target. If the target is not selectively expressed on tumor cells, even the most perfect ADC or IT design will not be able to rescue the therapeutic index of such a conjugate. This represents not only the first but also one of the most important challenges in immuno-conjugate research. In fact, it is believed that the "target space" addressable with highly potent ADCs and ITs may be limited to just a few dozen targets that based on tumor selective expression qualify for ADC or IT intervention strategies. Even for FDA-approved ADCs this remains a challenge. For instance, the HER2 antigen recognized by FDA-approved ADC Kadcyla® is also widely expressed at low levels on healthy cells or tissues and very unfavorably also on cardiomyocytes. HER2 expression on healthy cells represents an even higher risk for the ADC Kadcyla® than for the unconjugated FDA-approved therapeutic antibody Herceptin® (trastuzumab), for which a cardiotoxic effect is already documented. Therefore, the therapeutic index of an ADC or IT, in first instance, is very significantly influenced by the ratio of target expression on tumor cells versus target expression on healthy cells. This ratio should ideally be as high as possible. Thepen and Barth are summarizing the use of CD64 (Fc gamma receptor I) exclusively expressed on monocytes and macrophages for the development of recombinant immunotoxins. By selective destruction of M1-type macrophages, these agents might allow treatment of monocyte-derived leukemia as well as different chronic inflammatory diseases.

**Target Internalization** Once a target for ADC and IT strategies has been identified, the target needs to be able to internalize into the cancer cell, to ensure intracellular trafficking into endosomal and lysosomal compartments allowing the toxophore to be released inside the tumor cell. Not every target has a sufficient internalization rate allowing transport adequate amounts of the toxophore into the cancer cell to effect its destruction. Here is a clear advantage of immunotoxins versus antibody drug conjugates. The protein toxins fused to antibody fragments in immunotoxins (e.g., bacterial diphtheria toxin or pseudomonas exotoxin) act catalytically on their intracellular substrate, and they are often effective for cell killing, even if only a very small number of molecules are internalized and translocated to the cytosol. Therefore, ITs are better suited to address cancer targets that are expressed at low levels and/or with a potentially low internalization rate.

However, in some cases, target internalization is a function of the epitope that is bound by the targeting antibody or antibody fragment. There are many examples showing that antibodies binding different epitopes on a selected target can have a huge impact on the internalization rate for an ADC or IT.

Alternatively, and this has mostly been postulated in the ADC space, it is possible to design linkers in such a way that they are cleaved in the tumor microenvironment, without the need for target internalization, thereby effecting killing of the tumor cells and also neighboring supportive tumor stromal and vascular tissues that support tumor growth. However, the balance between effecting efficient release of a toxic payload at the site of the tumor, but not prematurely in circulation is quite a challenging task.

**Properties of the Targeting Moiety** In addition to identifying the right target, in terms of tumor selective expression and internalization capabilities, the targeting moiety itself needs to exhibit, what is known in the field as, favorable "developability" properties. This essentially boils down to assuring favorable physiologic properties of the ADC or IT upon systemic application of the drugs. In general terms, the drugs should have low immunogenicity and appropriate serum half-life or pharmacokinetic properties in vivo. Many years of research and development of therapeutic antibodies have led to a wealth of knowledge that nowadays strongly influence the design, the engineering, and the selection of a final antibody candidate as a basis for antibody-based targeted therapy. Antibodies selected for ADC and IT strategies will at least be humanized or developed outright as fully human antibodies. However, selected binders will probably additionally be subjected to the removal of additional T-cell epitopes to minimize their immunogenic potential in humans. At the same time, other sequence liabilities will be addressed with the aim to increase thermal and pH stability, to reduce propensity for aggregation, especially at high concentration. Eventually, all the optimization of the biophysical and physiological parameters of the antibody moiety needs to be balanced with the expression levels of the antibody in fermentation, so that it can also be manufactured at commercial scale with acceptable cost of goods (COGS). In other words, even if "Finding the right target" and "Target internalization" as described above are properly addressed, unfavorable properties of an antibody can be detrimental for the development of an effective ADC or IT.

**Selection of Toxin Payload** In addition to the selection of the right target and antibody for an ADC or IT, the choice of the toxophore as the active pharmaceutical ingredient (API) is as important. While in the field of recombinant ITs only a limited number of bacterial and plant toxins have been employed (e.g., pseudomonas exotoxin, diphtheria toxin, gelonin), the choice of toxophores for ADCs is significantly larger and more complex, because any high-potency API chemical structure can potentially be coupled to an ADC. However, for recombinant ITs, it is not only the choice of protein toxin, but also the variant thereof, which can extensively be engineered, in order to optimize its potency and to reduce its risk of immunogenicity. The chapter by Kreitmann et al. from the laboratory of Ira Pastan at NIH highlights advances in the development of recombinant ITs

demonstrating the activity of BL22 and its derivative moxetumomab pasudotox for the treatment of CD22-related B-cell disorders. In the field of ADCs, the majority of ADCs in clinical evaluation carry tubulin-inhibiting payloads of the maytansin or dolastatin/auristatin class, derived from the bark of an African shrub *Maytenus ovatus,* or a sea slug *Dolabella auricularia*, respectively. These toxins interfere with tubulin polymerization and therefore with the re-organization of the intracellular cytoskeleton, which is required for cell proliferation and cell motility (i.e., cancer cell invasiveness). Therefore, these drugs target primarily rapidly proliferating tumor cells. Alternatively, ADCs may comprise DNA damaging agents that can also lead to destruction of non-proliferating cells, by interfering with proper transcription of genes. The first ADC that had been FDA approved for the treatment of CD33-positive acute myeloid leukemia (AML), Mylotarg® (gemtuzumab ozogamicin), contained the DNA damaging toxin calicheamicin as a payload. The chapter by H.P. Gerber and P. Supra provides detailed background about the calicheamicin toxin platform for ADCs and describes the use of this payload for the development of anti-CD22 ADCs that are subject to clinical development activities at Pfizer. In addition to the calicheamycin DNA damaging toxin platform, most recently so-called pyrrolo-benzo-diazepines (PBDs) have entered into the arena of ADCs. The PBD toxin platform is nowadays employed by many ADC researchers for developing next-generation ADCs, both academically and commercially. John Hartley from UCL, one of the key opinion leaders in the PBD toxin field, has contributed a chapter describing the current state of the art in this field. However, other toxin platforms, either DNA damaging (duocarmycins, anthracyclines) or targeting other cellular pathways, e.g., alpha-amanitin binding to RNA polymerase II and III, are also being explored in newer generation ADCs.

**Linker Functionality** Another highly critical component of an ADC is the linker structure that covalently attaches the toxophore to the targeting moiety. On one hand this linker needs to ensure that premature release of the toxin (e.g. in circulation) is avoided. On the other hand, the linker should efficiently release the toxin upon binding to the cancer cells and/or upon internalization of the ADC/IT inside the cancer cell. First-generation ADCs, including the two FDA-approved ADC products Kadcyla® (trastuzumab-emtansine) for the treatment of HER2-positive breast and ovarian cancer and Adcetris® for CD30-positive Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL), use chemical linkers that are classified as "non-cleavable" or "cleavable," respectively, which couple the toxin randomly to either lysine or cysteine residues. The functional aspects of these traditional chemical linker approaches, but more importantly the state of the art of newer approaches for chemical conjugation, with respect to stability and site specificity, are outlined in the contributions by Pabst and colleagues from Abzena and Nanna and colleagues from the group of Christoph Rader from The Scripps Research Institute (TSRI).

Recombinant immunotoxins comprise the toxins as fusions to the antibody or antibody fragment. The link between the two fusion partners therefore is a normal peptide bond or a spacer peptide. In these drugs, it needs to be assured that the way

of fusion either does not interfere with the catalytic function of the protein toxin and/or that a specific release/translocation is effected by intracellular proteases.

Similarly, next-generation ADCs generated by site-specific conjugation using enzymatic approaches, as outlined in the chapter by Beerli and Grawunder, are characterized by the formation of stable peptide or amide bonds that require the action of specific or non-specific proteases, in order to optimally release the toxophore in cancer cells. Clearly, without an optimal linker technology, which confers the proper spatial and temporal release of the toxin from an ADC or immunotoxin, a favorable therapeutic index cannot be achieved.

**Conclusion** The field of ADC and immunotoxin development has entered into a new era. Novel "next-generation" ADCs and ITs employing different linker technologies, both chemical and enzymatic, different small molecular toxins, and engineered version of protein toxins, are increasingly entering into preclinical and clinical evaluation. All chapters in this book on "next-generation" ADCs and immunotoxins contributed by eminent researchers and key opinion leader in the field of ADC and IT development provide an outlook on exciting novel therapeutic principles and formats of immuno-conjugates that will hopefully soon be able to cure human disease in a safer and more effective manner.

Basel, Switzerland — Ulf Grawunder
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# Contents

# Chapter 1
# Chemical Assembly of Antibody-Drug Conjugates

**Alex R. Nanna, William R. Roush, and Christoph Rader**

**Abstract** Antibody-drug conjugates (ADCs) are a rapidly expanding class of pharmaceuticals for the treatment of cancer. Currently, two ADCs are FDA approved and over 60 are in clinical trials. Although many ADCs involve non-specific drug attachment via lysine and cysteine residues, the desire to produce homogeneous ADCs has led to the development of several site-specific chemical assembly strategies. The first site-specific ADCs are just beginning to enter clinical trials and are expected to have improved efficacy with reduced toxicity.

**Keywords** Antibody-drug conjugate • drug conjugation • site-specific • homogeneous • orthogonal reactivity • chemical conjugation

## 1.1 Introduction

Antibody-drug conjugates (ADCs) are emerging as a promising class of cancer therapeutics. The strategy of conjugating highly cytotoxic drugs to tumor-targeting antibodies has already been proven effective with two FDA-approved ADCs, the first for the treatment of Hodgkin lymphoma (brentuximab vedotin; Adcetris®; Seattle Genetics) (Senter and Sievers 2012) and the second for the treatment of HER2+ breast cancer (ado-trastuzumab emtansine; Kadcyla®; Genentech) (Lambert and Chari 2014; Amiri-Kordestani et al. 2014). In addition to these ADCs, there are over 60 in clinical trials and many others in preclinical

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development (Lambert 2015). The effectiveness of ADCs is explained by their mode of action, which begins with the antibody selectively binding to a receptor expressed on cancer cell surfaces. After binding, the ADC is internalized into an endosome, trafficked to a lysosome, and ultimately processed in a manner that causes drug release. The drugs then cause cell death by effecting important intracellular targets (e.g., tubulin or DNA). Since more drug is delivered to target cells and less to nontarget cells, side effects associated with off-target cytotoxicity are reduced, thereby broadening the therapeutic index of ADCs.

Although the concept of ADCs seems relatively simple, each component has drastic effects on the overall efficacy of the therapeutic as a whole. The first component is the antibody, which has to bind specifically to a cell surface receptor that, ideally, is amply overexpressed on targeted cancer cells to minimize toxicity to normal cells and tissues. The receptor must also be internalized after the ADC binds for successful cytotoxic drug delivery. The second component is the cytotoxic drug, which has to have in vitro cytotoxicity at subnanomolar concentrations due to the very small amount of drug ultimately reaching the intracellular target, estimated to be less than 2% (Teicher and Chari 2011).

Besides the antibody and drug being important components, the method of attaching the two has also been challenging due to the limited functional groups present in antibodies that can be utilized for conjugation. The need for orthogonal reactivity has led to several approaches for drug conjugation. These strategies can be divided into two categories: those that use natural amino acids (i.e., cysteine or Cys, selenocysteine or Sec, and lysine or Lys) and those that use unnatural amino acids (i.e., *p*-acetyl-phenylalanine or *p*-acetyl-Phe and *p*-azidomethyl-Phe) (Fig. 1.1).

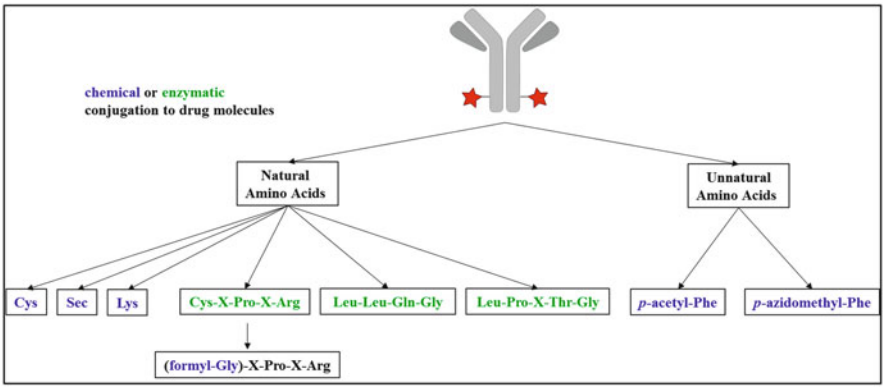


**Fig. 1.1** Overview of strategies to prepare antibody-drug conjugates (ADCs). Chemical conjugation (*blue*) or enzymatic processes (*green*) are used for drug attachment. The focus of this chapter will be on chemical conjugation methods. Three natural amino acids are used because they possess a nucleophilic functional group: thiol (Cys), selenol (Sec), and amino (Lys). Enzymatic modification of Cys can also form an aldehyde (formyl-Gly) for conjugation. Two other enzymatic approaches are used for direct conjugation of a suitably derivatized drug molecule [as in the case of transglutaminase (Dennler et al. 2014; Lhospice et al. 2015; Strop et al. 2013) and sortase A (Beerli et al. 2015)]. Two unnatural amino acids are used for ADC preparation, which possess unique functional groups for conjugation: *p*-acetyl-Phe (keto) and *p*-azidomethyl-Phe (azide)

Although there are several enzymatic conjugation strategies to prepare ADCs, this chapter will focus only on chemical conjugation strategies. We refer the reader to an excellent review that includes enzymatic conjugation strategies (Agarwal and Bertozzi 2015) as well as a more comprehensive review (Chari et al. 2014).

## 1.2 Conjugation via Natural Amino Acid Residues

Currently, all ADCs in clinical trials and both FDA-approved ADCs use Cys or Lys for drug conjugation. The use of these natural amino acids has been the most popular because there is, in general, no need to reengineer the antibody for drug attachment. The drawback to their use is that they typically lead to non-site-specific drug conjugation, which results in a complex mixture of ADCs with 0–8 drugs per antibody (Hamblett et al. 2004; Junutula et al. 2010; Feng et al. 2014). Selenocysteine (Sec) is the other natural amino acid being pursued in ADC development (Li et al. 2015; Vire et al. 2014). Although antibody reengineering is required, the use of Sec makes producing ADCs with a discrete number of drugs (i.e., site-specific ADCs) possible. Site-specific ADCs are expected to have more predictable pharmacokinetic and pharmacodynamic properties and are expected to translate more quickly from preclinical to clinical investigations. The final strategy using natural amino acids utilizes an enzyme, formylglycine-generating enzyme (FGE), to convert Cys that is present in a particular sequence into a formylglycine residue. This enzymatically generated unnatural amino acid is then used to produce site-specific ADCs. All these topics will be discussed in the sections that follow.

### *1.2.1 Random Conjugation at Native Cysteine Residues*

Cys has been used the most extensively for ADC preparation because it is present in the form of four interchain disulfide bonds in the IgG1 molecule, which is the most common antibody isotype. These covalent bonds act to stabilize the IgG1 heterotetramer composed of two heavy and two light chains, but a common strategy has been to reduce them and conjugate to the free thiols using maleimide linkers. The maleimide group serves as a Michael acceptor for the Cys-SH group to form a thiosuccinimide linkage. Several ADCs use this chemistry for drug conjugation, but the exact linker structure varies depending on the drug used. A prominent example of this conjugation strategy is represented in brentuximab vedotin, one of the two FDA-approved ADCs (Fig. 1.2). The cytotoxic compound used is monomethyl auristatin E (MMAE), a potent tubulin binder with a half maximal inhibitory concentration ($IC_{50}$) in the subnanomolar range. After reducing the disulfide bonds in the antibody, a maleimide-containing drug linker is added to produce the ADC. This procedure results in a heterogeneous mixture of ADCs containing 0–8 drugs per antibody, but predominately containing the ADC with a drug-to-antibody

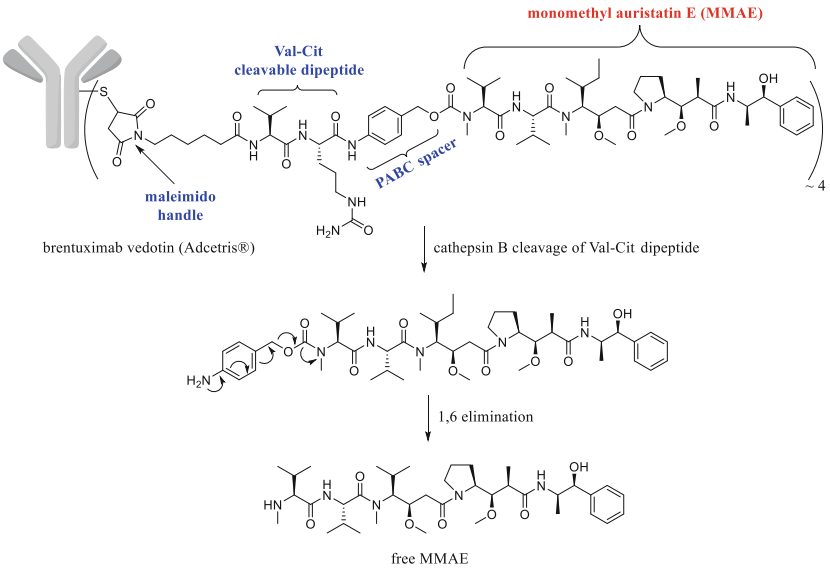


**Fig. 1.2** Structure of brentuximab vedotin (anti-CD30), currently FDA approved for the treatment of Hodgkin lymphoma

ratio (DAR) of 4 (Hamblett et al. 2004). The linker contains a cleavable valine-citrulline (Val-Cit) dipeptide attached to a *p*-aminobenzyloxycarbonyl (PABC) group. The dipeptide is stable during circulation, but cleaved once the ADC is internalized and transported to the lysosome where it is exposed to cathepsin B. After peptide cleavage, the PABC group undergoes 1,6 elimination to release free MMAE (Doronina et al. 2003). This ADC targets CD30, an antigen highly expressed on Hodgkin lymphoma and several other B-cell cancers (Deutsch et al. 2011).

SYD985 (Synthon) (Fig. 1.3) is an ADC that uses a duocarmycin derivative to target HER2+ breast cancer, which accounts for approximately 20% of total cases (Owens et al. 2004). The duocarmycins are a class of potent toxins that bind to the minor groove and alkylate DNA (Boger and Johnson 1995). For antibody attachment, a duocarmycin derivative was prepared by functionalizing the phenol that plays a crucial role in forming the active drug. A bisamine cyclization module, a unit used extensively in prodrugs (de Groot et al. 2001; Amir et al. 2003), was incorporated, so a more stable carbamate would be formed with a Val-Cit-PABC cleavable linker. Carbamates are generally more stable than carbonates, which would have resulted if the duocarmycin was attached directly to the PABC spacer. Once the dipeptide is proteolytically cleaved, the cyclization module displaces the drug, and the phenol is free to promote the cyclization into the active form of the toxin. The active compound contains an electrophilic cyclopropane that is prone to attack by adenine in DNA. SYD985 has an average number of 2.8 drugs per antibody and targets HER2 with trastuzumab, the same antibody used in the

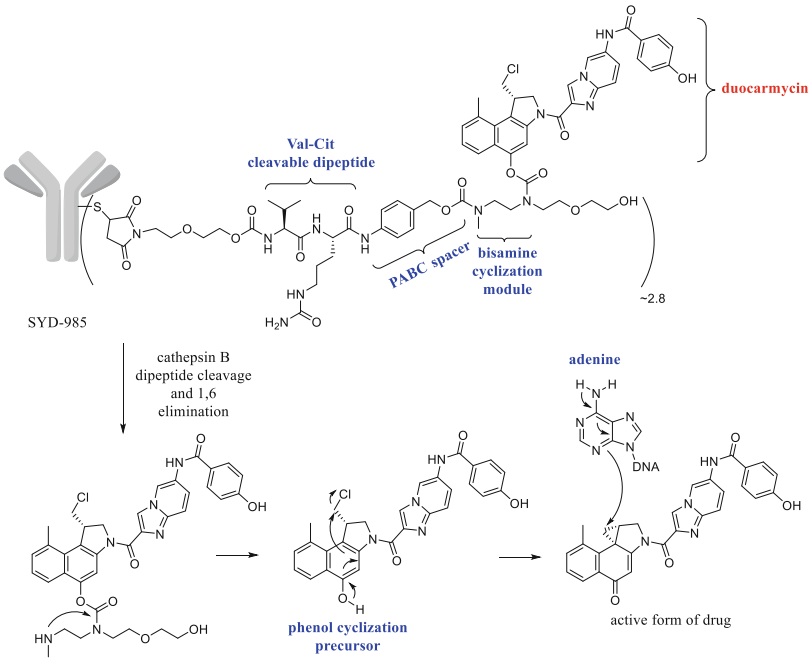


**Fig. 1.3** Structure of SYD-985 (anti-HER2) and mechanism of drug release

FDA-approved ADC for the treatment of HER2+ breast cancer (ado-trastuzumab emtansine) (Elgersma et al. 2015). When compared to ado-trastuzumab emtansine, SYD985 is more potent in vivo especially in low-expressing HER2 models (van der Lee et al. 2015). Currently SYD985 is undergoing a phase I clinical trial for the treatment of HER2+ solid tumors (NCT02277717).

Another class of ADCs utilizes maleimide linkers to conjugate SN-38, a potent topoisomerase I inhibitor (Rivory et al. 1996). Sacituzumab govitecan (IMMU-132; Immunomedics) (Fig. 1.4) is an ADC targeting TROP2 (trophoblast cell surface antigen), a target expressed in epithelial cancers (Trerotola et al. 2013). Several different linkers were screened including one that had a Phe-Lys cleavable peptide and PABC spacer, so SN-38 could be released in a similar fashion as MMAE in brentuximab vedotin (Govindan et al. 2009). However, when the Phe was removed and tested alongside the original, both had virtually the same efficacy in vitro and in vivo. This suggested that the primary method of drug release was hydrolysis of the acid-sensitive carbonate (Cardillo et al. 2011). This process occurs at low pH, such as in the tumor microenvironment or after internalization and trafficking to lysosomes. Recently, IMMU-132 completed a phase I clinical trial for the treatment of solid metastatic cancers (Starodub et al. 2015) and is currently in a phase II clinical trial for the treatment of triple-negative breast cancer (NCT02161679). It has also recently demonstrated efficacy in several TROP2-expressing tumor models (Cardillo et al. 2015). In addition to IMMU-132,

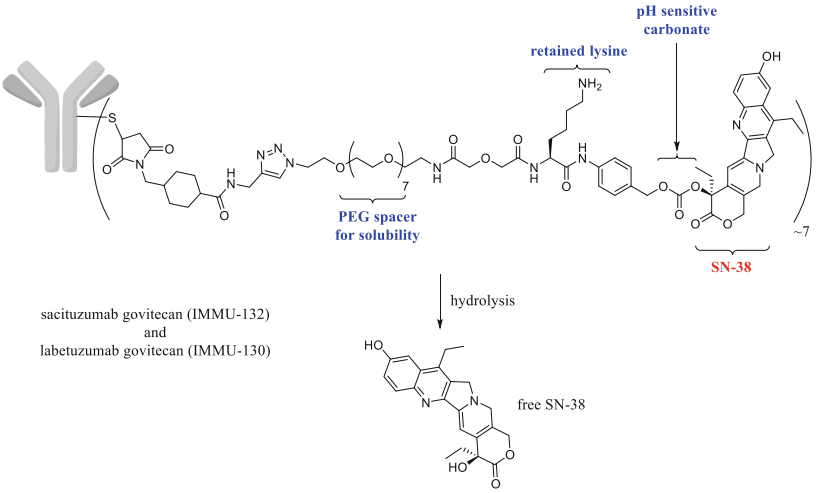


**Fig. 1.4** Structures of sacituzumab govitecan (anti-TROP2) and labetuzumab govitecan (anti-CEACAM5)

labetuzumab govitecan (IMMU-130; Immunomedics) is another ADC using this drug conjugation strategy but targets carcinoembryonic antigen cell adhesion molecule 5 (CEACAM5), a target that is also overexpressed on a variety of carcinomas. The chemistry used for drug attachment is identical to that of IMMU-132 and both have a DAR around 7. IMMU-130 is currently in a phase I/II clinical trial for the treatment of colorectal cancer (NCT01605318). It should be noted that SN-38 has only single-digit nanomolar cytotoxicity as a free drug making it less toxic than most ADC drugs (typically in the picomolar range). The lower toxicity may help to widen the therapeutic window of these ADCs (Govindan et al. 2014).

Despite being around 50- to 100-fold less toxic than the auristatins, doxorubicin has also been used to prepare ADCs. The most clinically advanced doxorubicin ADC is milatuzumab doxorubicin (IMMU-110; Immunomedics) (Fig. 1.5), which targets CD74, an antigen expressed on 90% of B-cell cancers (Burton et al. 2004). The ADC was prepared using a maleimide-modified doxorubicin containing a pH-sensitive hydrazone for release. Eight drugs on average were conjugated to each antibody, and the resulting ADC was highly potent when tested in vivo using a human multiple myeloma xenograft model. The ADC also had acceptable toxicity at doses up to 30 mg/kg when tested in cynomolgus monkeys (Sapra et al. 2005). Milatuzumab doxorubicin is currently being evaluated in a phase I/II clinical trial for the treatment of non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL) (NCT01585688).

All the ADCs discussed so far use cleavable linkers, meaning the drug is released from the antibody by a molecular cleavage process, such as hydrolysis or proteolysis. However, several ADCs do not have cleavable linkers and instead rely on complete antibody degradation inside of target cells. The drug is released still

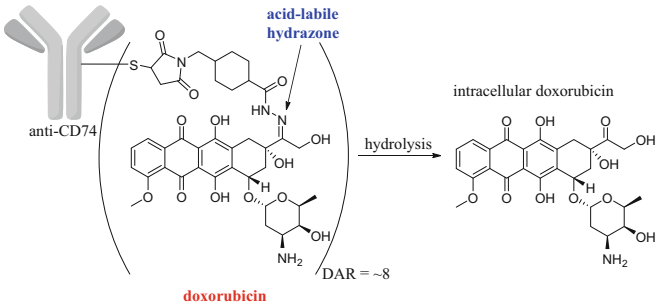


**Fig. 1.5** Structure of milatuzumab-doxorubicin (anti-CD74)

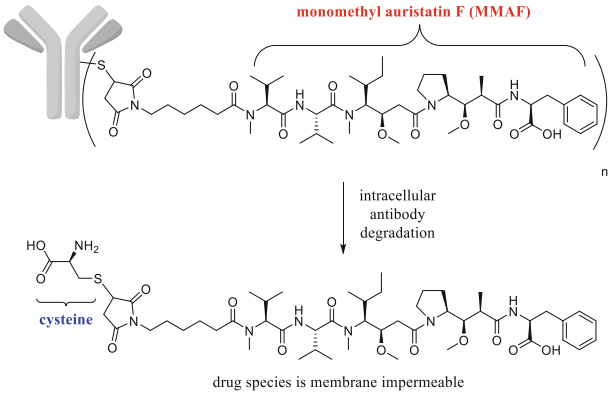


**Fig. 1.6** General structure of MMAF ADCs

attached to the original amino acid that comprised the connection point to the antibody. Noncleavable linkers are used extensively in several ADCs that are currently being investigated in clinical trials (Fig. 1.6). The maleimide group is used for Cys conjugation, but monomethyl auristatin F (MMAF) is used due to its exceptionally high potency.

Interestingly, free MMAF is 100-fold less toxic than free MMAE in vitro. This is thought to be due to the unprotected carboxylic acid at the C-terminus, which hinders cell permeability (Doronina et al. 2006). Five such ADCs are in phase II clinical trials using this strategy and they all share a common structure. It is important to note that after antibody degradation, the active drug species is charged owing to its connection to the antibody-derived amino acid, which may help prevent drug leakage out of the targeted cells and prevent off-target toxicity.

### 1.2.2 Site-Specific Conjugation at Engineered Cysteine Residues

An important consideration is that all the ADCs discussed up to this point are heterogeneous mixtures containing 0–8 drugs per antibody, which leads to polypharmacological, pharmacokinetic, efficacy, and toxicity properties. For example, antibodies with high drug loading are more hydrophobic, tend to be eliminated more quickly, and have higher toxicity (Hamblett et al. 2004). These factors make generating ADCs with defined drug loading highly desirable. An important method for accomplishing this involves Cys substitution on the antibody. These additional Cys residues do not negatively affect binding to the target antigen and can be selectively activated for drug conjugation. This THIOMAB technology demonstrated equal efficacy and decreased toxicity, despite having a decreased drug loading (DAR = 1.6) when compared to a traditional heterogeneous ADC (DAR = 3.1) (Junutula et al. 2008).

The concept of drug conjugation to engineered cysteines has been extended to using different toxins including the pyrrolobenzodiazepine (PBD) dimers, which are highly potent DNA cross-linkers (Smellie et al. 2003). The conjugation strategy uses a valine-alanine cleavable dipeptide and a maleimidocaproyl group for Cys attachment (Fig. 1.7). These compounds are currently being used in two site-specific ADCs, both of which have recently entered phase I clinical trials. The first, SGN-CD33A (Seattle Genetics), is being investigated for the treatment of acute myeloid leukemia (AML, NCT01902329). This ADC is very homogeneous (DAR = 1.9) and was generated using an engineered Cys at position 239 on the heavy chain (IgG1, S239C) (Kung Sutherland et al. 2013). The second, SGN-CD70A (Seattle Genetics), is being used for the treatment of renal cell carcinoma and lymphoma (NCT02216890). These ADCs are the first site-specific ADCs to have entered clinical trials.

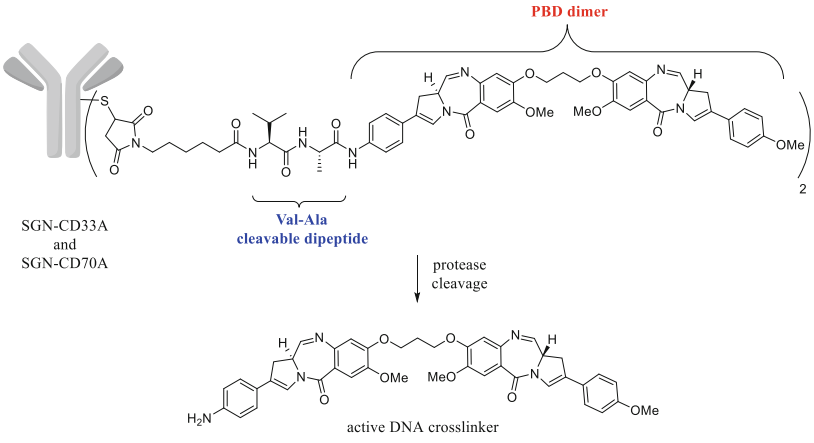


**Fig. 1.7** Structures of SGN-CD33A and SGN-CD70A

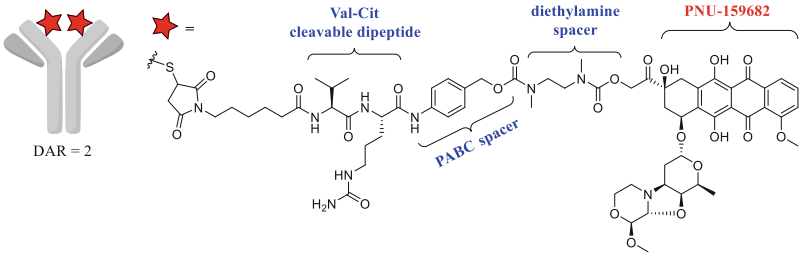


**Fig. 1.8** Structure of site-specific anthracycline-based ADC (anti-CD22)

Another ADC based on THIOMAB technology was generated by conjugating PNU-159682, a potent anthracycline with an $IC_{50}$ in the picomolar range, to two engineered Cys in the heavy chain (IgG1, A114C). This ADC has a DAR of nearly 2 (Quintieri et al. 2005) (Fig. 1.8) and targets CD22, which is expressed in NHL and acute lymphoblastic leukemia (ALL). The protease-cleavable Val-Cit-PABC module is used along with a diethylamine spacer. The dipeptide is presumably cleaved, but the actual structure of the active toxin generated in the cells is unknown at this time. This ADC was tested against MMAE-resistant cell lines and was more potent than the corresponding ADC with MMAE, demonstrating that PNU-159682 may help combat inherent or acquired ADC resistance (Yu et al. 2015).

### *1.2.3 Stabilization of Maleimide Linkage*

Although maleimide chemistry has been widely used to prepare ADCs, a significant amount of drug is lost systemically in circulation presumably via retro-Michael reactions (Alley et al. 2008; Shen et al. 2012) (Fig. 1.9a). This process can lead to decreased efficacy and an increase in off-target toxicity. A strategy has been developed to minimize this issue by introducing an amino group adjacent to the maleimide, which promotes ring hydrolysis (Fig. 1.9b). Once hydrolyzed, the elimination reaction can no longer occur. ADCs containing this modified linker were shown to have improved activity with less toxicity when tested in vivo (Lyon et al. 2014).

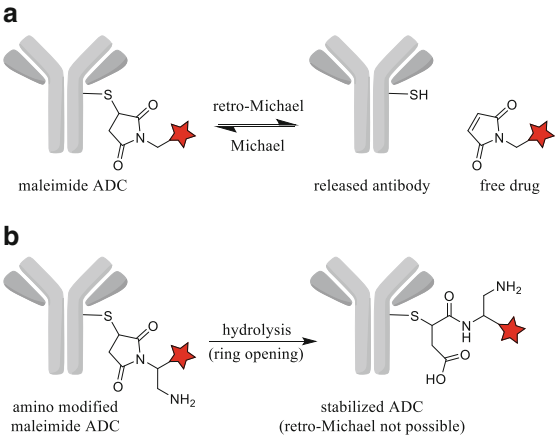


**Fig. 1.9** Retro-Michael reaction of maleimide conjugates leading to release (**a**) and stabilized maleimide ADCs via ring hydrolysis (**b**)

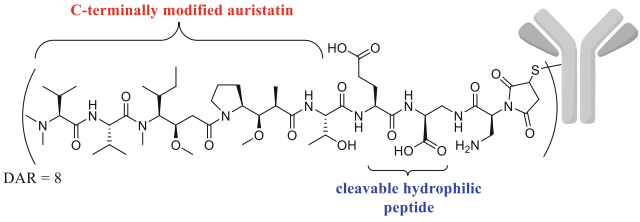


**Fig. 1.10** Structure of homogeneous ADC with hydrophilic spacer

### *1.2.4 Hydrophilic Spacers*

Although ADCs with high drug loading often have higher potency in vitro, they have decreased efficacy in vivo due to higher clearance rates (Hamblett et al. 2004). A recent strategy has circumvented this by preparing homogeneous ADCs using hydrophilic spacers. Antibodies were first completely reduced using excess tris (2-carboxyethyl)phosphine (TCEP). Conjugation was then carried out using a maleimide-auristatin derivative incorporating a threonine and hydrophilic cleavable peptide at the C-terminus (Fig. 1.10). ADCs with a DAR of 8 were obtained that had superior activity in vivo, when compared to the corresponding ADC with a hydrophobic Val-Cit-PABC cleavable linker. It should also be noted that PEG spacers also have effects on hydrophobicity, and optimizing these linkers was shown to improve in vivo efficacy with homogeneous ADCs with high drug loading (Lyon et al. 2015).

### 1.2.5 Cysteine-Bridged Conjugation

Another strategy using Cys conjugation involves reduction of the interchain disulfide bonds and cross-linking the two reduced thiols. This approach does not require any antibody modification and increases the stability of the final construct because the two polypeptide chains remain covalently bridged. Three reagents have been developed for use in this approach. The first is a bis-sulfone reagent, which undergoes a series of Michael and elimination reactions to conjugate MMAE (Fig. 1.11) (Badescu et al. 2014). ADCs with a predominant DAR of 4 (>78%) (Fig. 1.12) were tested alongside one of the FDA-approved ADCs (ado-trastuzumab emtansine). The bridged ADC was more stable and more potent in vivo (Bryant et al. 2015).

The second reagent for the generation of Cys-bridged ADCs is dibromomaleimide, which was originally reported to be an effective reagent for protein pegylation (Jones et al. 2012). ADCs have now been generated using this conjugation handle with a DAR of 4 (Behrens et al. 2015) (Fig. 1.13a). A third reagent is a dibromopyridazinedione with two clickable sites (Fig. 1.13b). This reagent is unique in that two compounds can be attached per disulfide bond, meaning eight compounds can be attached in total per antibody. The cross-linking group has an alkyne for attaching the 1st compound using a strain-promoted alkyne-azide cycloaddition (SPAAC) under copper (Cu)-free conditions and a normal alkyne for attaching the 2nd compound using Cu-catalyzed click chemistry (Lallana et al.

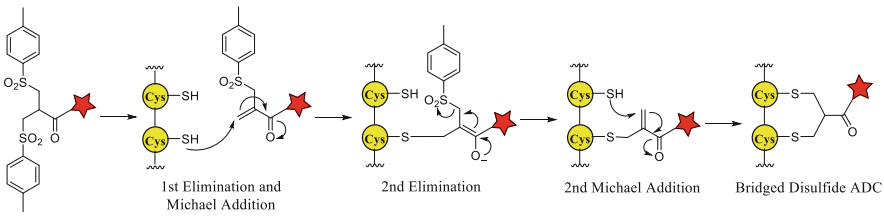


**Fig. 1.11** Structure of bis-sulfone reagent and mechanism leading to ADC formation

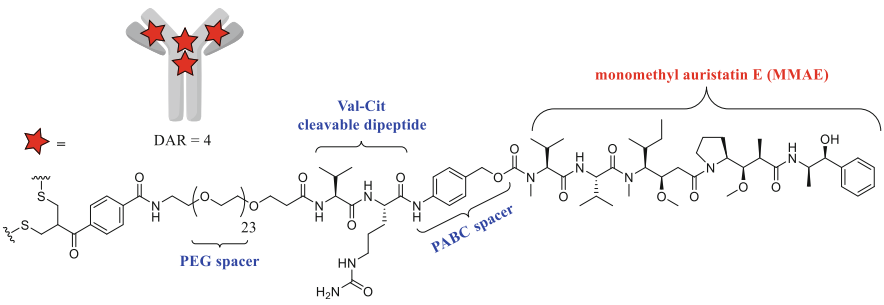


**Fig. 1.12** Structure of ADC (anti-HER2) derived from bis-sulfone drug conjugation

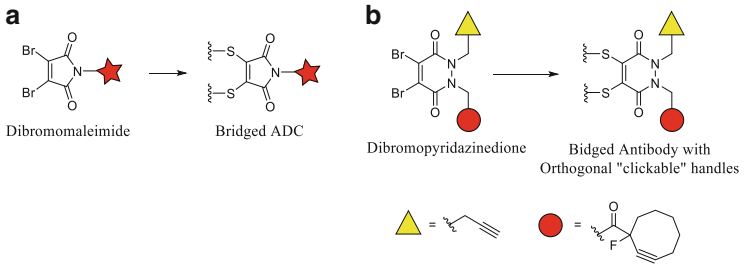


**Fig. 1.13** Structure of dibromomaleimide (**a**) and dibromopyridazinedione (**b**) reagents used to prepare Cys-bridged ADCs. The dibromopyridazinedione handle has two clickable alkynes for the attachment of two different compounds

2011; Jewett and Bertozzi 2010). This strategy was used to attach four doxorubicin molecules along with four fluorophore molecules. This ADC was shown to selectively kill HER2-expressing cancer cells when trastuzumab (anti-HER2) was used as the antibody (Maruani et al. 2015). Interestingly, this approach opens up the possibility of attaching two drugs with different modes of action as a means of overcoming ADC drug resistance.

### *1.2.6 Site-Specific Conjugation at Engineered Selenocysteine Residues*

Selenocysteine (Sec) is the 21st natural amino acid and is much more reactive than Cys as a nucleophile. With a pKa of 5.2, Sec can be used for selective conjugation under slightly acidic conditions where the other nucleophilic amino acids (i.e., Cys and Lys) are unreactive. Sec is incorporated by recoding the opal stop codon (UGA) with a Sec incorporation sequence (SECIS) in the 3′ untranslated region of the mRNA. Although incorporation efficiency (20%) is modest, an affinity tag can be incorporated downstream of the stop codon to purify only Sec-containing protein (Hofer et al. 2008). Sec has been incorporated into several antibody formats, including IgG1, and has been selectively labeled with maleimide, iodoacetamide (Li et al. 2014; Hofer et al. 2009), and methyl sulfone oxadiazole linkers (Patterson et al. 2014) (Fig. 1.14). Sec is first reduced using dithiothreitol (DTT), and the conjugation is performed at a pH of 5.2 to prevent cross-reactivity with Cys. Currently, up to two Sec can be incorporated into each antibody making a DAR of 2 possible (Li et al. 2014). Conjugation of methyl sulfone oxadiazoles to engineered Sec and Cys residues produced conjugates with higher stability than maleimide conjugates. This conjugation strategy also avoids maleimide exchange with the free Cys-34 residue of human serum albumin (Li et al. 2014). SELENOMAB and THIOMAB technology has also been combined to generate

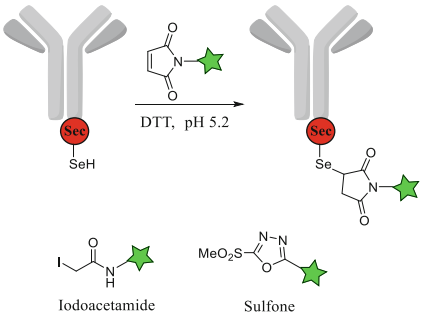


**Fig. 1.14** Site-specific selenocysteine (Sec) conjugation using maleimide, iodoacetamide, and methyl sulfone oxadiazole linkers with fluorophores (*green star*)

antibodies with engineered Sec and Cys residues that can be orthogonally conjugated, providing a platform for dual warhead ADCs (Li et al. 2015).

## *1.2.7 Random Conjugation at Native Lysine Residues*

Though not as common as Cys, there are still over ten ADCs in the clinic that use Lys for drug conjugation. Work has shown that ~40 Lys, half from the heavy and half from the light chain, are available for conjugation when preparing an ADC (Wang et al. 2005). Despite this high number, only 0–8 drugs per antibody have been reported, which is most likely due to other factors such as surface accessibility making only certain Lys residues susceptible to conjugation. There are currently two main classes of drugs used for Lys conjugation: maytansinoids and calicheamicins.

### 1.2.7.1 Maytansinoid ADCs

ADCs using Lys drug conjugation have been developed using maytansinoids, a class of highly potent antimitotic compounds ($IC_{50}$ values in the picomolar range) that inhibit tubulin polymerization (Chari 2008). Maytansinoid ADC preparation requires two chemical components. The first is a heterofunctional linker that contains an *N*-hydroxysuccinimide (NHS) ester on one end and an activated disulfide or maleimide on the other. The ester reacts with Lys of the antibody to form stable amide bonds. The other end of the linker is either a disulfide bond if a cleavable linker is desired (SPDP, SPDB, SPP, and SMPP) or a maleimide for the noncleavable version (SMCC) (Fig. 1.15a). The disulfide bond can be reduced by thiols in target cells resulting in drug release. Glutathione is thought to be the major thiol involved in this reduction since it is found at high concentration (0.5–10 mM) in the cytoplasm (Wu et al. 2004), but at much lower levels in plasma (~2 μM) (Mills and Lang 1996). The rate of reduction can be tuned by adding methyl groups in alpha position to the thiol in the heterofunctional linker. Noncleavable linkers are

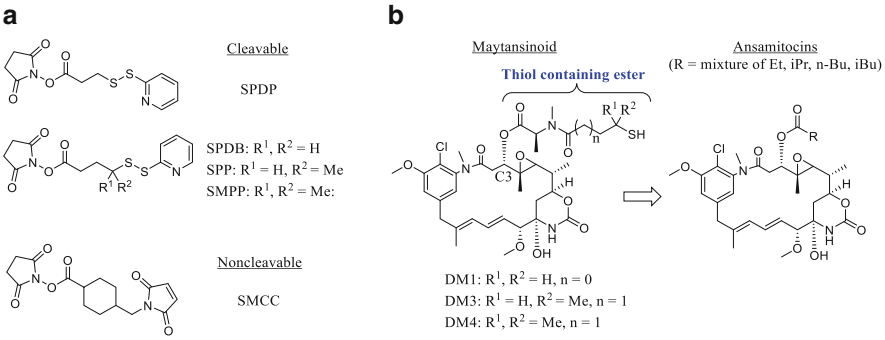


**Fig. 1.15** Heterobifunctional linkers used to prepare maytansinoid ADCs (**a**) and maytansinoid derivatives used for antibody conjugation (**b**)

thought to remain intact and are released still attached to the Lys of the original antibody after it is degraded inside target cells (Erickson et al. 2012).

The second component is the maytansinoid drug, which requires modification of the natural product to include a thiol group for attachment to the antibody. This process involves refunctionalization of ansamitocins, which are obtained from the microorganism *Actinosynnema pretiosum* that produces them in a mixture of C3 esters containing predominately the isobutyrate ester (80% of mixture). After generating the free alcohol at C3, the drug is then coupled to a series of thiol-containing carboxylic acids. The amount of steric hindrance was varied around the thiol using methyl group substitution in three derivatives (DM1, DM3, and DM4) (Fig. 1.15b). All these derivatives were shown to be highly potent when tested against cancer cell lines with DM3 and DM4 about threefold and 12-fold more cytotoxic than DM1.

The procedure for preparing the maytansinoid ADCs begins by combining the heterofunctional linker with the antibody to achieve Lys attachment (Fig. 1.16). The maytansinoid is then added to displace 2-thiopyridine and form the ADC. The same strategy is used to prepare the noncleavable version, except that the thiol group of the drug undergoes Michael addition to the maleimide to form a thioether-linked ADC. On average, 3–4 drugs are conjugated to each antibody in both the cleavable and noncleavable formats (Widdison et al. 2006).

Ideally, the disulfide bond should be stable in circulation to prevent premature cleavage and off-target toxicity. To compare the effect of steric hindrance on the rate of drug release, several maytansinoid ADCs were prepared with varying amounts of hindrance on the drug or the linker side of the disulfide bond (Fig. 1.17). The antibody used for this study was huC242, a humanized antibody against CanAg which is an antigen highly expressed on the surface of several solid tumors. All the conjugates displayed similar cytotoxicity in vitro with a half maximal effective concentration ($EC_{50}$) between 3.5 and 15 pM, despite the stability of the disulfide bond varying greatly. For instance, when two methyl groups were alpha to the disulfide bond on both sides, the stability was increased

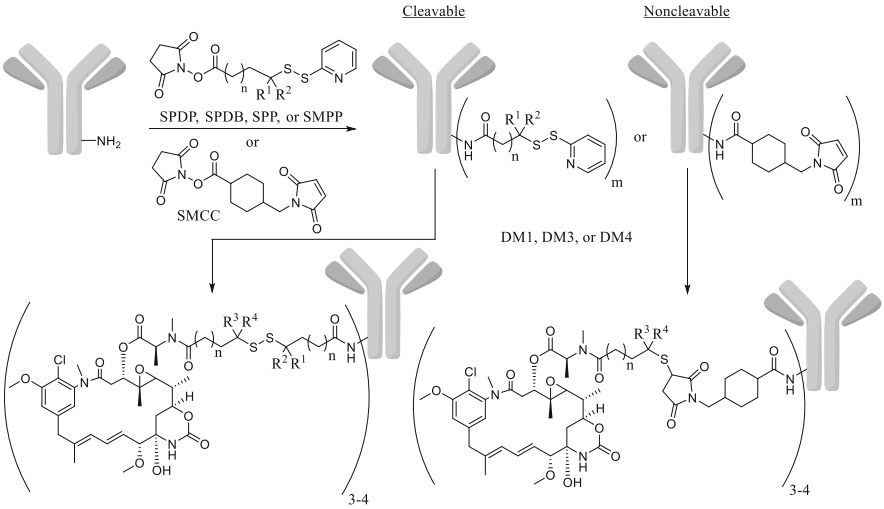


**Fig. 1.16** Preparation of cleavable (i.e., disulfide-containing) and noncleavable maytansinoid ADCs

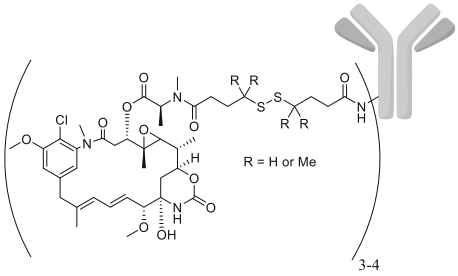


**Fig. 1.17** Maytansinoid ADCs with varying hindrance around disulfide bond

by over 22,000-fold, when compared to the form without any methyl groups. The stability of the disulfide bond increased as the steric hindrance increased regardless if it was on the drug or linker side.

To determine if the stability had an effect on off-target toxicity, bystander cells (antigen negative) and target cells (antigen positive) were mixed at different ratios and treated with each ADC. All of the ADCs containing disulfide bonds were able to kill bystander cells at 1 nM, which was the concentration found to kill all of the antigen-positive cells, suggesting drug leakage out of target cells. However, the ADC with the noncleavable linker did not kill any bystander cells. These results are consistent with previous studies and can be explained by the metabolic fate of the ADCs (Fig. 1.18). After internalization, the ADCs are first proteolytically broken down in lysosomes to release the maytansinoid still attached to Lys. The metabolism stops here for the noncleavable linker, but continues for the disulfide metabolites

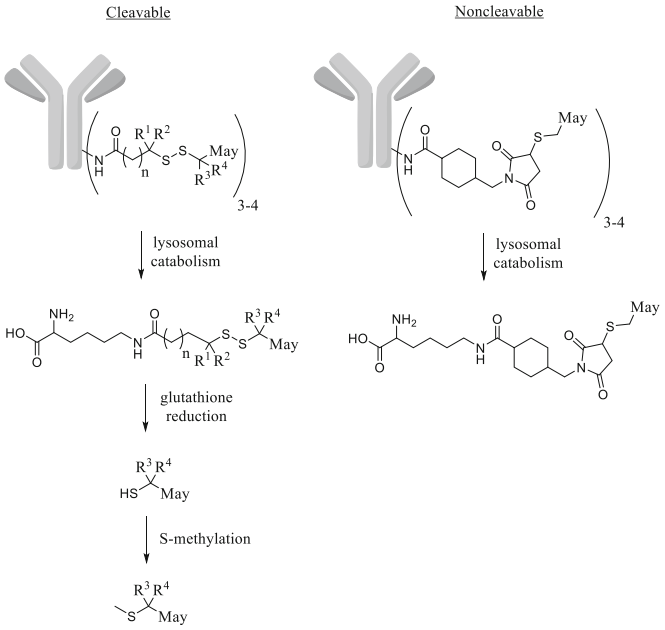


**Fig. 1.18** Metabolic fate of maytansinoid ADCs. Adapted from (Chari et al. 2014)

which are reduced by glutathione to give the original maytansinoid (DM1 or DM4). The maytansinoids can then be methylated by S-methyltransferase to form S-methyl-DM1 and S-methyl-DM4. The difference in bystander killing is because the noncleavable linker metabolite is charged, making it membrane impermeable. In contrast, the uncharged metabolites from the cleavable antibody linker construct are capable of diffusing out of target cells and into bystander cells (Erickson et al. 2010).

When the stability of these ADCs was evaluated in vivo, an increase in the stability of the disulfide bond was correlated with a higher half-life. The most stable ADC evaluated in vivo had a half-life of 218 h, which was over 14 times longer than the ADC with the least hindered disulfide. One would predict that the ADC with the longest half-life would be the most effective in vivo. Surprisingly, this was not the case with the ADCs having intermediate stabilities being the most effective. The most stable ADCs, including the noncleavable version, were the least effective. This result suggests that diffusion out of target cells into surrounding tissue may be an important mechanism for tumor eradication (Kellogg et al. 2011). It should also be noted that the therapeutic effect of ADCs may be increased by the cytotoxic compounds stimulating dendritic cells to induce antitumor immunity (Martin et al. 2014; Muller et al. 2014).

Although the in vivo efficacy of these ADCs appears to be best when a disulfide linker is used, this is not the case for all target cancers. A prominent example of this is represented by FDA-approved ado-trastuzumab emtansine (Kadcyla®), which uses a noncleavable linker with DM1 (Fig. 1.19). The noncleavable linker (SMCC)

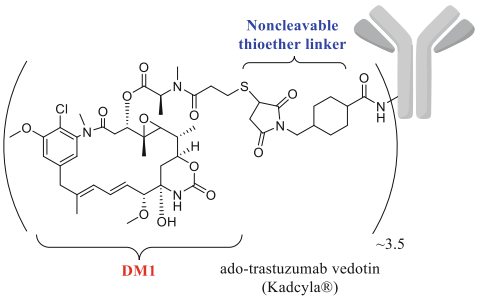


**Fig. 1.19** Structure of ado-trastuzumab vedotin (anti-HER2)

and cleavable disulfide (SPP) antibody constructs were found to have equal antitumor activity in vivo (Erickson et al. 2012). However, the noncleavable linker was ultimately chosen for development because it was better tolerated by twofold (Lewis Phillips et al. 2008). This ADC has an average DAR of 3.5 and is used for the treatment of HER2+ metastatic breast cancer following prior treatment with trastuzumab and chemotherapy (Wakankar et al. 2010).

### 1.2.7.2 Calicheamicin ADCs

Another drug class used for Lys conjugation is the calicheamicins, which are highly cytotoxic antibiotics that bind to the minor groove of DNA and cause double-strand breaks (Zein et al. 1988; Lee et al. 1992). These highly reactive compounds have a unique structure that is crucial for their mode of action. Calicheamicin $\gamma_1$I is the most well-known member of this antibiotic class. It contains two domains: an enediyne core that is involved in cleaving DNA and a tetrasaccharide tail, which anchors the molecule in the minor groove of DNA (Zein et al. 1988, 1989) (Fig. 1.20). Once anchored, the trisulfide group is cleaved by glutathione to give a free thiol (Fig. 1.20, S1), which then cyclizes to form a dihydrothiophene (Fig. 1.20, S2). This intramolecular cyclization imposes strain on the enediyne causing a Bergman cycloaromatization to form a diradical (Fig. 1.20, S3). The diradical abstracts hydrogen atoms from the deoxyribose backbone of DNA, causing double-strand breaks. Calicheamicin is one of the most potent antitumor agents known, displaying picomolar activity in several tumor cell lines (Long et al. 1989). However, as with other drugs used in ADCs, the therapeutic window was too narrow to find utility as a free drug.

NAc-γ-calicheamicin DMH, a hydrazine containing derivative of calicheamicin (Fig. 1.21), was used for early work on antibody attachment. After periodate oxidation of carbohydrates on the antibody to form reactive aldehydes, the calicheamicin derivative was added to form a hydrazone linkage. The hydrazone is prone to acid hydrolysis in the lysosomes of target cells. These "carbohydrate conjugates" also contained a disulfide bond with two methyl groups flanking one side for increased stability. This bond is necessary for the drug's mechanism of action and also acts as a second mechanism for drug release via reduction

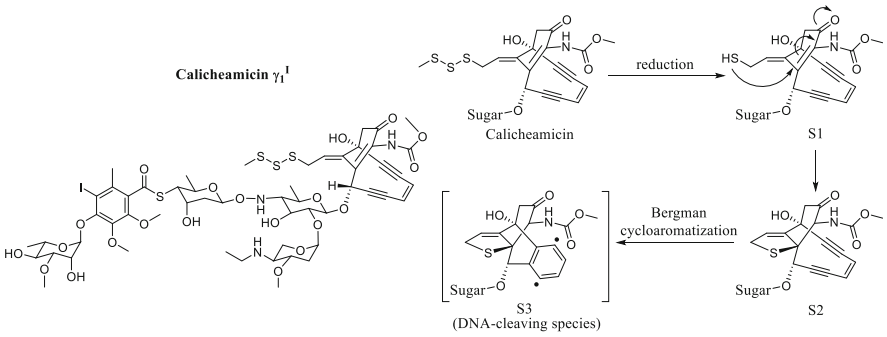


**Fig. 1.20** Structure of calicheamicin $\gamma_1$I and mechanism leading to diradical DNA-cleaving species

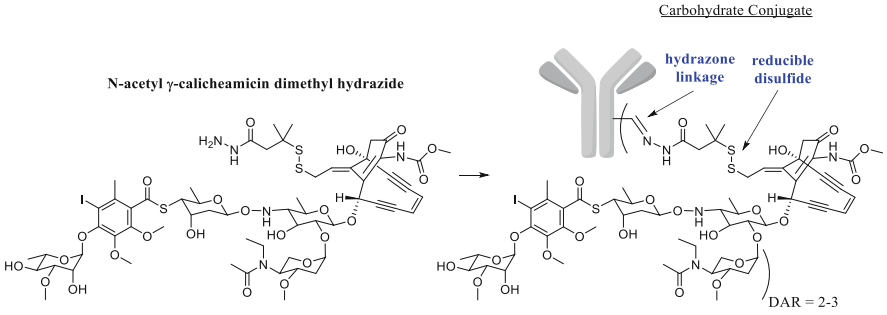


**Fig. 1.21** Structures of NAc-γ-calicheamicin DMH and ADC derived from aldehyde conjugation

(Fig. 1.21) (Hinman et al. 1993). Additional work showed that the hydrazone linker was not necessary for activity. Subsequently, an activated ester was used for direct conjugation to Lys residues of the antibody (Fig. 1.22). These "amide conjugates" had equivalent activity, but had higher selectivity in vitro and less toxicity in vivo, suggesting premature drug release occurred with the hydrazone linker. Both these attachment strategies resulted in ADCs with 2–3 drugs per antibody (Hamann et al. 2005).

Identical attachment strategies were used during the development of gemtuzumab ozogamicin (Fig. 1.23), an ADC targeting CD33 for the treatment of AML. However, in this case, the carbohydrate conjugate was 7000-fold more potent and 100-fold more selective than the amide conjugate toward antigen-expressing cells. The carbohydrate conjugate was also more active in vivo where it produced nine tumor-free survivors out of ten, whereas the amide conjugate gave zero tumor-free survivors (Hamann et al. 2002). The carbohydrate conjugate was therefore chosen for further development and a humanized IgG4 anti-CD30 antibody was developed. Ultimately, a modified linker had to be developed because several oxidation attempts for drug attachment resulted in low drug loading. This linker used an activated ester for lysine attachment with a ketone for hydrazone

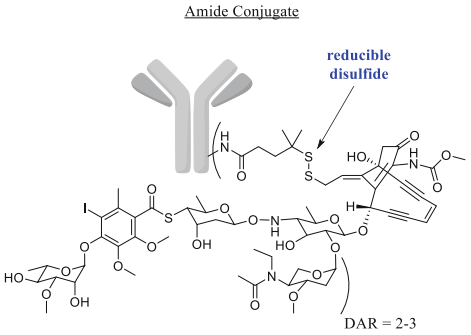


**Fig. 1.22** Structure of calicheamicin amide conjugate

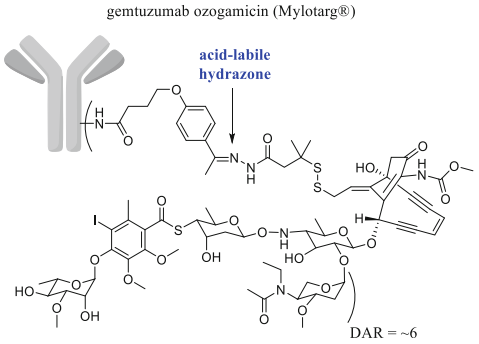


**Fig. 1.23** Structure of gemtuzumab ozogamicin (anti-CD33)

formation. After screening several linkers, one was chosen because it was highly stable at pH 7.4, but almost completely hydrolyzed at pH 4.5 making it ideal for ADC incorporation. This ADC had an average DAR of 3, but 50% of the mixture was unmodified antibody and the other 50% had ADC with a DAR of 6 (Kunz 2004). This ADC was FDA approved in 2000 for the treatment of AML (gemtuzumab ozogamicin; Mylotarg®; Wyeth), but was voluntarily withdrawn due to limited clinical activity and safety concerning the rate of fatal toxicity (Bornstein 2015).

Two other calicheamicin ADCs are being pursued clinically. The first, inotuzumab ozogamicin (CMC-544; Pfizer), targets CD22, which is expressed on most B-cell cancers including NHL, CLL, ALL, and hairy cell leukemia (Olejniczak et al. 2006). This ADC uses identical linker chemistry to Mylotarg and has 5–7 drugs per antibody with less than 10% of unconjugated antibody (DiJoseph et al. 2004). Inotuzumab ozogamicin is currently being evaluated in several clinical trials, the most advanced of which is a phase III study for the treatment of ALL (NCT01564784). The second is PF-06647263 (Pfizer) which targets ephrin-A4, a receptor tyrosine kinase overexpressed on triple-negative breast cancer and ovarian cancer. PF-06647263 outperformed cisplatin and doxorubicin in triple-negative breast cancer and ovarian patient-derived xenograft

models and is currently being evaluated in a phase I clinical trial (NCT02078752) (Damelin et al. 2015).

## 1.3 Conjugation via Unnatural Amino Acids

Even though methods are emerging to generate site-specific ADCs using natural amino acids, a complementary strategy has been the introduction of unnatural amino acids with unique reactivities. Typically, two unnatural amino acids are introduced per antibody (owing to its homodimeric architecture) to produce ADCs with a DAR of 2. Currently two unnatural amino acids have been used to generate site-specific ADCs, *p*-acetyl-Phe and *p*-azidomethyl-Phe.

### *1.3.1 Site-Specific Conjugation at Engineered* p*-Acetyl-Phe Residues*

*p*-Acetyl-Phe contains a keto group that can be selectively labeled using N-alkoxy-amine functionalized drugs (Fig. 1.24). For antibody incorporation in mammalian cells, the amber stop codon (UAG) is substituted at the desired location, and an orthogonal mutant *Escherichia coli* aminoacyl-tRNA synthetase (aaRS) along with an amber suppressor tRNA is used to insert *p*-acetyl-Phe at the amber stop codon (Liu et al. 2007). Initial proof of principle studies used trastuzumab and substituted an alanine (A121) in the heavy chain for *p*-acetyl-Phe. The *p*-acetyl-Phe-containing antibody was stably expressed in Chinese hamster ovary (CHO) cells in good yield (>300 mg/L). A C-terminally modified auristatin derivative was the drug chosen

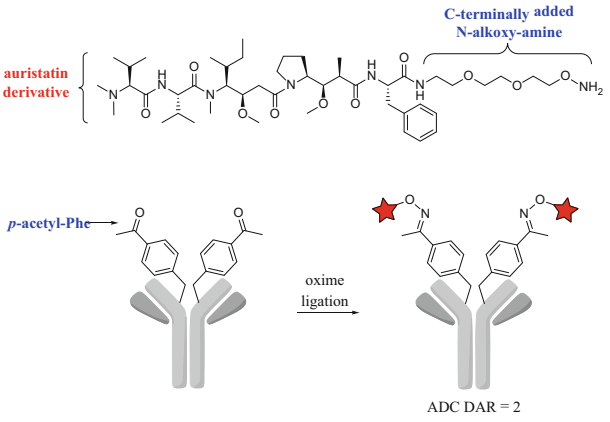


**Fig. 1.24** Structure of N-alkoxy-amine auristatin derivative used to prepare site-specific ADCs with engineered *p*-acetyl-Phe

because previous work indicated that these compounds have increased toxicity and are better tolerated when compared to cleavable N-terminally modified auristatins (Doronina et al. 2008). Oxime ligation produced highly stable ADCs with a DAR of 2 with >95% efficiency. These ADCs were highly specific and potent when tested in vitro and in vivo (Axup et al. 2012).

### 1.3.2 Site-Specific Conjugation at Engineered p-Azidomethyl-Phe Residues

The incorporation of *p*-azidomethyl-Phe has also been used to prepare site-specific ADCs. A cell-free system was optimized using a variant of the *Methanococcus jannaschii* tyrosyl tRNA synthetase to insert *p*-azidomethyl-Phe at the S136 position of the heavy chain of trastuzumab. MMAF was conjugated using a dibenzocyclooctyne (DBCO) handle so that strain-promoted azide-alkyne cycloaddition (SPAAC) copper-free click chemistry could be used for antibody attachment (Fig. 1.25). ADCs with DARs ranging between 1.2 and 1.9 could be prepared depending on the aminoacyl-tRNA synthetase used. All ADCs were highly potent when tested in vitro against HER2+ cells (Zimmerman et al. 2014).

### 1.3.3 Site-Specific Conjugation at Engineered Formylglycine Residues

Although enzymatic strategies are not the focus of this review, formylglycine-generating enzyme (FGE) has been used to introduce aldehyde tags for drug attachment. FGE oxidizes Cys when present in a particular peptide sequence (CXPXR) to

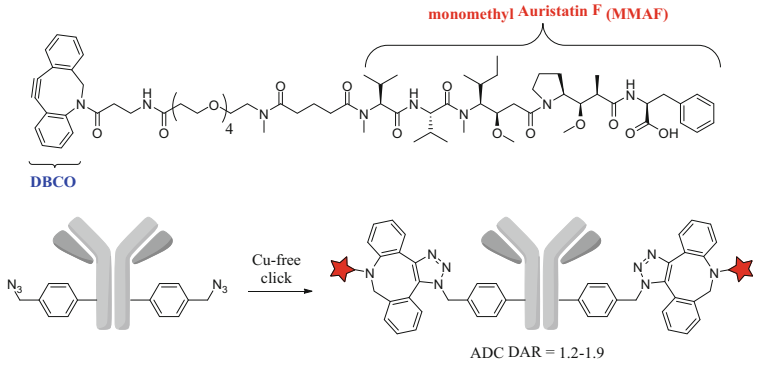


**Fig. 1.25** Structure of MMAF derivative with DBCO handle and preparation of ADCs using copper-free click chemistry

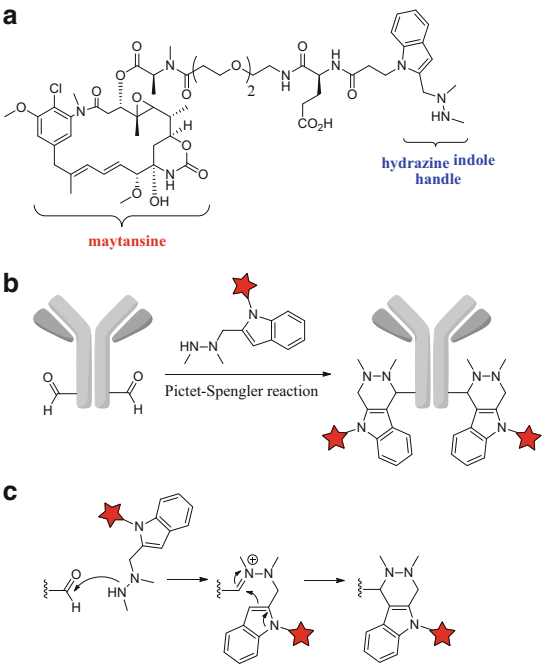


**Fig. 1.26** Structure of maytansine derivative with hydrazine-indole handle (**a**), preparation of site-specific ADCs using FGE inserted tags (**b**), and mechanism of drug conjugation (**c**)

form an aldehyde functional group (Rabuka et al. 2012). Like *p*-acetyl-Phe, N-alkoxyamines have been used for conjugation. However, a more stable linker was developed for ADC preparation that uses hydrazino-iso-Pictet-Spengler chemistry. A maytansine drug analogue was prepared with a hydrazine indole handle for antibody attachment (Fig. 1.26). Initially a hydrazinium ion forms, which then reacts with the nucleophilic indole to form a stable C-C bond. Several antibodies were prepared by inserting the aldehyde handle at different sites of trastuzumab: one internal site on the light chain, seven internal sites on the heavy chain, and the final site at the C-terminus of the heavy chain. Ultimately, the C-terminus site proved to be the best attachment site. The resulting ADC was highly potent in vitro and in vivo and had an improved safety profile when compared to FDA-approved ado-trastuzumab emtansine (Drake et al. 2014).

## 1.4 Conclusions

The development of ADCs has progressed rapidly in recent years. Although early strategies relied on nonspecific drug attachment, focus has shifted toward the development of site-specific attachment strategies. There are now several promising strategies relying on unique chemistries to produce homogeneous ADCs, and

the first of these are just now entering clinical trials. Whether or not these ADCs will actually be approved faster because of improved efficacy and decreased toxicity remains to be answered. The fate of several heterogeneous ADCs in clinical trials will also be an area of interest, especially considering homogeneous ADCs will now be compared directly in clinical trials. Future work in this field will most likely be directed toward new site-specific technologies and the use of new cytotoxic compounds with novel modes of actions and linker technologies.

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# Chapter 2
# Preclinical Evaluation of ADCs Delivering Highly Potent Pyrrolobenzodiazepine (PBD) Dimers

**John A. Hartley**

**Abstract** The pyrrolobenzodiazepines (PBDs) are tricyclic natural products that exert their biological activity by binding and covalently bonding in the minor groove of DNA. An important development in the history of the PBDs was the synthesis of nonnatural PBD dimers that have the ability to form highly cytotoxic DNA cross-links. Rational structural modification can produce PBD dimers with exquisite potency making them ideal candidates for the 'warhead' component of antibody drug conjugates. Two different sites on PBD dimers have been utilised to attach linkers, N10 and C2, and antibody PBD conjugates (APCs) of both types have shown impressive in vitro and in vivo activity against both haematological malignancies and solid tumours. Several APCs including vadastuximab talirine and rovalpituzumab tesirine have progressed into early phase clinical trials.

ADCs delivering PBD dimer payloads represent a novel mode of action in the ADC area. An important feature of the highly cytotoxic DNA interstrand cross-links produced by the PBD dimers is their persistence in cells. This contributes to their potency and also to their ability to affect slowly proliferating target cells, including cancer stem cells. The PBD dimers are a highly flexible platform with many advantages over existing payloads. These include the ability to target low copy number antigens and to exploit low drug-antibody ratios due to the high warhead potency. APCs are active in tumour types inherently resistant to other warhead classes and against multidrug-resistant tumours.

**Keywords** Pyrrolobenzodiazepine dimer • PBD dimer • Vadastuximab talirine • Rovalpituzumab tesirine • Antibody PBD conjugate

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## 2.1 Background to the PBDs

The pyrrolobenzodiazepines (PBDs) are tricyclic natural products (Fig. 2.1) originally discovered in cultures of *Streptomyces* (Leimgruber et al. 1965). They were presumably evolved as a form of chemical defence and exert their biological activity by binding in the minor groove of DNA with a selectivity for 5′-purine-guanine-purine sequences and forming a covalent bond to the guanine base at its exocyclic amino group (Hertzberg et al. 1986; Hurley et al. 1988). The first PBD to be isolated and characterised, anthramycin (Fig. 2.1), was shown to have a broad spectrum of antitumour activity in vivo and as a result was tested clinically (Korman and Tendler 1965). It was found to have activity against gastrointestinal and breast cancers, lymphomas and sarcomas without significant haematological toxicity. Its clinical use was limited, however, due to dose-limiting cardiotoxicity.

The biological activity of the natural compounds originally established a structure-activity relationship, and subsequently, numerous synthetic PBD compounds have been synthesised introducing different chemical substituents into the A- and C-rings of the tricyclic core structure (Antonow and Thurston 2011). As a result, fully synthetic PBD monomer compounds can be produced with enhanced in vitro cytotoxicity and DNA-binding affinity and lacking the cardiotoxicity of anthramycin.

An important development in the history of the PBDs was the synthesis of nonnatural PBD dimers. It was rationalised that linking two PBD molecules together in an appropriate manner with a suitable tether could result in a molecule capable of spanning a greater number of base pairs of DNA whilst retaining the favourable inherent properties of the natural products such as cellular uptake and trafficking to the nucleus. In addition, a PBD dimer would have the potential to form two covalent bonds to guanine bases in the DNA minor groove and thus the ability to form highly cytotoxic DNA cross-links (Bose et al. 1992; Smellie et al. 1994, 2003). This led to the development of the optimised structure SJG-136 (later named SG2000) in which two C2-exo-methylene PBD monomer units are linked via their aromatic A-ring phenol C8 positions by a flexible propyldioxy tether (Fig. 2.2) (Gregson et al. 2001).

SG2000 was found to have potent in vitro cytotoxic activity against human tumour cell lines, and the drug was highly active in vivo against ten tumour models performed by the US National Cancer Institute, including tumour-free responses in

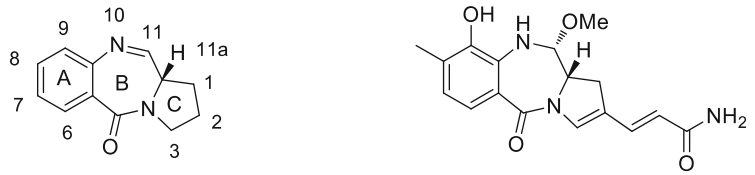


**Fig. 2.1** The basic pyrrolobenzodiazepine tricyclic structure and the natural product anthramycin

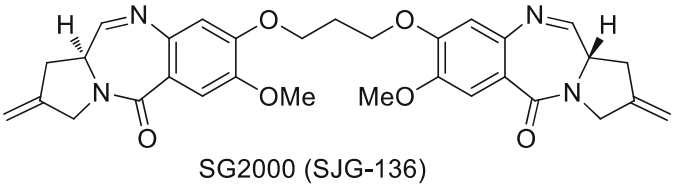


**Fig. 2.2** The structure of PBD dimer SG2000

six models (Hartley et al. 2004; Alley et al. 2004). The drug was also active in mice bearing human ovarian cancer xenografts that have acquired resistance to the conventional DNA cross-linking antitumour drug cisplatin (Hartley et al. 2004). Based on the large body of data showing activity and tolerability in preclinical models, SG2000 entered early phase clinical trials in 2004. Three different dosing schedules were evaluated against solid tumours and one against haematological malignancies. The conclusion from these studies was that SG2000 had activity in patients with solid tumours or advanced leukaemias with manageable toxicity (Kadia et al. 2009; Puzanov et al. 2011).

The formation of DNA interstrand cross-links by SG2000 was confirmed in studies in naked DNA, cells in culture and in tumours in vivo (Gregson et al. 2001; Hartley et al. 2004). In addition, DNA cross-linking was measured in patients during Phase I studies (Puzanov et al. 2011). The highly cytotoxic interstrand cross-links produced by PBD dimers including SG2000 have several characteristics that distinguish them from those produced by conventional cross-linking chemotherapeutic agents such as the nitrogen mustards (e.g. melphalan, chlorambucil, cyclophosphamide) and platinum drugs (e.g. cisplatin, carboplatin, oxaliplatin). In addition to binding in a different groove of DNA, the cross-links produced span a larger number of base pairs. For example, the interstrand cross-link produced by SG2000 is formed preferentially in the DNA sequence 5′-PuGATCPy-3′ (where Pu is purine and Py is pyrimidine), with the covalent adduct spanning between the guanines in the central four base pairs (Fig. 2.3). This sequence selectivity results not only in a more limited number of cross-links but also a much higher proportion of the critical cytotoxic interstrand cross-link compared to other types of collateral DNA damage. An important feature of the resulting PBD cross-links is their minimal distortion of the structure of the DNA (Gregson et al. 2001), a property which appears to be responsible for their lack of recognition, and therefore persistence, in cells (Hartley et al. 2004; Clingen et al. 2005). This persistence contributes to the potent activity against tumour cells, including those that only proliferate slowly.

The potency of PBD dimers can be enhanced by rational structural modification. For example, the corresponding dimer to SG2000 but containing a 5-carbon rather than a 3-carbon tether between the PBD monomer units (SG2057, Fig. 2.4) spans an extra base pair of DNA and has tenfold enhanced cross-linking efficiency and superior in vitro cytotoxic potency (Gregson et al. 2004). In in vivo models,

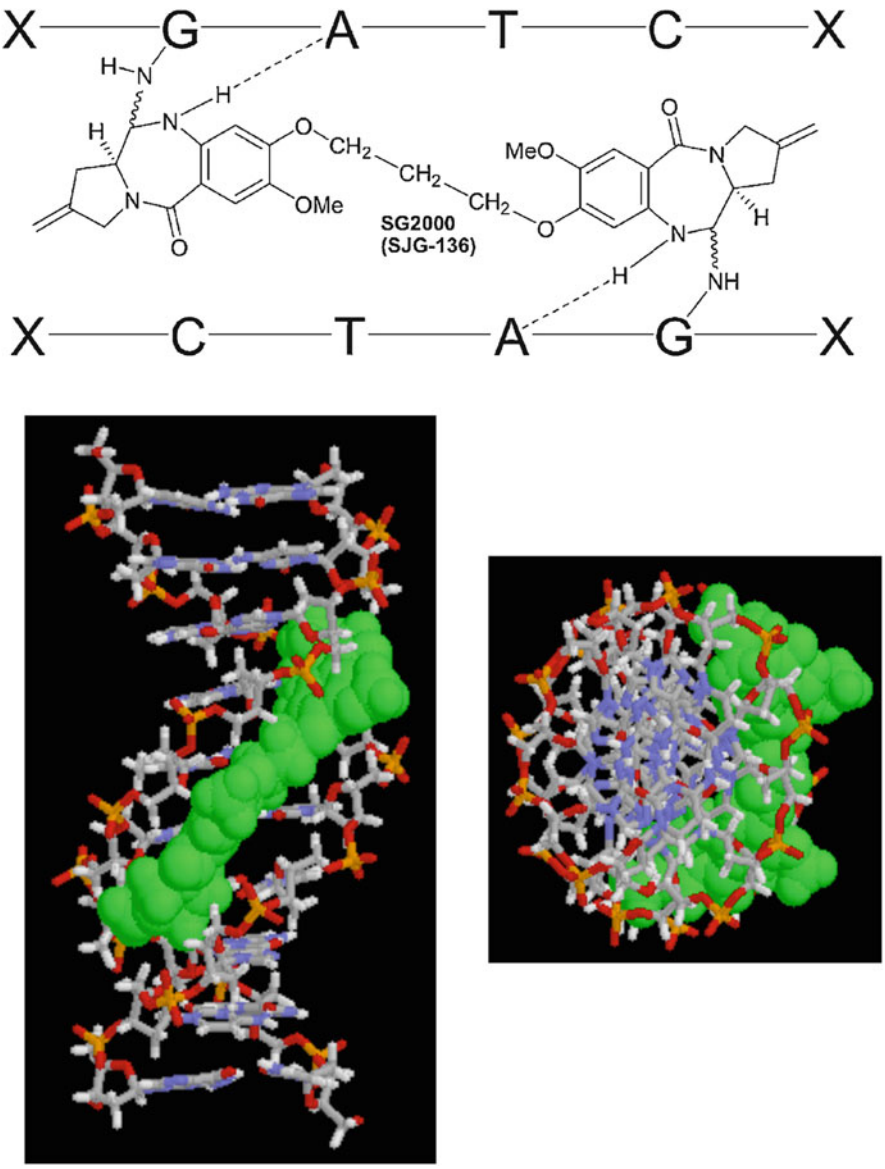


**Fig. 2.3** Schematic representation of the DNA interstrand cross-link formed by SG2000 (*top*) and models showing SG2000 (*green*) binding and cross-linking in the minor groove of DNA revealing minimal distortion of the DNA structure

SG2057 showed a greater tumour growth delay and response rate compared to SG2000 (Hartley et al. 2012). Structure-activity relationships with synthetic PBD monomers revealed that in vitro potency could also be enhanced by C2-unsaturation in the pyrrole C-ring (Antonow et al. 2010). An example is

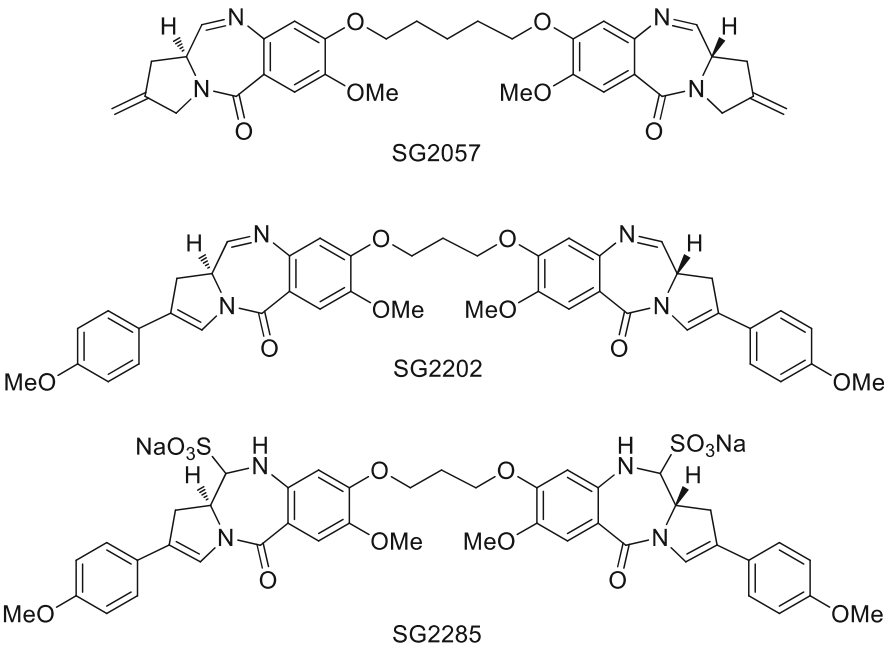


**Fig. 2.4** Structure of PBD dimers SG2057, SG2202 and SG2285

SG2202 (Fig. 2.4) where the two PBD monomer units retain an endo-exo unsaturation motif with resulting cytotoxicity now exhibited in the picomolar or, in some cell lines, sub-picomolar range (Howard et al. 2009). Development of this agent was challenging, however, due to solubility issues. To overcome this, SG2202 was converted into the C11/C11′ bisulfite adduct prodrug SG2285 (Fig. 2.4). This compound is freely water soluble and retains the in vitro potency of SG2202 (Howard et al. 2009). It is highly efficient at producing DNA interstrand cross-links in cells, which form more slowly than by SG2202 as would be expected due to the time needed for elimination of the bisulfite groups from the prodrug to generate the active drug (Hartley et al. 2010). SG2285 is also highly active in vivo against hard-to-treat human cancer xenografts including LOX-IMVI melanoma, SKOV-3 ovarian, PC3 prostate and Bx-PC-3 pancreatic models (Hartley et al. 2010).

The delivery of a highly active PBD dimer as a prodrug resulted in an agent with very different cellular and whole animal pharmacokinetic properties compared to SG2000. The data also suggested an increased therapeutic index in vivo. The ability to generate PBD dimer molecules that display such exquisite potency, that have multiple potential sites of attachment and that can be inactivated with an appropriate modification suggested that they may have a potential role in strategies designed to target, and then release, a highly cytotoxic warhead directly at a tumour site. An obvious example of this is as the ‘warhead’ component of antibody drug conjugates.

## 2.2 Antibody PBD Conjugates (APCs)

Two different sites on PBD dimers have been utilised to attach linkers: N10 and C2 (Fig. 2.5). Since the N10 position is involved in covalent binding to DNA, linker attachment at this point produces a prodrug, as well as an ADC payload. As a result, however, attachment at the N10 position requires a linker that becomes completely traceless following cleavage. In contrast, attachment at C2 can allow a flat substituent such as a phenyl ring within the DNA minor groove and which, as described above, can potentially enhance PBD activity.

For proof of concept, antibody drug conjugates utilising the well-characterised antibody trastuzumab (Herceptin) have been synthesised using either of the two attachment sites on potent PBD dimers with cleavable valine-alanine dipeptide linkers (Hartley et al. 2013). Stochastic conjugation was to cysteine residues on the antibody resulting in an average drug to antibody ratio (DAR) of around 2.5. Activity was evaluated in vitro against Her2-expressing cell lines demonstrating potent, selective cytotoxicity. The APCs showed durable complete regressions in vivo at 1 mg/kg (i.v. qwk × 3) and significant growth delays at 0.3 mg/kg against human breast cancer Her2-expressing BT474 xenografts. Tumour-free survivors and immunological selectivity were also obtained after a single dose of 1 mg/kg and superiority to T-DM1 (DAR 3.2) in this model at one tenth of the administered

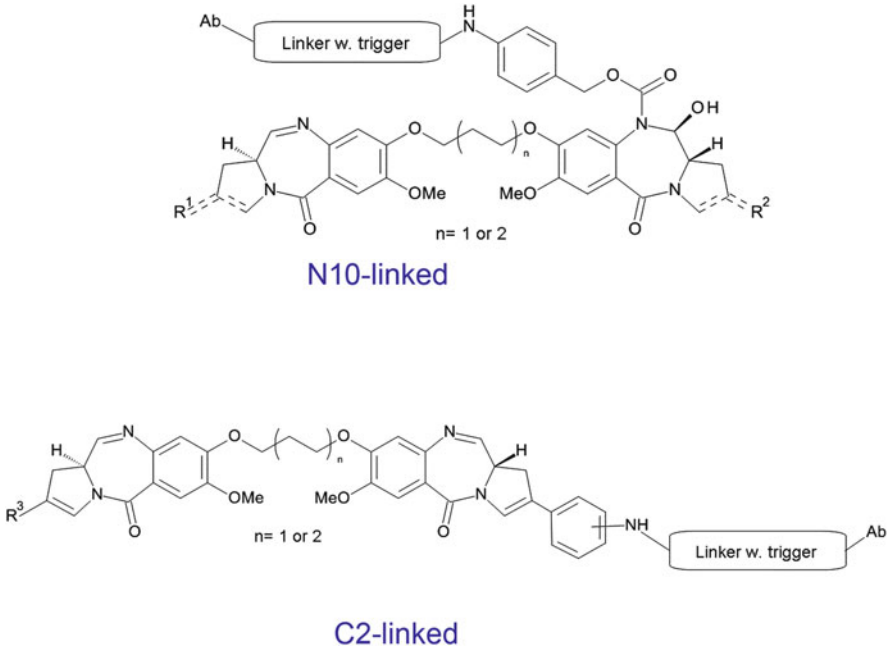


**Fig. 2.5** Attachment of linkers to a PBD dimer can be through the N10 position (*top*) or C2 position (*bottom*)

dose. A trastuzumab C2-linked APC was also highly active in the lower Her-2 expressing human breast carcinoma xenograft model JIMT-1. Significant growth delays were again observed at 0.3 mg/kg (i.v. qwk × 3) with durable regressions, including 7/10 tumour-free survivors, at 1 mg/kg.

### 2.2.1 C2-Linked APCs

Two further C2-linked antibody PBD conjugates have been reported, both delivering the same PBD dimer, SGD-1882 (Fig. 2.6). This warhead is highly potent against both solid tumour and haematological cell lines, including those known to express the MDR-1 (P-glycoprotein) efflux pump. In both cases the PBD is attached to the antibody using a maleimidocaproyl valine-alanine dipeptide linker via a C2-*para* aniline residue (Fig. 2.6).

In the first conjugate, the antibody is the anti-CD70 hIF6 with site-specific conjugation to engineered cysteines at position 239 on the heavy chain of the antibody (Jeffrey et al. 2013). This allowed consistency in drug loading and low levels of aggregation compared to stochastic conjugation through endogenous interchain cysteines on this antibody. The resulting APC was shown to be highly active and immunologically specific towards CD70-positive non-Hodgkin lymphoma (NHL) and renal cell carcinoma (RCC) cell lines (Jeffrey et al. 2013). Furthermore, the agent had a pharmacokinetic profile similar to the parent antibody and was resistant to drug release in circulation. In vivo, the APC gave impressive antitumour activity against CD70-positive human tumour xenograft models in mice when given as two weekly doses. Complete responses were observed at 0.3 mg/kg in mice bearing Caki-1 RCC tumours and at 0.1 mg/kg in the highly sensitive MHH-PREB-1 NHL model. In disseminated models, $hIFb_{239C}$-PBD (SGN-CD70A) at two weekly doses of 0.1 mg/kg was able to cure 10/10 mice with ACHN RCC, and a single dose of 0.1 or 0.3 mg/kg showed a survival advantage against Raji NHL. Importantly, the APC was tolerated in mice at a dose of 2.5 mg/kg.

The second C2-linked APC is SGN-CD33A (Kung Sutherland et al. 2013). In this APC the humanised anti-CD33 antibody h2HI2 was again engineered to contain cysteine at position 239 on the heavy chain with conjugation to SGD-1882 via the maleimidocaproyl valine-alanine linker. SGN-CD33A was highly active against all twelve CD33-positive AML cell lines tested, regardless of MDR or p53 status. This

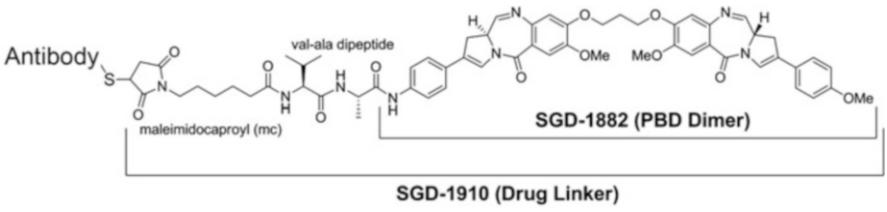


**Fig. 2.6** Structure of the PBD dimer SGD-1882 linked via its C2 position to a maleimidocaproyl valine-alanine dipeptide linker

was in contrast to gemtuzumab ozogamicin (GO), the CD33-targeting ADC that delivers calicheamicin as the warhead, which showed a higher mean $IC_{50}$ and was only active in 8/12 lines. SGN-CD33A was also active (and superior to GO) in 15/18 primary AML samples, with activity demonstrated across the entire cytogenetic risk spectrum (unfavourable, intermediate and favourable) (Kung Sutherland et al. 2013).

The ADC also demonstrated complete and durable responses against subcutaneous AML models and potent antileukaemic activity against disseminated models. In sensitive models, single doses as low as 30 μg/kg could reduce tumour growth with 100 μg/kg giving complete responses in all mice. In drug-resistant models, doses up to tenfold higher were required to give comparable results, whereas GO was not active in these models at the schedules tested (Fig. 2.7). Specificity was

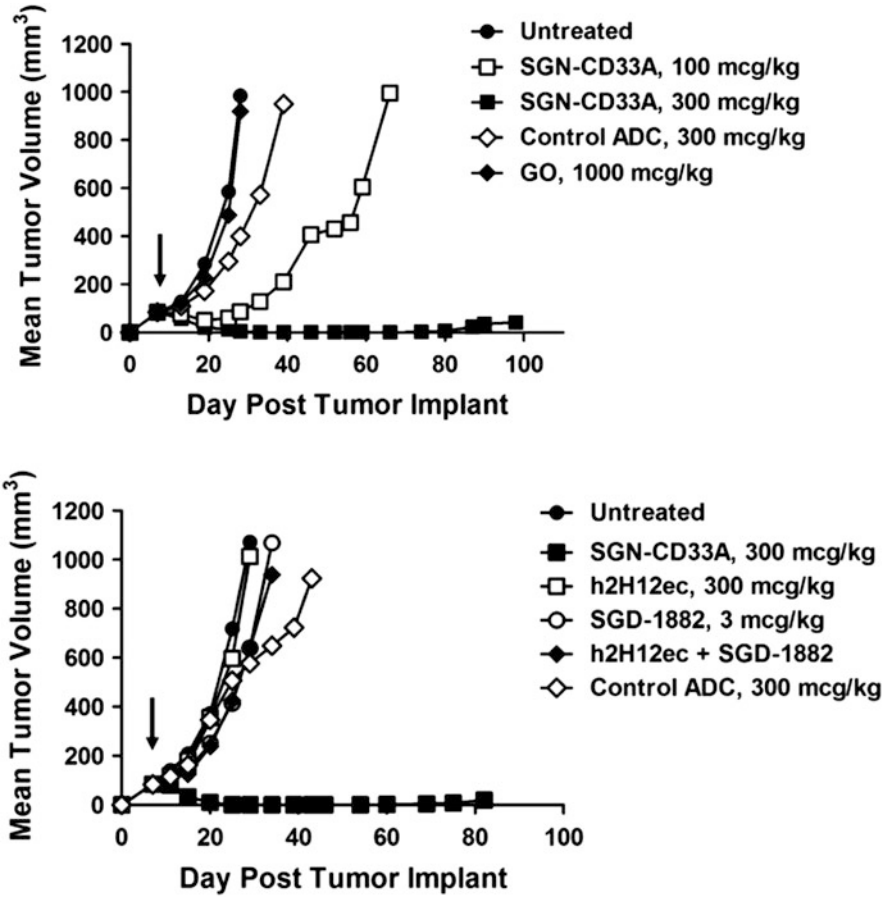


**Fig. 2.7** Single-dose anti-tumour activity of SGN-CD33A against MDR+ TF1-α cells grown as subcutaneous model in SCID mice. Control ADC is a non-binding control ADC, GO is gemtuzumab ozogamicin, h2H12ec is the naked antibody, and SGD-1882 is the warhead. Data are from Kung Sutherland et al. (2013)

demonstrated by lack of activity for the unconjugated antibody and equivalent dose of the free warhead or an unconjugated mixture of the two (Fig. 2.7) (Kung Sutherland et al. 2013).

### 2.2.2 N10-Linked APCs

Several N10-linked PBD-containing ADCs have also been described. ADCT-301 is an ADC consisting of the human IgG1 anti-CD25 antibody HuMax®-TAC stochastically conjugated via a cathepsin-cleavable valine-alanine linker to the PBD dimer warhead SG3199 (Fig. 2.8) with a DAR of 2.3 ± 0.3 (Flynn et al. 2016). This APC has picomolar affinity for the IL-2R-α, comparable to that of the naked antibody. It has potent and highly selective cytotoxicity against CD25-expressing haematological cancer cell lines, and in vivo a single-dose i.v. administration showed curative activity at 0.5 mg/kg against anaplastic large-cell lymphoma Karpas 299 xenografts. Activity was markedly superior to CD30-targeting Adcetris® (brentuximab vedotin). This result was impressive given that the cell line expresses CD30 at levels nearly three times the level of CD25 and Adcetris targets the tubulin binder auristatin E with a DAR of 3.4. ADCT-301 is tolerated in mice at 9 mg/kg.

Increased expression of delta-like 3 (DLL3), a dominant inhibitor of notch signalling normally expressed during development in the Golgi, was discovered in patient-derived (PDX) small-cell lung cancer (SCLC) and large-cell neuroendocrine carcinoma (LCNEC) tumours (Saunders et al. 2015). This finding was confirmed in primary tumours, with DLL3 protein shown to be expressed on the surface of cells, but not on normal adult tissues. A DLL3-targeted ADC, SC16LD6.5, was developed comprising of a humanised monoclonal antibody conjugated to warhead SG3199 as in Fig. 2.8. This APC, with a mean DAR of 2, induced durable regressions in vivo across multiple PDX models (Fig. 2.9). Efficacy correlated with DLL3 expression and responses were observed in PDX

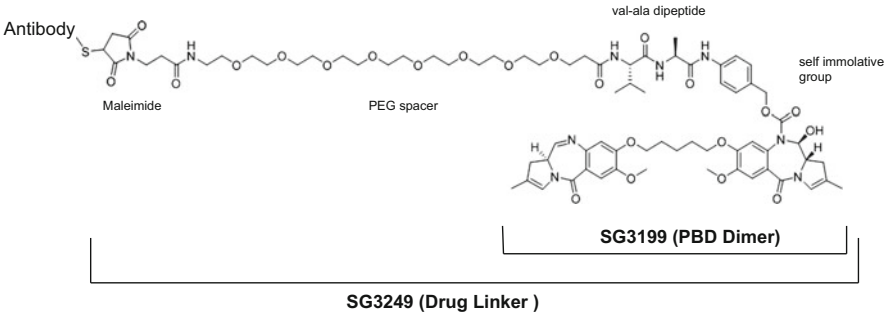


**Fig. 2.8** Structure of the PBD dimer SG3199 linked via its N10 position to a maleimidocaproyl valine-alanine dipeptide linker

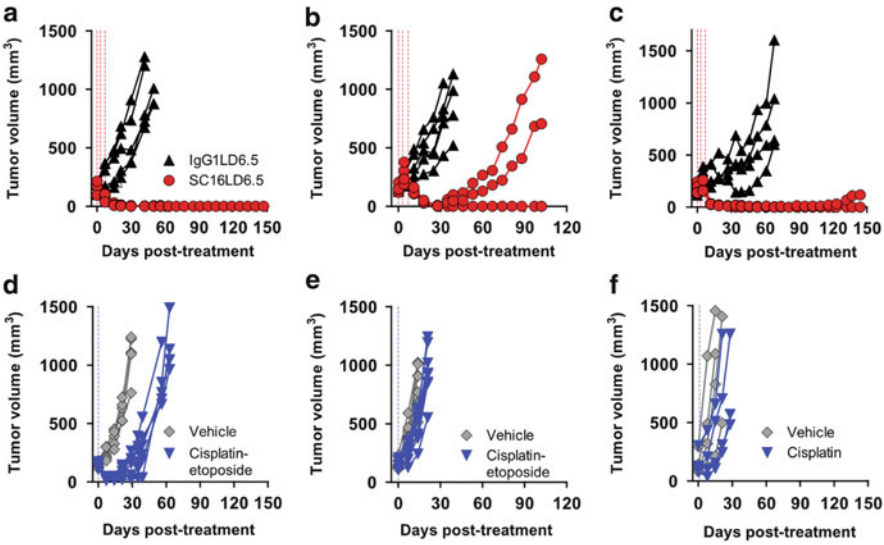


**Fig. 2.9** Demonstration of in vivo efficacy with SC16LD6.6. Mice bearing SCLC LU64 (**a** and **d**), SCLC LU86 (**b** and **e**) or LCNEC LU37 (**c** and **f**) PDX tumours were treated with control nontarget ADC IgG1lD6.5 or SC16LD6.5 (1 mg/kg) (**a**–**c**) on a Q4Dx3 regimen or vehicle or standard of care chemotherapy of cisplatin (5 mg/kg) on the day of randomisation and etoposide (8 mg/kg, where indicated) on the day of randomisation and the two subsequent days (**d**–**f**). Data are from Saunders et al. (2015)

models from patients with both limited and extensive-stage disease and were independent of sensitivity to standard cytotoxic chemotherapy (Saunders et al. 2015).

hLL2-PBD is an ADC composed of the humanised antihuman CD22 antibody epratuzumab stochastically conjugated via the same cleavable linker to the same warhead as ADCT-301 and SC16LD6.5. Specific and potent activity was demonstrated against CD22-expressing human Burkitt lymphoma and human diffuse large B-cell lymphoma-derived cell lines (Zammarchi et al. 2015). In mice, hLL2-PBD has a blood half-life of approximately 9 days, and in vivo a single-dose administration was curative against Daudi and Ramos human xenograft models at 0.3 and 1 mg/kg, respectively. At equivalent doses it was markedly superior to an anti-CD22 ADC delivering auristatin E (Hu10F4-vcMMAE) in the Ramos model.

KTNO182A, an anti-KIT PBD-containing ADC, was reported to have potent kit-dependent antitumour activity in vitro and dose-dependent tumour growth inhibition and regression in a range of kit-expressing xenografts in vivo (Lubeski et al. 2015).

## 2.3 Emerging Clinical Data

Several PBD-containing ADCs have progressed into the clinic. The first clinical data to emerge was from an interim analysis of a Phase I trial of SGN-CD33A in patients with CD33-positive acute myeloid leukemia (Stein et al. 2014). Monotherapy with the drug has demonstrated dose-dependent activity in AML patients with adverse risk factors. Subsequently, SGN-CD33A plus hypomethylating agent has been found to be a well-tolerated regimen with a high remission rate in frontline unfit AML. As a result, SGN-CD33A (now named Vadastuximab talirine) is currently in the Phase 3 CASCADE study, a randomized, double-blind, multi-center trial designed to evaluate the drug in combination with hypomethylating agents in previously untreated AML patients.

Recently, initial results from the first-in-human trial of an APC targeting a solid tumour have been reported (Rudin et al. 2017). SC16LD6.5, now named Rovalpituzumab teserine, demonstrated encouraging single agent antitumour activity with a manageable safety profile in patients with recurrent small cell lung cancer previously treated with one or two chemotherapeutic regimens, including a platinum-based regimen. The efficacy of the agent is currently being further evaluated in the Phase 2 TRINITY study in subjects with relapsed or refractory DLL3-expressing small cell lung cancer.

Table 2.1 summarises the APCs that had entered the clinic by the end of 2016. The promising clinical data starting to emerge suggests that the impressive pre-clinical data with both C2- and N10-linked APCs may translate into real patient benefit in the clinic.

**Table 2.1** APCs that have entered clinical trial by the end of 2016

| Company | ADC | Target | Indication | PBD | Linkage | Name |
|---|---|---|---|---|---|---|
| Seattle Genetics | SGN-CD33A | CD33 | AML | SGD-1882 | C2 ValAla | Vadastuximab talirine |
| Seattle Genetics | SGN-CD70A | CD70 | NHL RCC | SGD-1882 | C2 ValAla | |
| Stemcentrx | SC16LD6.5 | DLL3 | SCLC | D6.5 (SG3199) | N10 ValAla | Rovalpituzumab tesirine |
| ADC Therapeutics | ADCT-301 | CD25 | Lymphoma AML | SG3199 | N10 ValAla | |
| ADC Therapeutics | ADCT-402 | CD19 | B-NHL B-ALL | SG3199 | N10 ValAla | |
| Seattle Genetics | SGN-CD123A | CD123 | AML MDS | SGD-1882 | C2 ValAla | |
| Seattle Genetics | SGN-CD19A | CD19 | B-cell malignancies | SGD-1882 | C2 ValAla | |
| Seattle Genetics | SGN-CD352A | CD352 | Multiple myeloma | SGD-1882 | C2 ValAla | |

## 2.4 Properties and Advantages of APCs

The PBD dimers offer a novel warhead mechanism of action in the ADC arena, exploiting a completely different cellular target to the more widely used auristatin and maytansinoid tubulin inhibitors and a different mode of DNA damage to other DNA interacting warheads such as calicheamicin. These other payloads are structurally complex and can only be used in the context of a targeted therapy such as an ADC. In contrast, the PBD dimers are relatively easy to make through robust and scalable synthetic routes, and a member of the class has demonstrated a therapeutic index in patients as a stand-alone agent.

The fully synthetic PBD dimer structure provides a number of sites for linker attachment, two of which have been described, and a whole range of conjugation and linker chemistry options. The greater potency of some PBD dimer warheads compared to the tubulin inhibitors provides the important ability to target low copy number antigens and to exploit ADCs with a lower drug-antibody ratio (typically around 2) than traditional auristatin and maytansinoid-containing ADCs (typically close to 4). Indeed, the level of delivery of PBD warhead is often 10- to 100-fold lower than for other classes which may be particularly important for the treatment of solid tumours where ADC penetration may be limited. Furthermore, PBD dimers are active in tumour types, e.g. colon, which are inherently resistant to tubulin binder drugs.

Rational synthetic modification of the PBD dimer structure can, however, enable potency to be modulated over several orders of magnitude. It can also be used to modulate the biophysical properties of PBD dimer payloads. Initial experience with warhead SGD-1882 suggested that the hydrophobic properties of the PBD dimer required site-specific cysteine conjugation to minimise aggregation (Jeffrey et al. 2013). Later generation payloads, however, can be efficiently employed in stochastic cysteine conjugation settings as demonstrated in ADCT-301 and hLL2-PBD described above. Alternative conjugation strategies are also possible, as clearly shown by the site-specific conjugation of PBD dimer payloads using click cycloaddition chemistry to genetically encoded azide-containing amino acids within antibodies (VanBrunt et al. 2015).

The studies with SGN-CD33A demonstrate impressive activity in AML models with a multidrug-resistant phenotype (Kung Sutherland et al. 2013). Similarly, SGN-CD70A is highly active in MDR+ models of renal cell carcinoma and non-Hodgkin lymphoma (Sandall et al. 2014). Although P-glycoprotein expression can influence in vitro and in vivo activity of some PBD dimers (Guichard et al. 2005), it is dependent on the particular PBD structure. Indeed, many potent PBD dimer warheads are not significant P-glycoprotein substrates (Mao et al. 2015). This is an important advantage over the warheads used in the two clinically approved ADCs Adcetris® and Kadcyla® where P-glycoprotein overexpression can lead to acquired resistance.

Membrane permeability is a key factor in determining bystander cell killing, an important consideration in solid tumours with heterogeneous cellular antigen target

expression. PBD-containing ADCs with cleavable linkers demonstrate highly efficient bystander killing of antigen-negative cells in models of mixed antigen-positive and antigen-negative cell both in vitro and in vivo (Flynn et al. 2016; Li et al. 2015). In contrast, less permeable payloads such as monomethylauristatin F or auristatin T killed only the antigen-expressing cells (Li et al. 2015).

The mechanism of action of the PBD dimers is through the formation of highly cytotoxic DNA interstrand cross-links. Following treatment of cells with the free warhead, cross-links form rapidly reaching a peak within 2 h as determined by a modification of the single-cell gel electrophoresis (comet) assay. In contrast, following a 2-h exposure of target antigen-expressing cells to an APC consisting of PBD warhead conjugated via a cathepsin-cleavable linker, the cross-links do not reach a peak until 4–8 h (Flynn et al. 2016). This is due to the time required for cellular uptake and processing to release free drug within the target cell.

An important feature of the highly cytotoxic DNA interstrand cross-links produced by the PBD dimers is their persistence in cells (Hartley 2011). This contributes to their potency and also to their ability to affect slowly proliferating target cells, including cancer stem, or tumour-initiating, cells. This was recently illustrated by an ADC in which a PBD dimer payload was site specifically conjugated to an antibody against a tumour-associated antigen overexpressed on many carcinomas on both bulk tumour and cancer stem cells (Harper et al. 2015). In vivo, the APC was efficacious against target-positive solid tumour carcinoma models, producing long-term regressions. The ADC was more potent than those delivering tubulysin or auristatin tubulin binder payloads. Importantly, the PBD-containing ADC significantly reduced the cancer stem cell population of tumours in vivo unlike the tubulin inhibitor-containing ADCs. Additionally, the APC was efficacious against tumours that had acquired resistance to a tubulysin-based ADC (Harper et al. 2015).

Evidence for the effective targeting of tumour-initiating cells by APCs was further provided by the preclinical studies with SC16LD6.5 (Saunders et al. 2015). Limiting dilution of cells in PDX serial transplantation experiments indicated that the lack of tumour recurrence after APC exposure resulted from the targeting of tumour-initiating cells expressing the target DLL3.

The DNA damage produced by the PBD dimers has been shown to induce a robust DNA damage response in the form of histone 2AX phosphorylation at serine 139 (γ-H2AX) (Arnould et al. 2006; Wu et al. 2013). Dose-dependent increases in the levels of γ-H2AX were observed in target cells with both SGN-CD33A and SGN-CD70A (Kung Sutherland et al. 2013; Sandall et al. 2014). As would be expected, the response to the ADC was delayed compared to the free warhead.

The ability to measure the critical cytotoxic lesion produced by PBD dimers using the modified comet assay and also the resulting DNA damage response as γ-H2AX foci provides two assays that can be used as pharmacodynamic markers in vivo. Indeed, both assays have been validated for use in clinical samples and have been previously used in the clinical development of SG2000 as a nontargeted agent (Puzanov et al. 2011; Wu et al. 2013). The measurement of cross-linking in tumour cells can be used to demonstrate appropriate targeting and confirm mechanism.

Such a pharmacodynamic endpoint is not available for other warhead types such as tubulin inhibitors. In addition, the more sensitive γ-H2AX assay can be used to measure delivery, and therefore potential toxicity, to nontarget tissue.

Following DNA cross-linking, there is a cascade of cellular events leading to cell cycle arrest and apoptotic cell death. Cell cycle arrest in the G2 phase has been shown for PBD dimers (Hartley et al. 2004), and this has been confirmed for PBD-containing ADCs (Kung Sutherland et al. 2013; Sandall et al. 2015). Treatment of SGN-CD33A or free warhead SGD-1882 results in marked increases in formation of the apoptotic marker cleaved PARP (Kung Sutherland et al. 2013). SGN-CD70A activated the DNA damage-sensing protein kinases ATM and ATR within 24 hours in antigen-expressing cell lines (Sandall et al. 2015). Downstream checkpoint kinases Chk1 and Chk2 are subsequently activated, and caspase activity closely mirrors phosphorylation of Chk2. Elevated levels of pATM, pATR, pChk2 and γ-H2AX were also detected in xenograft tumours following SGN-CD70A treatment (Sandall et al. 2014).

The critical involvement of the DNA damage pathway in the cellular response to the PBD was confirmed using small-molecule inhibitors of ATM, ATR and Chk1/3 which were all synergistic with SGN-CD70A (Sandall et al. 2015). Interestingly, synergistic combinations of SGN-CD70A and DNA damage kinase inhibitors or an Mps1 inhibitor decreased the number of cells in G2 whilst increasing the number of polyploidy cells. These data suggest the induction of mitotic catastrophe, a mechanism that has been previously described to result from DNA interstrand cross-linking in mammalian cells under certain circumstances (Osawa et al. 2011).

Both SGN-CD33A and the PBD dimer warhead SGD-1882 produced dose-dependent increases in phosphorylated p53 in CD33-expressing HEL 92.1.7 cells (Kung Sutherland et al. 2013). However, cytotoxic effects of SGN-CD33A were observed in a wide panel of AML cell lines regardless of p53 status. This is consistent with previous studies on the PBD dimer SG2000 where the cytotoxicity of primary B-CLL cells was not related to p53 status (Pepper et al. 2004) suggesting a p53-independent mechanism of cell killing unlike other cross-linking agents, such as chlorambucil where marked resistance is observed in p53 non-expressing or p53 mutant cells.

Previously, the combination of PBD dimer SG2000 and fludarabine was found to be synergistic in chronic lymphocytic leukaemia cells (Pepper et al. 2007). Fludarabine-mediated suppression of the excision repair enzyme ERCC1 contributed to the observed cytotoxic synergy. Cells defective in the XPF-ERCC1 endonuclease, a component of nucleotide excision repair and also the processing of DNA interstrand cross-links, or defective in homologous recombination, have been shown to exhibit increased sensitivity to PBD dimers (Clingen et al. 2005). SGN-CD33A was also found in preclinical studies in AML to deliver significantly improved antitumour activity when combined with therapies that are commonly used in treating myeloid malignancies (Sutherland et al. 2014). For example, SGN-CD33A in combination with hypomethylating agents 5-azacytidine (Vidaza) and 5-aza-2-deoxycytidine (Decitabine) was synergistic in MDR-positive cell lines when treated simultaneously, and this was confirmed in vivo. Interestingly,

exposure of AML cells to hypomethylating agents appeared to up-regulate CD33 levels on the cells, the target antigen of the ADC, which could explain the synergistic interaction. In addition, SGN-CD33A in combination with cytarabine demonstrated greater activity in preclinical models of MDR-positive AML. Increased cell killing was associated with synergism in the activation of DNA damage sensor and apoptosis pathways (Sutherland et al. 2014).

## 2.5 Conclusions

ADCs delivering PBD dimer payloads represent a novel mode of action in the ADC area. They are a highly flexible platform with many advantages over existing payloads. The emerging clinical data suggest that the impressive preclinical results in both haematological malignancies and solid tumours may translate into clinically important drugs within the next few years.

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# 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 p$K_a$ 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.

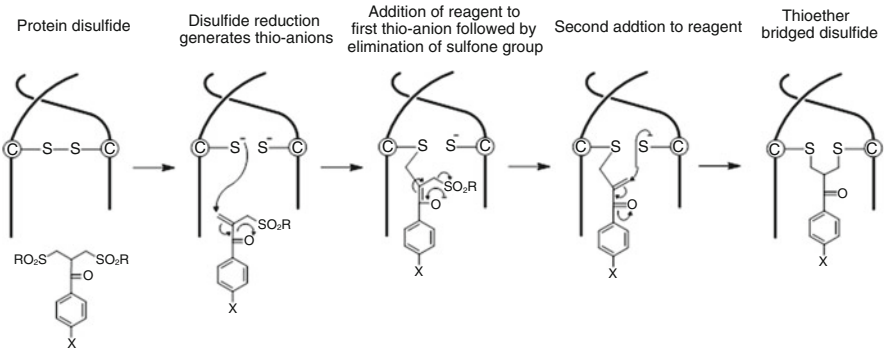


**Fig. 3.1** General reaction scheme of the sequential bis-alkylation reaction of a bis-sulphone linker

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).

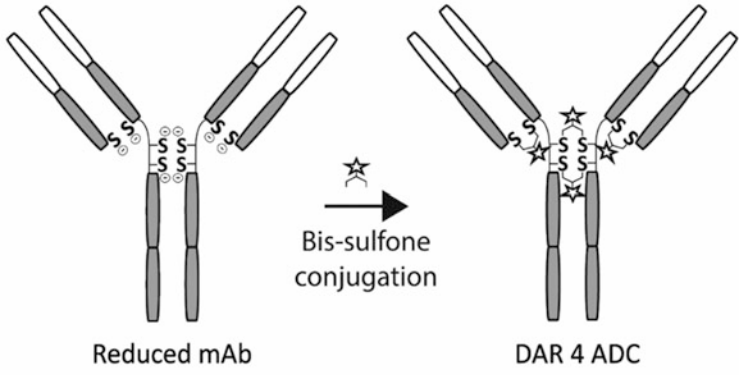


**Fig. 3.2** Conjugation of bis-sulphone ADC reagents to an IgG1 type antibody at the four interchain disulphides to give an ADC with a DAR of 4 as the major product

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

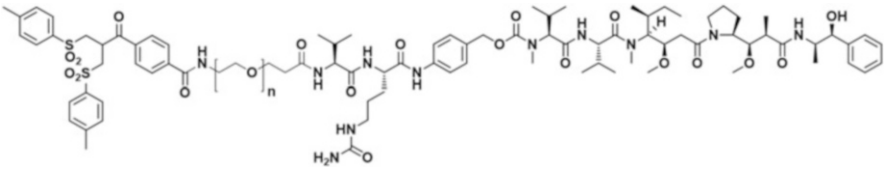

**Fig. 3.3** Example bis-sulphone reagent consisting of (*left* to *right*) a bis-sulphone linker, a PEG unit to aid solubility, an enzymatically cleavable self-immolative linker (valine-citrulline-*p*-aminobenzyloxycarbonyl, *PAB*) and a cytotoxic payload (monomethyl auristatin E, *MMAE*). Reprinted 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

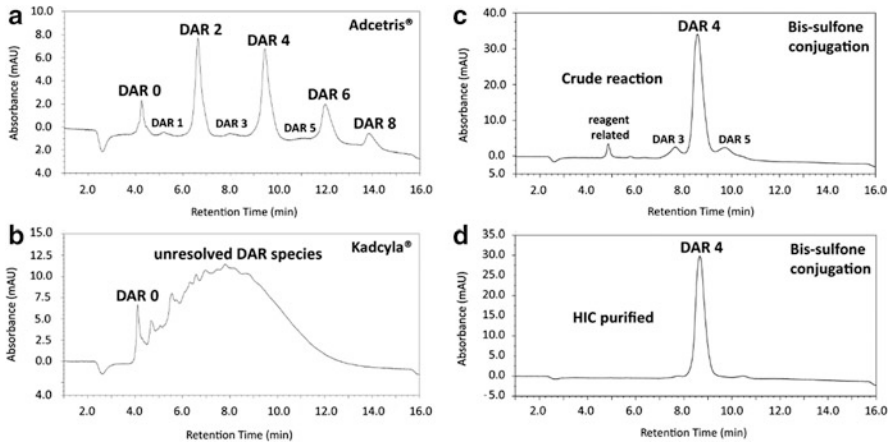


**Fig. 3.4** HIC profiles for (**a**) Brentuximab vedotin (Adcetris®), (**b**) Trastuzumab emtansine (Kadcyla®), (**c**) example crude reaction mixture for a bis-sulphone val-cit-PAB-MMAE reagent conjugated to brentuximab to give a DAR 4 as the major product, (**d**) Bis-sulphone conjugated brentuximab-MMAE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

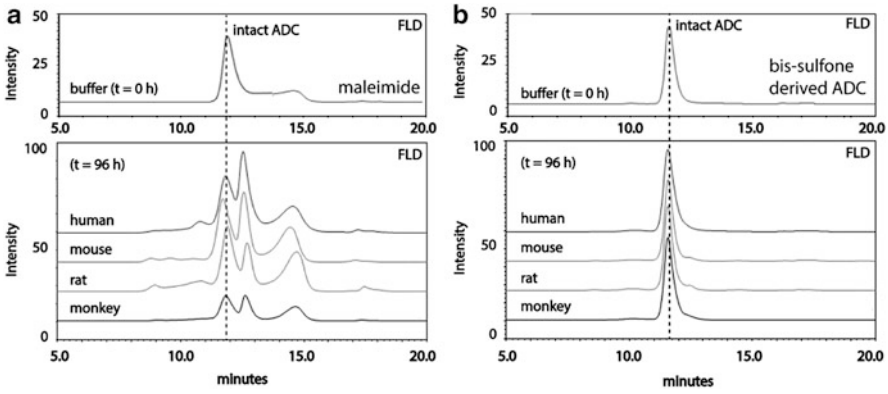


**Fig. 3.5** Stability of conventional maleimide ADC (**a**) versus bis-sulphone-derived ADC (**b**) in different sera. ADCs were prepared with a fluorescent dye as a mock payload to allow analysis by size-exclusion chromatography. The bis-sulphone-derived ADC showed excellent stability after 96 h human, 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

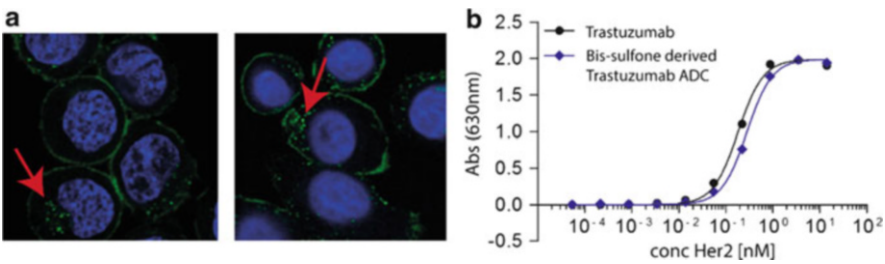


**Fig. 3.6** (**a**, *Left image*) Internalisation of a bis-sulphone-derived trastuzumab-FITC conjugate into *Her2* positive SK-BR-3 cells. (**a**, *Right image*) Internalisation of trastuzumab, detection with an anti-human mAb conjugated to Alexa Fluor® 488. (**b**) *Her2* target binding, comparison 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

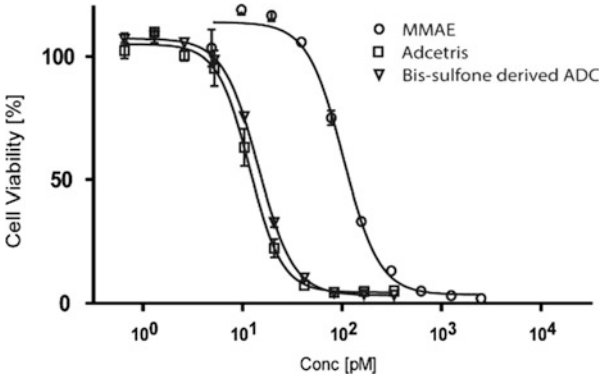


**Fig. 3.7** In vitro cell viability assay in an antigen positive cell line (Karpas 299) for a bis-sulphone-derived brentuximab conjugate (DAR 4), a maleimide-derived brentuximab MMAE conjugate (average DAR 4, Adcetris®) and MMAE. Both conjugates showed a comparable in vitro potency, superior to unconjugated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

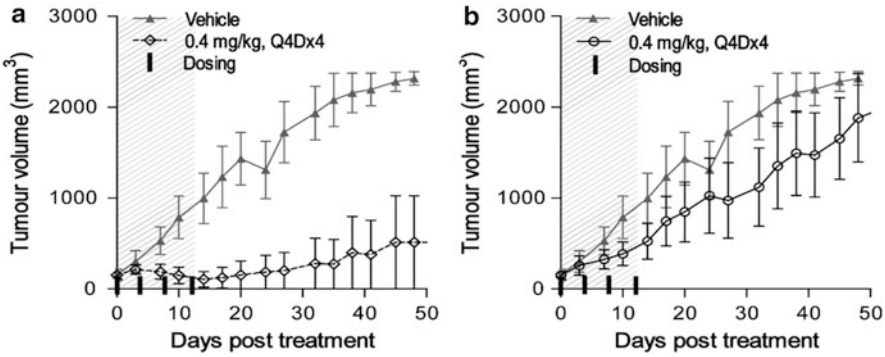


**Fig. 3.8** In vivo efficacy in a Karpas 299 SCID mouse xenograft model. The bis-sulphone conjugated brentuximab-MMAE conjugate (*left*) showed better efficacy compared to the maleimide conjugated brentuximab-MMAE conjugate Adcetris® (*right*)

## 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.

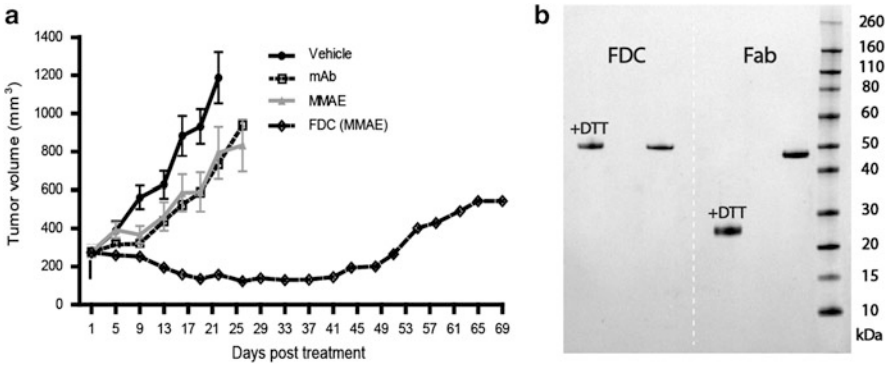


**Fig. 3.9** (**a**) Evaluation of in vivo efficacy of a bis-sulphone-derived Fab MMAE conjugate (DAR 1) in a BT-474 SCID mouse xenograft model showing tumour regression and 5/10 tumour free survivors. (**b**) SDS-PAGE analysis of Fab and a bis-sulphone-derived Fab MMAE conjugate under reducing and non-reducing 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).

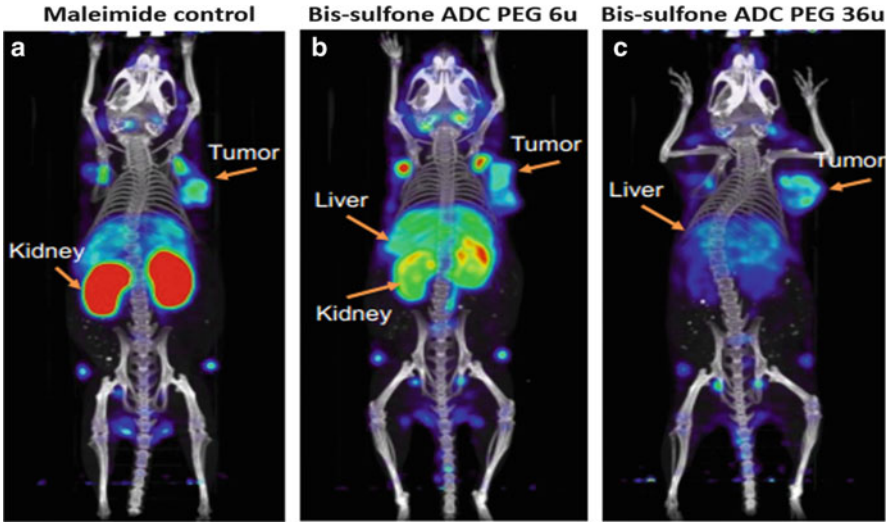


**Fig. 3.10** PET images, provided courtesy of ImaginAb, Inc. U.S., showing SCID mice 48 h after administration with $^{89}$Zr-labelled minibody conjugates prepared with (**a**) malemide–desferrioxamine reagent (**b**) bis-sulphone–desferrioxamine reagent containing a 6 repeat unit PEG (**c**) bis-sulphone–desferrioxamine 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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# Chapter 4
# Calicheamicin Antibody-Drug Conjugates for Liquid and Solid Tumor Indications

**Hans-Peter Gerber, Marc Damelin, and Puja Sapra**

**Abstract** Antibody-drug conjugates (ADCs) for solid tumor indications have traditionally employed microtubule disrupting agents. We recently demonstrated utility of calicheamicin, a double-strand DNA break inducing payload, when conjugated to an antibody targeting a cell surface antigen expressed on the surface of cancer stem cells (CSCs) or tumor-initiating cells (TICs) and Ephrin-A4 (EFNA4). When tested in preclinical models of breast and ovarian cancer, the hydrazone linker-based calicheamicin conjugate (PF-06647263) displayed robust antitumor activity, and the compound is currently being evaluated for clinical benefit to cancer patients. Calicheamicin is a member of a highly potent enediyne class of deoxyribonucleic acid (DNA)-damaging cytotoxic natural products with a unique mechanism of action that involves scission of DNA. Two additional calicheamicin-based ADCs are in late-stage clinical development, including the ADC, inotuzumab ozogamicin, targeting CD22-positive, liquid tumors including NHL and ALL, and the CD33-targeting gemtuzumab ozogamicin, targeting AML.

The recent expansion of the utility of calicheamicin conjugates to solid tumors may have a broader impact on ADC development, as their mechanism of action leading to tumor cell death is entirely different from microtubule disrupting agents. In particular, calicheamicin impacts quiescent or dormant cells as well as cycling cells, whereas microtubule inhibitors impact only cycling cells. Herein, we review the mechanism and key pharmacological findings of calicheamicin conjugates targeting liquid and solid tumors and discuss potential areas for future development of calicheamicin conjugates.

**Keywords** Antibody drug conjugates • Calicheamicin • Tubulin inhibitor • Cancer stem cells • Oncology

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## 4.1 Mechanism of Action of Calicheamicin as an ADC Payload

Calicheamicin is a member of a highly potent enediyne class of deoxyribonucleic acid (DNA)-damaging cytotoxic natural products with a unique mechanism of action that involves scission of DNA. N-Ac-γ-calicheamicin DMH is a derivative of γ-calicheamicin (Hinman et al. 1993; Lee et al. 1992; Zein et al. 1988) and is the cytotoxic agent in two antibody-drug conjugates in development, inotuzumab ozogamicin (anti-CD22) and gemtuzumab ozogamicin (anti-CD33). Calicheamicins bind DNA in the minor groove and induce double-strand DNA breaks and apoptosis independent of cell cycle progression (Bernstein 2000; Damle 2004; Dedon and Goldberg 1992; Thorson et al. 2000). In addition, calicheamicin has been reported to inhibit the formation of transcription initiation complexes (Drak et al. 1991; Ho et al. 1994; Ikemoto et al. 1995). Due to its remarkable structural and conformational properties, calicheamicin demonstrates surprisingly high DNA binding specificity among all the enediynes isolated to date. It binds DNA in the minor groove with a preference for the oligopyrimidine-oligopurine sequences (T-C-C-T).d(A-G-G-A) and has been reported to inhibit transcription in vivo with similar sequence specificity (Drak et al. 1991; Ho et al. 1994; Ikemoto et al. 1995).

While calicheamicin is too potent to be administered as a free drug, it can be conjugated to an antibody in order to enhance tumor-specific delivery. The antibody-drug conjugate (ADC) binds to a target cell, internalizes into the cell, and then is routed through the endosomes and lysosomes in the cell, where the calicheamicin prodrug is released by hydrolysis of the hydrazone group in the acidic environment of the lysosomes of the antigen-positive target cells [reviewed by Shor et al. (2015)]. The calicheamicin moiety becomes activated after reductive cleavage of the disulfide bond by intracellular thiol groups, generating the highly active bi-radical intermediate. The activation of calicheamicin to calicheamicin γ1 is attributed to the high intracellular concentration of glutathione, a cytoplasmic thiol cofactor. Glutathione is a thiol-containing tripeptide that is present in micromolar concentrations in the blood, whereas its concentration in the cytoplasm or nucleus is in the millimolar range (Bellomo et al. 1992). Once in the nucleus, activated cytotoxin intercalates into DNA and causes double-strand DNA break formation, leading to induction of apoptosis and cell death (Ellestad 2011; Shor et al. 2015).

The focus during the initial efforts to develop calicheamicin-based ADCs was on acid-cleavable hydrazone linkers that are relatively stable at neutral pH (bloodstream pH 7.3–7.5), but undergo hydrolysis once internalized into the mildly acidic endosomes (pH 5.0–6.5) and lysosomes (pH 4.5–5.0). The disulfide linkage connecting the calicheamicin moiety with the hydrazone linker is further stabilized by two methyl groups to prevent premature release of calicheamicin (Hamann et al. 2002), as illustrated in the blue-colored linker structure in Fig. 4.1.

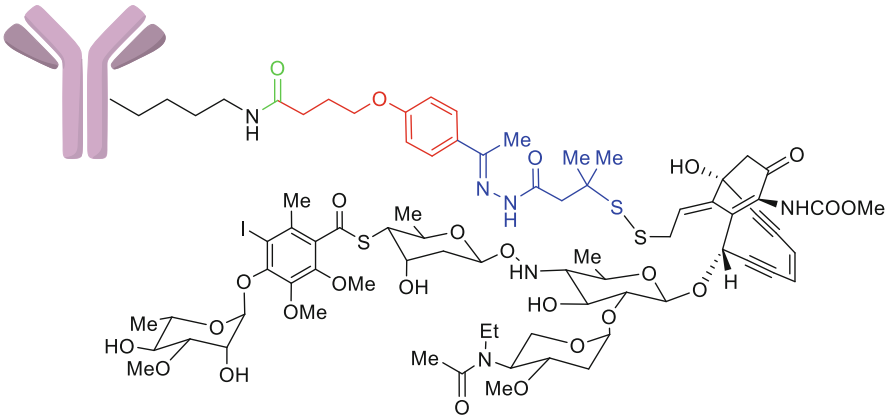

**Fig. 4.1** Molecular structure of calicheamicin conjugates. Calicheamicin conjugates consist of an acid-cleavable hydrazone linker (*blue*), a stabilized disulfide linkage (also *blue*) connecting the calicheamicin moiety (*black*) with the hydrazone linker that is further stabilized by two methyl groups (Me, *blue*), to prevent premature release of calicheamicin in circulation

The calicheamicin conjugates currently being tested in the clinic consist of this bifunctional linker, composed of an acid-labile, hydrazone-based linker and a stabilized disulfide linker, to allow for release of the prodrug calicheamicin in the reductive, intracellular environment. In preclinical models, the hydrazone linkage produced ADCs with higher potency than the corresponding amide-bearing conjugates, which still contained the stabilized disulfide linkage, but lacked the hydrazone functionality (Hamann et al. 2002). These findings provided evidence that the stabilized disulfide bond alone is insufficient for efficient release of the drug in the target cell and that the hydrazone group is required to maximize potency.

## 4.2 Preclinical Activities of Inotuzumab Ozogamicin

Inotuzumab ozogamicin is a calicheamicin-based ADC that targets the CD22 antigen, a cell surface transmembrane protein that is member of the immunoglobulin superfamily. The function of CD22 is not entirely clear, but it appears to be involved in the regulation of B-cell function and survival (Hoelzer 2014). CD22-positive cancers have been targeted with ADCs or immunotoxins for a number of reasons. CD22 is expressed in most B-lymphoid malignancies, including indolent non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), and hairy cell leukemia (HCL) (Olejniczak et al. 2006). In mature ALL, up to 90–100% of B-ALL cases showed CD22 expression. In addition to its consistent expression in B-cell cancers, CD22 is rapidly internalized by endocytosis with half-life of less than 1 h upon ligand or anti-CD22 mAb binding

and is not shed in the extracellular environment (de Vries et al. 2012; Gerber et al. 2013; John et al. 2003). B-cell specific expression pattern of CD22 also suggests that the impact of treatment with CD22-targeted agents on long-term immune functioning is expected to be minimal. Altogether, these properties make CD22 an ideal antigen for targeting with ADC therapies.

Inotuzumab ozogamicin and the parent, unconjugated G544 antibody were both characterized for binding to their targets and their biological activity in vitro. These studies established high binding affinity and specificity of inotuzumab ozogamicin for CD22, cell internalization and trafficking to lysosomes, induction of DNA damage, and cytotoxicity in CD22-positive tumor cell lines (DiJoseph et al. 2004a). Both inotuzumab ozogamicin and the parent G544 antibody bound to a human CD22-Fc protein fragment with high affinity (Kd = 150 pM). Furthermore, the antibody was determined to bind normal B-cells in human whole blood but not the B-cells, granulocytes, monocytes, or T-cells from mouse, rat, rabbit, or cynomolgus monkey (DiJoseph et al. 2004a). The G544 antibody internalized efficiently in B-cell acute lymphoblastic leukemia (ALL) and B-cell non-Hodgkin's lymphoma (NHL) cell lines and trafficked to the lysosomes, as evidenced by co-localization with the lysosomal marker LAMP-1. A mechanism-of-action study demonstrated induction of double-strand DNA breaks in B-cell ALL cells after incubation with inotuzumab ozogamicin, consistent with the primary mechanism of action of calicheamicin. It was also confirmed that G544, an IgG4 antibody, did not mediate complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC) (DiJoseph et al. 2004a). Inotuzumab ozogamicin elicited highly potent cytotoxicity against multiple B-cell origin cancer cell lines in a target-dependent and calicheamicin-dependent manner (DiJoseph et al. 2006). B-NHL cell lines showed a higher level of CD22 expression as compared to B-ALL models. Despite this finding, B-cell ALL cell lines exhibited greater sensitivity to both inotuzumab ozogamicin and unconjugated N-Ac-y-calicheamicin-DMH than B-NHL cells, suggesting potential intrinsic differences in how NHL and ALL cells may respond to inotuzumab ozogamicin (Dijoseph et al. 2007).

Having characterized inotuzumab ozogamicin activity in vitro, several major objectives were pursued in vivo including examination of (1) antitumor efficacy as a single agent in B-cell ALL models, (2) antitumor efficacy as a single agent in B-cell lymphoma models, and (3) antitumor efficacy of inotuzumab ozogamicin in combination with, in comparison to, or following relapse with chemotherapy or rituximab treatment in B-cell lymphoma models.

The therapeutic potential of inotuzumab ozogamicin in B-cell ALL was investigated in REH B-cell ALL xenografts grown subcutaneously (SC) or as disseminated tumors in immunodeficient nude mice (Dijoseph et al. 2007). When administered to nude mice with established SC xenografts of REH ALL over a range of 0.42–6.57 mg/m$^2$, which corresponds to 0.14 and 2.19 mg/kg, respectively, inotuzumab ozogamicin showed dose-dependent activity with complete tumor regressions at 6.57 mg/m$^2$. In a disseminated model of ALL, treatment with inotuzumab ozogamicin effectively suppressed tumor growth in mice, leading to

100% survival at the 3.3 mg/m$^2$ dose (Dijoseph et al. 2007). In addition, inotuzumab ozogamicin strongly inhibited the engraftment of human CD45-positive leukemic B-cells in mice with disseminated disease.

Inotuzumab ozogamicin was also evaluated for its antitumor effects in subcutaneously implanted (SC) B-cell lymphoma xenografts and disseminated B-cell lymphoma tumor models. Treatment with inotuzumab ozogamicin led to a dose-dependent regression of SC Ramos or RL B-cell lymphoma xenografts in athymic nude mice. Antitumor efficacy was evident irrespective of the size of the tumor before the initiation of therapy, including masses as large as 2 g (equivalent to 10% of the body weight) (DiJoseph et al. 2004b). The minimum efficacious dose at which some tumor growth inhibition was observed with inotuzumab ozogamicin treatment in Ramos and RL models was as low as 0.42 and 0.81 mg/m$^2$, which translates to 0.14 and 0.27 mg/kg of ADC dose level, respectively. Complete tumor regressions were observed at 6.57 mg/m$^2$ in Ramos tumors and 4.92 mg/m$^2$ in RL tumors. In addition, inotuzumab ozogamicin was highly efficacious in the systemically disseminated lymphoma model, where a dose as low as 1.65 mg/m$^2$ given within 3 or 9 days after tumor cells administration resulted in survival of 100% of mice during the observation period of the study (DiJoseph et al. 2004b).

Antitumor effects of inotuzumab ozogamicin were assessed in comparison to chemotherapy or rituximab and in resistant or relapsed tumors following chemotherapy (DiJoseph et al. 2011) or rituximab (DiJoseph et al. 2006) treatment. In these comparative studies, neither CHOP nor CVP treatment regimens were as effective as inotuzumab ozogamicin in inducing sustained tumor suppression. In addition, when the chemotherapy-resistant tumors were treated with inotuzumab ozogamicin, tumor growth was inhibited. Rituximab alone was less effective in preventing growth of established tumors compared to inotuzumab ozogamicin, but treatment of rituximab-resistant tumors with inotuzumab ozogamicin resulted in tumor growth inhibition (DiJoseph et al. 2006).

The combination of inotuzumab ozogamicin with CHOP or CVP chemotherapy resulted in more pronounced antitumor activities than either inotuzumab ozogamicin or chemotherapy alone (Dijoseph et al. 2007). The combination of rituximab and inotuzumab ozogamicin only showed improved survival over single-agent treatment when tested during early stages of tumor development (treatment started on 1 day after tumor injection), but it was ineffective when tumors were allowed to establish. One of the remaining key questions regarding the antitumor activity of inotuzumab ozogamicin is whether the tumor cells that mediate resistance to CHOP or CVP treatment express CD22 and whether these cells can effectively be targeted and killed by the calicheamicin conjugate. However, such analysis was not conducted in the context of Ramos tumors, representing B-cell NHL, or the REH B-cell ALL model. Therefore, additional work will need to be conducted to understand whether combining CVP or CHOP standard of scare treatment with inotuzumab ozogamicin yields more than additive anti-tumor activities.

## 4.3 Preclinical and Clinical Experience with Gemtuzumab Ozogamicin

Gemtuzumab Ozogamicin consists of a CD33 targeting antibody, conjugated to γ-calicheamicin dimethyl hydrazone (Hamann et al. 2002). The myeloid differentiation antigen CD33 is a cell surface, transmembrane protein expressed on leukemic blasts from 85 to 90% of AML patients (Dinndorf et al. 1986; Griffin et al. 1984). When tested preclinically, gemtuzumab ozogamicin induced selective cytotoxicity against CD33+ AML cells, effectively inhibited colony formation units (CFUs) in human AML specimen, and caused regression of CD33+ AML cell line xenografts in athymic mice (Hamann et al. 2002). Clinical trials conducted over the last decade demonstrated that gemtuzumab ozogamicin improves event-free survival or relapse-free survival for many but not all AML patients (Castaigne et al. 2012) as well as overall survival (Hills et al. 2014), which is consistent with the preclinical observations that CD33 is a valid target for only some AML subtypes. It is a matter of intense clinical investigation to identify the factors that determine the sensitivity of certain subtypes of AML to treatment with gemtuzumab ozogamicin. Survival data from clinical studies suggests that gemtuzumab ozogamicin may be more effective in patients with certain cytogenetic features which are different from the class subtypes of AML which previously has been classified based on morphologic features. Studies to date have not been able to determine whether gemtuzumab ozogamicin, besides acting on more mature CD33+ progeny, can directly kill CD33+ leukemia stem cells (LSCs) and whether long-term benefit is related to successful targeting of such LSCs.

Three models of mutational events related to phenotypic differentiation have been proposed to explain the variability in their response to treatment between subsets of AML patients. First, both the initial and subsequent mutational events underlying malignant transformation in AML occur at the level of pluripotent CD33− precursors. These AML types are captured under the term of "immature" leukemias, which are not effectively targeted by gemtuzumab ozogamicin (Fialkow et al. 1987; McCulloch 1983). Secondly, the initial mutation leading to AML is acquired in pluripotent HSCs, whereas the collaborating mutational event(s) leading to full AML transformation and subsequent clonal expansion occur only at a later stage, possibly at the level of committed CD33+ myeloid precursors (LSCs). It is speculated that at least some of the core-binding factor (CBF) leukemias belong to this second category. These CBF AML leukemias are defined by the presence of chromosomal aberrations disrupting one of the CBF transcription factor genes (Muller et al. 2008). The third model includes all mutational events and clonal expansions at the level of committed, CD33+ myeloid precursors. An example for this category may be acute promyelocytic leukemia (APL), where predominant involvement of committed CD33+ myeloid progenitors was reported (Grimwade and Enver 2004). In this particular AML subset, in which leukemias originate from a committed, CD33+ progenitor cell type, gemtuzumab ozogamicin appears to be highly active (Breccia et al. 2007; Lo-Coco et al. 2004). Another reason why

gemtuzumab ozogamicin may be highly active in APL is that the density of CD33 on the surface of this AML subtype is higher than in other AML subtypes (Pollard et al. 2012).

## 4.4 Ephrin-A4 (EFNA4): A Novel Target for Calicheamicin Conjugates

PF-06647263 is an anti-EFNA4 antibody-drug conjugate for the treatment of solid tumors (Damelin et al. 2015). EFNA4 (Ephrin-A4) is a novel ADC target that was found overexpressed on aggressive tumor cell populations called cancer stem cells (CSCs) or tumor-initiating cells (TICs). CSCs represent a framework for explaining the high rates of tumor recurrence after clinical treatment, and thus the therapeutic targeting of CSCs is expected to result in more durable clinical responses (Zhou et al. 2009). In preclinical models of TNBC and ovarian cancer, EFNA4 mRNA expression levels were enriched in CSCs compared to the less tumorigenic cells within the same tumors (Damelin et al. 2015). EFNA4 belongs to a family of signaling molecules comprised of ephrin ligands and Eph receptor tyrosine kinases [reviewed in Pasquale (2005, 2010)]. Ephrin-Eph signaling can be bi-directional (impacting both the ligand- and receptor-expressing cells) and regulates a broad range of biological activities such as neural development, cell patterning, angiogenesis, cell motility, and invasion. While therapeutic targeting of the Eph receptors has been explored, targeting of ephrin ligands has not been pursued to any great extent (Pasquale 2010), in part because it is generally not feasible to use small molecules to interfere with ligand binding selectively.

In the context of cancer, the expression of various ephrins and Ephs has been observed, and various functions were reported (Hafner et al. 2004; Pasquale 2010; Surawska et al. 2004). Due to ligand-receptor binding promiscuity as well as functional overlap, it has been difficult to precisely define the roles of each ephrin and Eph. There is evidence of roles for EFNA4 in axonal guidance during development and wound healing, epidermal differentiation, transendothelial migration of leukocytes, retinal neovascularization, and craniosynostosis (Du et al. 2012; Lin and Anseth 2011; Moss et al. 2005). The expression of EFNA4 in normal adult tissues is remarkably low; some expression has been observed in the colon and the skin (Hafner et al. 2004). Recently, EFNA4 was identified in a screen for genes that affected mammary cell growth and development (Burleigh et al. 2015), which is consistent with its enriched expression on breast CSCs.

The anti-EFNA4 ADC (PF-06647263) is comprised of the huE22 humanized IgG1 antibody and the N-Ac-γ-calicheamicin DMH linker-payload, the same as in inotuzumab ozogamicin and gemtuzumab ozogamicin. Notably, PF-06647263 has a lower drug/antibody ratio and a tighter loading distribution relative to inotuzumab ozogamicin and gemtuzumab ozogamicin, which may provide a safety advantage. Calicheamicin generates double-strand DNA breaks and therefore impacts both

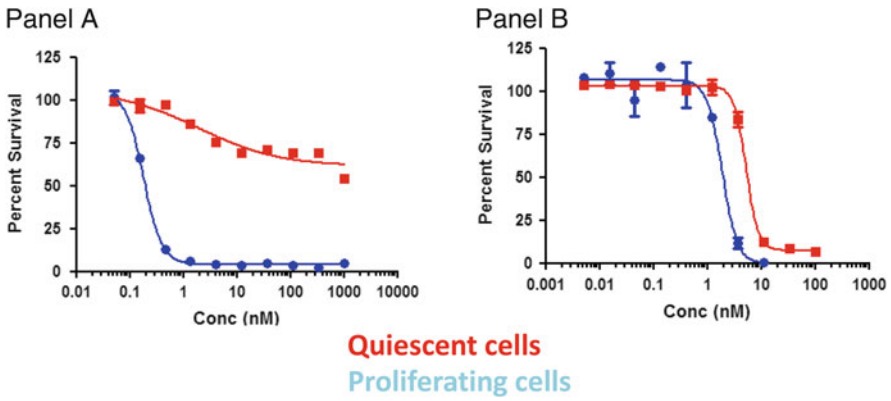


**Fig. 4.2** Difference in the mechanism of action between DNA targeting payloads (calicheamicin) and tubulin inhibitor payloads. The mechanism of action employed by calicheamicin to induce cancer cell death (panel B) is fundamentally different from the tubulin-binding class of cytotoxics (panel A), which constitutes the prevalent class of payloads among clinical stage ADCs. Calicheamicin generates double-strand DNA breaks and impacts both rapidly proliferating and quiescent or slowly proliferating cells (Hinman et al. 1993; Zein et al. 1988). Panel A displays the difference in the $IC_{50}$ values between microtubule inhibitors payloads incubated with nonproliferative cells (*red line*) and proliferative tumor cells (*blue line*). Panel B displays comparable $IC_{50}$ values of the DND targeting payload calicheamicin incubated with proliferative cells (*blue line*) and nonproliferative cells (*red line*)

rapidly proliferating and quiescent or slowly proliferating cells (Hinman et al. 1993; Zein et al. 1988). This mechanism of action might be particularly effective against CSCs which are thought to have a lower overall proliferation rate compared to the bulk tumor cells (Zhou et al. 2009). In contrast, most ADCs currently in the clinic employ microtubule inhibitor (MTI) linker-payloads that preferentially impact rapidly proliferating cells (Alley et al. 2010; Sapra et al. 2011), as illustrated in Fig. 4.2.

## 4.5 Antitumor Activity of the EFNA4-ADC Against CSCs in Triple Negative Breast Cancer (TNBC) and Ovarian Carcinomas (OVCA)PDX Models

PF-06647263 was administered to immune-compromised mice harboring subcutaneous human tumor xenografts, in particular patient-derived xenografts (PDX) (Damelin et al. 2015). PF-06647263 induced potent antitumor activity against breast cancer and ovarian cancer PDX models, and in some models, treatment of established tumors with PF-06647263 resulted in complete regressions of more than 200 days after the last ADC dosing. Notably, in comparison to inotuzumab

ozogamicin and gemtuzumab ozogamicin, PF-06647263 consistently achieved tumor regressions at approximately five- to tenfold lower dose levels compared to the activities observed in NHL models. Furthermore, in the majority of the tumor models tested, antitumor activity was observed at dose levels as low as 0.036 mg/kg. PF-06647263 elicited cytotoxicity in a target- and calicheamicin-dependent manner. Mechanism-of-action studies demonstrated that cytotoxicity was accompanied by DNA damage and apoptosis, consistent with the current understanding of calicheamicin's mechanism of action.

The in vivo efficacy data was generated in preclinical tumor models which are designed to mimic human tumors more closely than conventional cell line xenograft models [reviewed in Rosfjord et al. (2014)]. Specifically, PDX models, representing triple negative breast cancer and ovarian carcinomas, were established by direct implantation of freshly resected human tumor samples into immune-compromised mice. PDX models typically preserve the architecture and genotype of the human tumors from which they are derived to a greater extent than conventional cell line xenografts. Importantly, the CSC population appears to be maintained and propagated during serial transplantation of PDX models. In addition, the breast cancer PDXs were orthotopic, established in the mammary fat pads of the mice.

To confirm that tumor regressions were associated with a direct impact on Ephrin-A4-expressing CSCs, the tumorigenic potential of live tumor cells remaining after ADC exposure was assessed in an in vivo limiting dilution reimplantation assay. Using Poisson distribution statistics based on the frequency of no tumor growth in recipient animals 21 weeks posttransplant, CSC frequency was determined to be approximately threefold reduced in breast cancer tumors treated with PF-06647263 relative to the control ADC. These results demonstrate that PF-06647263 effectively reduced the CSC population in TNBC tumors, likely contributing to the sustained regressions observed in in vivo efficacy studies (Damelin et al. 2015).

As was observed with CSCs from TNBC xenografts, CSCs from MMMT and a subset of high-grade serous ovarian tumors were found to express elevated levels of the EFNA4 transcript versus the non-tumorigenic tumor cells and normal tissue cells. To determine whether elevated Ephrin-A4 expression in ovarian cancer PDX models could be leveraged to deliver therapeutically relevant doses of calicheamicin to tumor cells, mice harboring ovarian cancer PDX were randomized and treated with the standard-of-care therapeutic agent cisplatin, a control ADC, or PF-06647263. PF-06647263-induced significant tumor regression and/or tumor growth inhibition in the ovarian PDX models was tested. Moreover, the prolonged tumor regression lasting many months after a single round of treatment implies that even quiescent ovarian cancer CSCs can be impacted by Ephrin-A4-calicheamicin ADCs. These data establish the robust single-agent activity of PF-06647263 in both TNBC and ovarian cancer PDXs and provided rationale for the initiation of a Phase 1 clinical trial (clinicaltrials.gov identifier: NCT02078752).

## 4.6 Conclusions and Future Perspectives

The mechanism of action employed by calicheamicin to induce cancer cell death is fundamentally different from the tubulin-binding class of cytotoxic drugs, which represent the prevalent class of payloads among clinical stage ADCs (Mullard 2013). In contrast to spindle poisons like the auristatins or maytansines, which are most effective against rapidly proliferating cells, calicheamicin induces double-strand DNA breaks and apoptosis independent of cell cycle progression (Bernstein 2000; Damle 2004; Dedon and Goldberg 1992; Thorson et al. 2000). Such properties may be advantageous when targeting malignant cells that are not markedly different in their proliferation compared to normal, quiescent cells. The optimal combination of antibody, linker, and payload varies by tumor antigen and depends on many factors including the internalization and trafficking patterns of the antibody-antigen complex, the sensitivity of the antigen-bearing tumor cells to various classes of cytotoxic agent, and the expression levels of the antigen in cells from normal tissues.

Perhaps the most important commonality between these three calicheamicin-based ADC programs is the highly restricted expression of their target antigens in normal, solid tissues (Fig. 4.3). In particular, the absence of CD22 and CD33 in hematopoietic stem cells and their presence in hematological compartments that are rapidly and continually replenished [reviewed in Ricart (2011); Walter et al. (2012)] may allow the body to rapidly repair damage to normal tissue resulting from target-dependent toxicity. In contrast, for targets expressed at high levels on normal solid tissues with low reconstitution potential, damage may be irreparable. EFNA4 may strike the perfect balance in that its expression on normal solid tissues is minimal, and expression in tumors is elevated, especially on the more malignant CSCs.

Another potential common mechanism of action that may contribute to the clinical activity of calicheamicin conjugates is their potential ability to stimulate anticancer immunity. Depending on the initiation stimulus, cancer cell death induced by cytotoxic compounds can be immunogenic or nonimmunogenic. Cytotoxic compounds vary in their ability to induce immunogenic cell death and consequently anticancer immunity. The term “immunogenic cell death” (ICD) is used to indicate a particular form of cell death that engages the adaptive arm of the immune system [reviewed in Kroemer et al. (2013)]. Among a large array of antineoplastic agents, only a few cytotoxic compounds were found to induce ICD. Compounds inducing a high level of ICD include various anthracyclines such as doxorubicin, epirubicin, and idarubicin, which are commonly used against a variety of neoplastic malignancies (Casares et al. 2005; Fucikova et al. 2011; Obeid et al. 2007). The DNA targeting, alkylating agent oxaliplatin, a platinum derivative used in combination regimens to treat advanced colorectal carcinoma, induces ICD, while cisplatin does not (Martins et al. 2011; Tesniere et al. 2010). Based on the activities of other DNA targeting cytotoxic compounds, the question arises whether calicheamicin conjugates induce immunogenic cell death of tumor

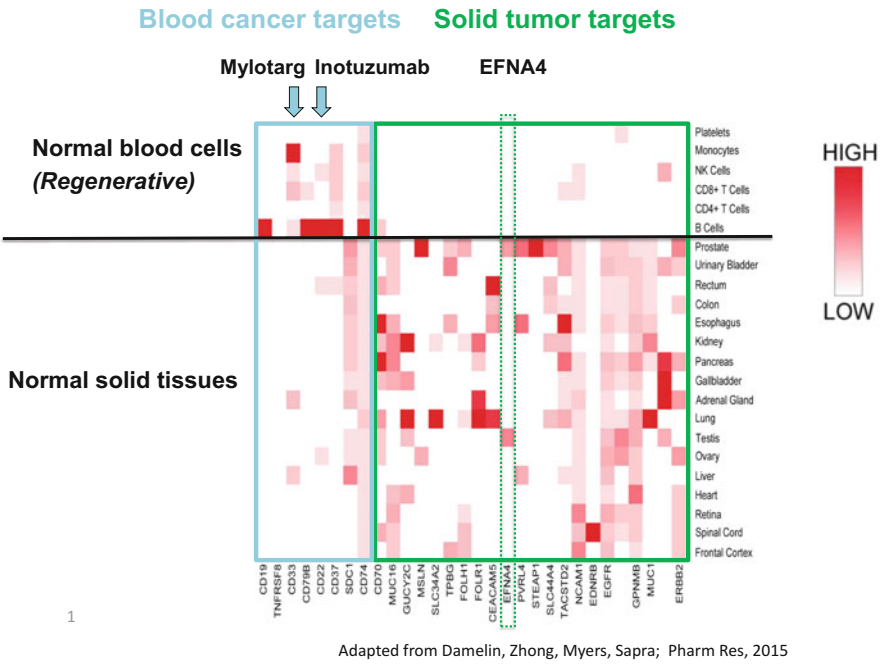


**Fig. 4.3** Normal tissue expression creates a barrier for ADCs with DNA-damaging payloads. An important commonality between calicheamicin-based ADC programs is the highly restricted expression of their target antigens in normal, solid tissues, to avoid normal tissue damage. The differences in expression levels of target antigens on normal tissues are shown in white (low expression levels) to *red* colors (high expression levels). Cell surface antigen expression levels of various ADC targets currently tested in the clinic (*x*-axis) which are on various normal tissues are displayed on the *y*-axis. Importantly, the target antigen expression levels of ADCs employing DNA targeting payloads appear to be are limited to hematopoietic lineages. Rapid recovery of hematopoietic lineages following ADC treatment, including CD22-positive B-cells or CD33-positive myeloid cells, is the foundation of the tolerable toxicity profiles of these ADCs in the clinic. Importantly, many solid tumor targets are expressed in normal tissues, including EGFR or Her2, which renders them less promising for targeting with ADCs employing DNA-damaging payloads, as they may induce tissue damages in normal tissues, potentially inducing dose-limiting toxicities of ADCs

cells. If so, the relative contributions of ICD to the overall antitumor activity of calicheamicin conjugates remain to be determined.

More recently, cancer stem cells (CSCs) in solid tumor and leukemia stem cells (LSCs) in liquid tumors were identified as promising therapeutic targets in oncology, as they may represent the "root problem" of cancer due to their self-renewal capabilities and their inherent resistance toward conventional anticancer therapeutics [reviewed in Nicolaou et al. (1993)]. CSC eradication is thought to be crucial for successful anticancer therapy resulting in durable responses. CSCs frequently display lower levels of steady-state proliferation compared to the more rapidly proliferating, fully differentiated cancer cells, which comprise the majority of

malignant cells within tumors (Zhou et al. 2009). The relative low proliferation rates of CSCs combined with the abundance of drug efflux mechanisms may account for the frequent relapse of cancer patients treated with conventional cytotoxics, including chemotherapy (Eramo et al. 2010). Thus, targeting of CSCs and other cells within tumors that do not proliferate rapidly may significantly contribute to malignancy. Therefore, the use of cell cycle-independent cytotoxic compounds, such as calicheamicin, may be advantageous.

CSCs represent cellular targets that, if effectively eliminated, may provide a profound impact on survival end points of cancer patients. Like their normal stem cell counterparts, CSCs likely contain a subpopulation of cells that are quiescent and thus are inherently resistant to cell cycle-dependent microtubule inhibitor payloads. Calicheamicin is a clinically validated toxin, able to kill cells independent of their status in the cell cycle (Bouchard et al. 2014). In this context, the EFNA4-ADC is an example of the strategy to attack CSCs with calicheamicin and is now in early clinical trials for solid tumors.

Given such activity of the calicheamicin payload when targeting cancer stem cells in solid tumors, the question arises to what extent the pharmacological activities of the two clinically more advanced calicheamicin conjugates, inotuzumab ozogamicin and gemtuzumab ozogamicin, are reflective of targeting of lymphoma and leukemia stem cells (LSCs), respectively. CD22 is expressed on the majority of B-lymphocyte malignancies, but it is not expressed in normal hematopoietic stems cells in individuals with B-cell malignancies. CD22 expression is also absent on other nonlymphoid or nonhematopoietic cells [reviewed in Ricart (2011)], as well as LSCs within the context of NHL tumors. Based on the relative expression levels of CD22 on patient samples, the relative contributions of targeting LSCs or more differentiated, pluripotent lymphoma stem cells by inotuzumab ozogamicin are likely to be minimal [reviewed in Shor et al. (2015)]. However, the paucity of current preclinical lymphoma models that contain functional LSCs for NHL and ALL will require additional model development to fully explore this aspect of LSC biology.

Similarly, it is well documented that CD33 is not expressed on HSCs, and therefore, repopulation of the hematopoietic compartment following gemtuzumab ozogamicin treatment is not impaired. However, CD33 is present on a subpopulation of common myeloid precursors within a subset of AML patients (Walter et al. 2012). It is important to emphasize that studies to date have not determined whether gemtuzumab ozogamicin, besides acting on more mature CD33+ progeny, can directly kill CD33+ LSCs in AML tumors in vivo and whether long-term benefit from gemtuzumab ozogamicin is related to successful targeting of LSCs (Walter et al. 2012).

In conclusion, the current limitations to develop preclinical models that maintain functional LSCs may account for our limited understanding of the role of LSCs when targeting CD22- and CD33-positive hematopoietic malignancies with calicheamicin conjugates. Standard tumor cell line models are prone to lose functional CSC populations, due to clonal selection during the in vitro propagation and expansion process [reviewed in (Rosfjord et al. 2014)]. In contrast, a therapeutic

**Table 4.1** PDX models use for testing of calicheamcin conjugates targeting solid tumors, and standard tumor cell line models used for testing of heme-onc targeting calicheamicin conjugates

| Tumor type | Model | Compounds tested | Key observations | References |
|---|---|---|---|---|
| B-cell ALL | REH B-cell disseminated and subcutaneous implants | Inotuzumab ozogamicin (CD22) | Complete tumor regressions | DiJoseph et al. (2007) |
| B-cell lymphoma | SC Ramos | Inotuzumab ozogamicin (CD22) | Complete tumor regressions at 6.57 mg/m$^2$ | DiJoseph et al. (2004b) |
| B-cell lymphoma | BCL | Inotuzumab ozogamicin (CD22) | Complete tumor regressions at 4.92 mg/m$^2$ | DiJoseph et al. (2004b) |
| B-NHL | BCL | Inotuzumab ozogamicin with CHOP or CVP chemotherapy | Improved survival of mice when combination treatment started early | DiJoseph et al. (2007) |
| CD33+ AML cell line | HL-60 | Gemtuzumab ozogamicin (CD33) | Complete tumor regression | Hamann et al. (2002) |
| TNBC PDX | BR-13 | EFNA4-ADC | Complete tumor regressions | Damelin et al. (2015) |
| Ovarian PDX | OVA-45 | EFNA4-ADC | Complete tumor regressions | Damelin et al. (2015) |

effect of EFNA4-calicheamicin ADCs was validated in PDX models, which maintain and propagate the CSC populations. Therefore, the relative contributions of targeting CSCs to the overall pharmacological activity of the calicheamicin conjugates targeting hematological malignancies will have to await the development of human patient-derived xenograft tumor models from patient biopsies representing AML, ALL, and NHL tumors. The development of such preclinical models will help to answer the question, whether DNA targeting cell cycle-independent calicheamicin payloads represents a key advantage for targeting of LSC in liquid tumor indications (Table 4.1).

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# Chapter 5
# Enzyme-Based Strategies to Generate Site-Specifically Conjugated Antibody Drug Conjugates

**Roger R. Beerli and Ulf Grawunder**

**Abstract** Antibody Drug Conjugates (ADCs) exploit the specificity of monoclonal antibodies for the targeting of highly potent small molecular weight toxins to cancer cells in order to selectively effect their destruction. It is expected that due to the targeting of ADCs to the tumor, these drugs will be associated with less side effects and a higher therapeutic index than standard chemotherapy. However, so far this promise of ADCs has only poorly translated into the clinic. Despite the fact that ADCs for cancer therapy have been developed for many decades, the field has experienced a number of failures of ADCs in clinical development, due to an unfavorable clinical benefit to safety relationship. The first ADC, Mylotarg®, an anti-CD33 ADC, approved for treatment of acute myeloid leukemia (AML) eventually had to be taken off the market 10 years post approval. To date only two ADCs, the anti-CD30 ADC brentuximab vedotin (Adcetris®) and the anti-HER-2 ADC trastuzumab-emtansine (Kadcyla®), are approved for cancer therapy. In fact, Kadcyla® is only approved as a second-line therapy in breast cancer due to a limited clinical benefit in comparison to standard therapy as a first-line therapy. There is an increasing body of evidence that first-generation ADCs, including the two marketed ADC products, that are generated by standard chemical conjugation, are associated with liabilities connected to the conjugation technologies employed, which have a negative impact on the therapeutic index and efficacy of these ADCs. Here, we describe novel conjugation approaches, with a specific focus on enzymatic conjugation technologies that aim at overcoming the limitations of first-generation ADCs, namely the heterogeneity of chemically conjugated ADCs and insufficient linker stability. While site-specific conjugation can also be achieved using novel chemical linker approaches, and while it is also possible to employ improved linkers in chemical conjugation technologies, there are additional compelling arguments for site-specific enzymatic conjugation of toxin payloads to antibodies.

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New developments and data related to the preclinical evaluation of such next-generation ADCs are discussed.

**Keywords** Sortase • Transglutaminase • Formylglycine converting enzyme • Split intein

## 5.1 Introduction

Antibody Drug Conjugates, or ADCs, are complex biological molecules that are designed to target highly potent small-molecular weight toxins to cancer cells by means of tumor-specific antibodies (Perez et al. 2014). As such, ADCs need to be distinguished from Immunotoxins, which represent fusion proteins between binding domains of antibodies and highly toxic protein toxin domains, e.g., pseudomonas exotoxin (PE) or diphtheria toxin subunit A (DT-A) from prokaryotes, that specifically act on higher eukaryotic cells (Alewine et al. 2015). Immunotoxins can be generated as recombinant proteins in prokaryotic expression systems. Such protein toxins are extremely toxic due to the fact that they act catalytically on their cellular target. Therefore, they can kill a cell, even if only a single or a few molecules are delivered into targeted cells. However, the drawback of such immunotoxins is that the protein toxins if they are of bacterial or plant origin, are highly immunogenic in humans. Although de-immunization strategies for bacterial protein toxins, like, e.g., PE (Mazor et al. 2016), are trying to address this issue, to date no immunotoxin has been approved for cancer therapy.

In contrast, ADCs usually are comprised of full-length antibodies with intact antibody structure, composed of two heavy and two light chains, that are conjugated to a number of small molecular weight toxins (often with a molecular weight below 1000–1500 Da). Traditionally, ADCs have been generated by coupling of such toxins to antibodies by means of chemical linkers that connect the toxin to desired amino acid side chains, mostly via the ε-amino group of lysine amino acid residues, or to thiol groups of cysteines. The latter often need to be generated from intra-chain disulphide bridges by mild reduction of the antibody.

The two ADC molecules that have been approved by the FDA for cancer therapy, Adcetris® (brentuximab vedotin) for CD30-positive lymphomas (e.g., Hodgkin Lymphoma) (Francisco et al. 2003) and Kadcyla® (trastuzumab-emtansine or T-DM1) for HER2-positive breast and ovarian cancer (Phillips et al. 2008), are generated by this conventional way of chemical conjugation to cysteine and lysine residues, respectively. It has been widely reported in the literature that the conventional conjugation of small molecular weight toxins to antibodies with chemical linkers is not trivial. In fact, the first FDA-approved anti-CD33 ADC product Mylotarg® (Naito et al. 2000), used for the treatment of CD33-positive acute myeloid leukemia (AML), suffered from insufficient covalent coupling of the toxin to the antibody, leading to significant release of toxin molecules in circulation,

which led to an unfavorable therapeutic index of this first-generation ADC. Eventually, Mylotarg®, which had been FDA-approved in 2000, was withdrawn from the market in 2010. Due to the safety concerns of this ADC, Mylotarg® was never approved for cancer treatment by the European Medicines Agency (EMA).

The quality and functionality of chemical linkers have subsequently been improved by medicinal chemists and many generations of either "non-cleavable" or "cleavable" linkers have been developed and incorporated into ADCs in development (Ducry and Stump 2010). "Cleavable" linkers usually refer to structural features incorporated into linkers that exhibit a certain propensity for either hydrolysis or reduction, depending on change of pH or redox potential, respectively (Jain et al. 2015). Cleavable linkers may also comprise a specific recognition sequences for intracellular proteases, like cathepsin-B (McCombs and Owen 2015). In contrast, "non-cleavable" linkers do not contain specific structures amenable to cleavage, and toxin-release is thought to be dependent on complete degradation of the ADC upon internalization and intracellular trafficking into late endosomal compartments. However, it has to be noted that the terms "cleavable" and "non-cleavable" do not appear to be absolutely clearly definable features, because also "non-cleavable" linkers are known to be subject to certain cleavage in circulation and before binding and internalization into targeted cancer cells (Drake and Rabuka 2015).

In this context, it has been recognized in the field that in particular classical maleimide containing linkers are subject to serum instability (Wei et al. 2016). Maleimide linkers are contained in the two approved ADC products Adcetris® (with a specific enzyme "cleavable" vcPAB linker) and Kadcyla® (with a sterically inhibited "non-cleavable" thioether containing SMCC linker). Maleimide linker chemistry is also employed in the majority of ADCs currently in advanced clinical trials (phases II and III). Scientists in Peter Senter's group at Seattle Genetics had reported this instability many years ago and suggested that a thio-succinimid linkage can be cleaved via a so-called Retro-Michael reaction by free thiol groups (Alley et al. 2008) (Fig. 5.1). In fact, the highest concentration of free thiol in human serum is provided by cysteine-34 of human serum albumin (HSA). Therefore, since 2008 it is already known that toxins conjugated to ADCs with maleimide linkers can be transferred to HSA, leading to undesired distribution of the toxic payload of ADCs via the circulation all over the body (Fig. 5.1).

However, this is only one of the issues known to be associated with conventional ADCs generated by chemical linking. A second issue is related to the fact that conjugation via lysine amino acid residues or via cysteine residues only allows a limited control over the site of conjugation or the number of toxins coupled to the antibody (the drug-to-antibody ratio, or DAR) (Panowski et al. 2014). For many toxins, it has been empirically determined that the "sweet-spot" for the average DAR ranges between three and four. This is reflected by the DARs of the two commercial products Adcetris® and Kadcyla®, which have been reported to be 3.6 and 3.5, respectively. Although it is theoretically possible to prepare fully conjugated ADCs via cysteine conjugation if toxins are coupled to all four reduced intrachain disulphide bridges of an $IgG_1$ antibody molecule, it has been found that ADCs with a DAR of 8 are more prone to aggregation, resulting in shorter serum half-lives

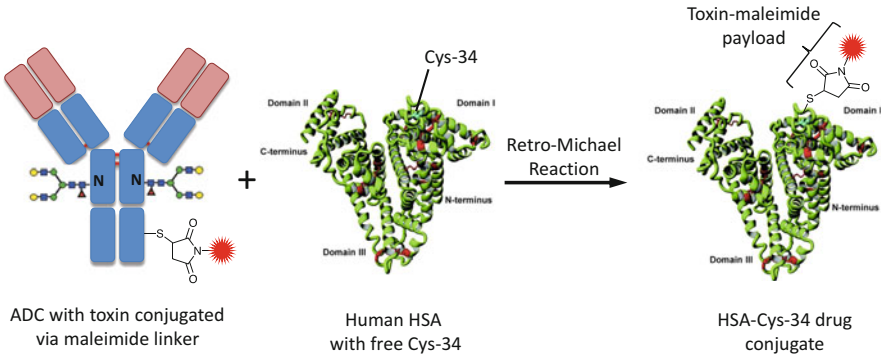


**Fig. 5.1** Proposed mechanism of the transfer of payload (*red-colored, spiked ball*) conjugated to an antibody (*left*) via a maleimide linker group to HSA. In the presence of free thiols, the thioether bond of the maleimide linker can be broken via a so-called Reverse Michael Reaction, and the maleimide-payload group can therefore be released from the ADC. The abundant serum protein, human serum albumin (HSA), contains an unpaired cysteine, cysteine-34, that provides a free thiol that acts as a nucleophile in this Reverse Michael Reaction. This way, a maleimide-payload group can covalently be transferred from the ADC to HSA, which is an undesired side product of conventional ADCs, generated by chemical conjugation with linkers comprising maleimide linker components (like commercial ADCs Kadcyla® and Adcetris®)

and generally unfavorable biophysical properties (Hamblett et al. 2004). Therefore, the therapeutic index of DAR 8 ADCs appears to be lower than ADCs with an average DAR in the 3–4 range. However, even cysteine-conjugated ADCs with an average DAR of around 3.5 contain higher DAR species with a DAR of up to 8.0, which exhibit the above-mentioned unfavorable biophysical and pharmacokinetic properties. In addition to instability of widely employed maleimide linkers in human serum due to a reverse Michael reaction, the faster degradation of higher DAR species adds to the undesired and premature release of highly potent toxins from such ADCs in circulation of patients. This adds to the observed toxin-specific side effects associated with particular toxin classes in many clinical trials, irrespective of the antibody targeting moiety (Saber and Leighton 2015).

Conversely, lower DAR species in chemically conjugated ADCs have a lower potency (Bryant et al. 2015), because they carry less of the toxic payload that can be delivered into targeted cancer cells upon ADC internalization. A heterogeneous, chemically conjugated ADC may even contain a certain fraction of entirely unconjugated antibodies which will act as "cold" competitors for properly conjugated ADCs, because they will occupy binding epitopes on targeted cancer cells. Depending on the tumor target and tumor type, lower DAR species are associated with significantly lower potency in tumor models in vivo (Hamblett et al. 2004). Because higher DAR ADCs tend to lose their payload with a faster rate than lower DAR ADCs, higher DAR species gradually convert into lower DAR species, which have a lower efficacy and thus have an unfavorable therapeutic index (Panowski et al. 2014).

All this makes it evident that while heterogeneous ADCs with variable DAR have been "good enough" in the past, there are strong arguments in favor of generating site-specifically conjugated, homogeneous ADCs, with an optimal DAR adjusted to the best therapeutic index for a given combination of target, antibody, and toxin payload.

To address the limitations associated with conventional maleimide chemical linker strategies outlined above, in recent years a variety of different chemical linker strategies have been developed with the objective to allow a more selective linkage of toxic payloads to selected amino acids in the antibody structure (Panowski et al. 2014). These strategies include the introduction of unique chemical handles for chemical conjugation, e.g., by incorporation of unnatural amino acids or site-directed mutagenesis. These and other possibilities are summarized in more detail in other publications (e.g., Sochaj et al. 2015; Schumacher et al. 2016), including contributions to this book.

An alternative to chemical conjugation is to exploit the inherently high substrate specificity of enzymes for conjugation of toxic payloads to antibodies. Enzymes, like antibodies, are proteins, and therefore, their structural integrity and function is best preserved under physiological pH and at low ionic strength. Therefore, it is expected that enzymatic conjugations will be more favorable for the preservation of structural and functional features of antibodies and eventually ADCs, than chemical conjugations that often need to involve unusual pH conditions or the use of organic solvents, in order to provide optimal conditions for linker-payload conjugations. Therefore, not only from the point of view of site-selectivity provided by enzymatic reactions, but also from the point of view of maximally preserving the structural integrity of ADCs, it is a compelling strategy to employ enzymes for the generation of homogeneous ADCs. In this book chapter, we will focus on the enzymatic approaches that are known in the ADC field and that have been published in peer-reviewed publications.

## 5.2 Enzymatic Conjugation Technologies Used for the Generation of Site-Specifically Conjugated ADCs

Enzymatic conjugation technologies for site-specific conjugation of small molecular weight payloads to antibodies may involve approaches which rely exclusively on the use of enzymes for performing the conjugation of a toxin payload to an antibody. Alternatively, some approaches have been developed, in which at least one or more steps of the conjugation involves the use of an enzyme or enzymes. This may be favorable to exploit the selectivity of an enzyme for introducing a chemical handle for conjugation, especially if the enzyme reaction would require high concentrations of an expensive linker-payload. Such platforms are often referred to as chemo-enzymatic conjugation technologies.

However, even in the case of purely enzymatic approaches, linker components with designed functionality may still be attached to the toxic payload. This can be performed by standard medicinal chemistry as part of the toxin synthesis or the generation of toxin derivatives. Most of the enzymatic conjugations discussed below can be used for both strategies.

### 5.2.1 *Strategies Involving Bacterial Transglutaminase (BTG) Enzyme*

Transglutaminases are a family of enzymes that catalyze the posttranslational modification of proteins by forming an isopeptide bond within or between proteins, in particular between the γ-carboxyamide group of glutamine residues and primary amines, such as the ε-amino-group of lysine residues (Lorand and Graham 2003; Eckert et al. 2013). The resulting isopeptide bond is highly stable and resistant to cleavage by proteases. The group of Schibli and colleagues at ETH-Zurich, Switzerland, were the first to use bacterial transglutaminase to conjugate payloads site-specifically to antibodies (Jeger et al. 2010; Dennler et al. 2013). They demonstrated that glutamine 295 (Q295) in the $C_{H2}$ domain of the human $IgG_1$ Fc region is specifically recognized as a substrate for bacterial transglutaminase after de-glycosylation of the antibody by PNGaseF treatment (Fig. 5.2). Because monoclonal antibodies are comprised of two identical IgH chains, this provides two conjugation sites in the Fc portion of an $IgG_1$ antibody. This concept was developed further by mutation of the asparagine 297 to glutamine in the $IgG_1$-Fc portion, which is the normal attachment site for N-linked glycosylation of $IgG_1$ antibodies. This N297Q mutation resulted in the generation of aglycosylated $IgG_1$ to which then four payloads per antibody could be coupled (2× Q295 and 2× Q297) (Jeger et al. 2010; Dennler et al. 2013) (Fig. 5.2).

In additional proof-of-concept experiments with bacterial transglutaminase and anti-CD30 antibody brentuximab, this concept was further validated by scientists from Innate Pharma (Lhospice et al. 2015). N297Q mutants of anti-CD30 antibody brentuximab providing four conjugation sites were conjugated with different vc-PAB-MMAE-derivatives containing different linkers via bacterial transglutaminase (BTG) (Fig. 5.2). In this study, it was shown that BTG-conjugated ADCs showed virtually no de-drugging in rat serum during a time period of 2 weeks. This translated into improved PK properties in comparison to Adcetris®. While the efficacies of Adcetris® and the site-specifically DAR4 BTG-conjugated ADC were comparable in a Karpas-299 Hodgkin lymphoma xenograft model at the standard dose of 1 mg/kg, the BTG-conjugated ADC showed improved efficacy at a lower dose of 0.3 mg/kg. Most notably, however, the maximal tolerated dose (MTD) of site-specifically BTG-conjugated ADCs could significantly be improved from 18 mg/kg for Adcetris to at least 60 mg/kg for the site-specifically BTG-conjugated ADCs. It is likely that the main reason for this increase in MTD

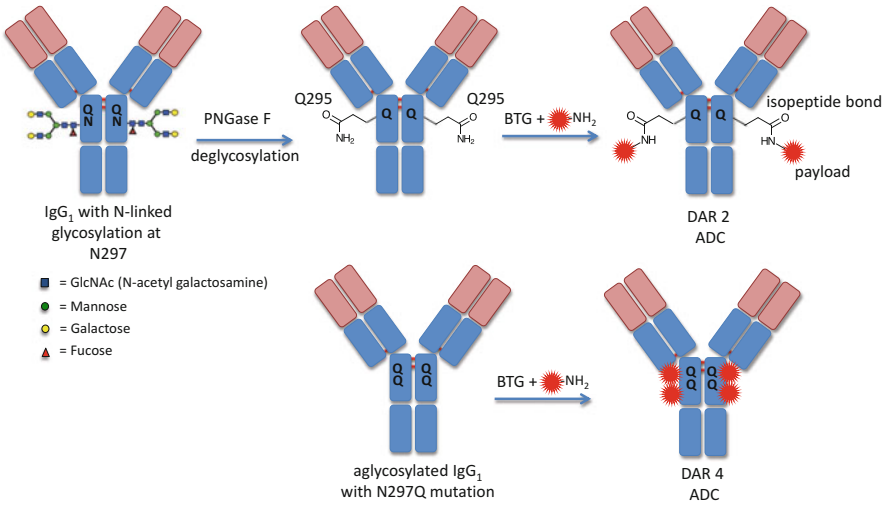


**Fig. 5.2** Representation of site-specific conjugation using endogenous glutamine Q295 (*top*), or Fc mutant N297Q (*bottom*) using bacterial transglutaminase (BTG) with a payload (*red-colored spiked ball*), modified with a primary amine function. Endogenous Q295 becomes accessible for BTG conjugation after deglycosylation with PNGase F, providing two attachment sites for the payload (*top*). If the N297 amino acid residue, which is the amino acid for N-linked glycosylation, is mutated to Q, then BTG can conjugate maximally four payloads site-specifically to generate a DAR4 ADC

and thereby expected therapeutic window is a consequence of the site-specific conjugation, which leads to favorable in vivo stability and PK properties. However, it cannot be entirely excluded that the lack of Fc-gamma receptor binding due to the N297Q mutation, which removes the N-linked glycosylation site, may be a compounding factor. Regardless of the mechanism leading to a higher MTD and expected higher therapeutic window, it is consistent that BTG-conjugated ADC accumulate to a lesser extent in liver, but to a higher extent in tumor tissue.

Because conjugation of primary amino group containing substrates requires a large molar excess of the substrate over the antibody to be conjugated, which increases the cost of goods in ADC manufacturing because larger amounts of high-potency toxin need to be manufactured, Lhospice and colleagues also explored a 2-step chemo-enzymatic conjugation approach to generate site-specifically conjugated ADCs (Lhospice et al. 2015). In this approach, in a first step a less expensive substrate with a linker containing an azide function is used as a handle for click chemistry and that is conjugated to the antibody using BTG enzyme. In a second step this intermediate was coupled to a DBCO-modified toxin payload comprising an alkyne function as a click-chemistry donor at essentially equi-molar ratio in order to generate site-specifically conjugated anti-CD30 ADCs with different linker-MMAE versions. As far as analyzed, ADCs generated by a 1-step BTG conjugation exhibited similar potencies and properties as ADCs

generated by the 2-step chemo-enzymatic conjugation, employing BTG conjugation in combination with click-chemistry coupling.

Additional data with site-specifically, BTG-conjugated ADCs have been reported by the Rinat group of Pfizer, which introduced the peptide sequence LLQG as a substrate sequence, in which the Q amino acid is specifically recognized by BTG (Strop et al. 2013) (Fig. 5.3). Dozens of positions of the artificial LLQT sequence were positioned in exposed loops of the antibody structure and have been screened for BTG enzyme conjugations. This resulted in the identification of eight positions in the IgH chain and three positions in the IgL chains, including the C-termini of each antibody chain, in which the LLQG tag was recognized by BTG

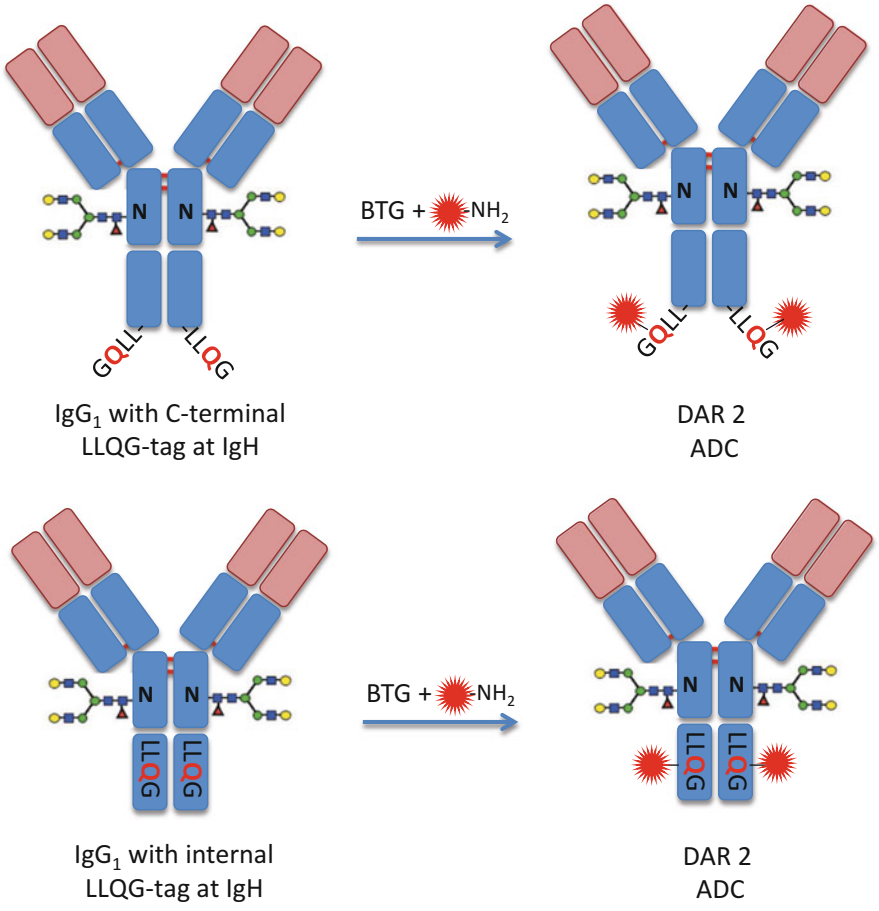


**Fig. 5.3** Bacterial transglutaminase (BTG) can also recognize a glutamine (Q) amino acid as a substrate for conjugation, if it is part of a LLQG amino acid sequence that can either be appended to the C-termini of Ig chains (*top*) or placed internally in the antibody sequence (*bottom*). In each case, two conjugation sites are provided that result in site-specifically conjugated DAR2 ADCs with an amine-modified payload (*spiked ball in red*)

and to which payloads could almost quantitatively be conjugated (Fig. 5.3). The stability and PK properties of site-specific DAR2-ADCs conjugated at different positions were analyzed in vivo in rats. These studies revealed that the specific location, or "microenvironment," of the LLQG conjugation tag had a significant influence on the stability of the DAR-2 conjugates. Selected ADCs with favorable stability and PK properties showed similar in vivo potency in tumor xenograft models as conventional chemically conjugated ADCs with a DAR of ca. 3.5, if identical antibody and payload were employed. This suggests that indeed site-specifically conjugated ADCs have an advantage over conventional heterogeneously conjugated ADCs (Strop et al. 2013). In an extension of this study, it was even found that the position and therefore the microenvironment of the conjugation site can lead to differential susceptibility of the payload for degradation and loss of activity (Dorywalska et al. 2015).

The studies with site-specifically BTG-conjugated ADCs show that this technology can be used to generate nearly homogeneous ADCs with defined stability and PK properties. Furthermore, the studies highlight that the site of conjugation can have a significant influence on the stability of the linkage between the antibody and the payload and even on the stability of the payload itself. This is additional indirect evidence that individual conventional chemically conjugated and heterogeneous ADCs will be subject to these positioning effects as well. It is unknown at this point of time whether aglycosylated ADCs as generated by the Innate Pharma group exhibit better or worse tox profiles than conventional ADCs, which normally comprise glycosylated antibodies.

### 5.2.2 *Use of Sortase Enzymes for Generating Homogeneous ADCs*

Another enzyme family that has widely been reported in the literature as a versatile tool for protein engineering is the family of the so-called sortase enzymes (Tsukiji and Nagamune 2009). Sortase enzymes are protein ligases that catalyze the formation of peptide bonds between two different proteins by way of a transpeptidation reaction. Sortase enzymes have originally been discovered in gram-positive bacteria as proteins that catalyze the exchange of virulence factors of proteoglycans expressed on the cell wall of gram-positive bacteria, like, e.g., *Staphylococci* and *Pneumococci* species (Spirig et al. 2011). This way the gram-positive bacteria change the set-up of their proteoglycan coat to evade an immune response of the infected host. Therefore, initially sortase enzymes have been and continue to be investigated as targets for antibacterial therapy. The first sortase enzyme that was discovered by the group of Schneewind at UCLA was the sortase A enzyme from *S. aureus* (Mazmanian et al. 1999). Soon after discovery of *S. aureus* sortase A enzyme, it was shown that this enzyme could robustly be expressed as a recombinant protein in *E. coli* and used to catalyze the ligation of proteins in vitro (Ton-That et al.

2000). Meanwhile, sortase enzymes have been identified in the genomes of many gram-positive bacteria, which, based on sequence homology, have been grouped into different classes (A-F) (Spirig et al. 2011). Therefore, hundreds of different sortase enzymes exist in nature, but only for some of them the substrate specificities have unequivocally been identified.

In order to catalyze the ligation of two proteins, one protein has to carry a penta-peptide motif (sortase motif), which in the case of sortase A from *S. aureus* is the amino acid sequence LPXTG, with X representing any of the 20 amino acids. The other protein has to have a stretch of glycine amino acid residues (usually three to five) with a free N-terminus. The first step of a sortase-mediated protein ligation is the binding of a sortase penta-peptide motif by the sortase enzyme, which then breaks the peptide bond between the 4th and 5th amino acid of the sortase motif (the peptide bond between threonine and glycine in the LPXTG sortase motif of sortase A of *S. aureus*) by formation of a thioester bond between the carboxylic acid group of the 4th amino acid and a cysteine in the active center of the sortase enzyme (cysteine-184 in sortase A of *S. aureus*). Upon formation of the covalent thioester intermediate with the 4th amino acid of the sortase motif, the 5th amino acid of the sortase motif and any sequences appended to its C-terminus are cleaved off. A suitable nucleophile, e.g., a protein with a glycine stretch at its N-terminus, can then attack the thioester bond via its free amino terminus to break the thioester bond, which releases the enzyme, and leads to the formation of a new peptide bond between the 4th amino acid of the sortase motif and the N-terminal glycine of the second polypeptide.

Following identification of sortase A enzyme, and demonstrating its use in protein engineering (Mao et al. 2004; Parthasarathy et al. 2007), many researchers have used sortase A from *S. aureus* for various applications in protein engineering, including protein–protein conjugation, circularization of peptides, and protein modification with glycine-modified dyes as well other lower molecular weight compounds (Antos et al. 2008, 2009a, b).

Only recently have sortase enzymes been used for the engineering of antibodies and antibody fragments. Initially, antibody fragments, like single-chain Fv ($scF_v$) and $F_{ab}$ fragments, have been used as substrates for sortase engineering (Möhlmann et al. 2011a; Ta et al. 2011; Madej et al. 2012). Levary and colleagues were the first to describe sortase-enzyme-mediated coupling of proteins to full-length antibodies by demonstrating that different proteins, including GFP, the plant toxin gelonin, and human serum albumin, could be coupled to the C-termini of LPETG-modified IgL chains with coupling efficiencies ranging between 35% and 85% (Levary et al. 2011). They have also shown that N-terminal conjugation of antibodies is possible, if a glycine-3-stretch is placed at the N-terminus of the IgH chain of an antibody. Swee and colleagues later also showed feasibility of coupling GFP to the C-termini of sortase-tagged IgH chains of a full-length anti-C-type lectin D205-specific antibody (αDEC205) (Swee et al. 2013).

Two studies have shown that immune-conjugates in which a plant toxin (gelonin) or bacterial toxins (incl. diphtheria toxin subunit A) are sortase conjugated to HER-2-specific $F_{ab}$-fragment or binding proteins result in potent immune-toxins

that can specifically kill HER-2-positive breast cancer cells in vitro (McCluskey and Collier 2013; Kornberger and Skerra 2014).

The concept of sortase-mediated conjugation of proteins, including toxin proteins, to antibody fragments or the IgH or IgL chains of full-length antibodies has been developed further by the authors and extended to the generation of site-specifically conjugated ADCs. We have demonstrated that potent small-molecular weight toxins, modified to contain five glycine residues as a handle for sortase enzyme conjugation, can efficiently be conjugated to both IgH and IgL chains of tumor-specific antibodies (Beerli et al. 2015) (Fig. 5.4). This technology was dubbed sortase-enzyme-mediated antibody conjugation, or SMAC-Technology™,

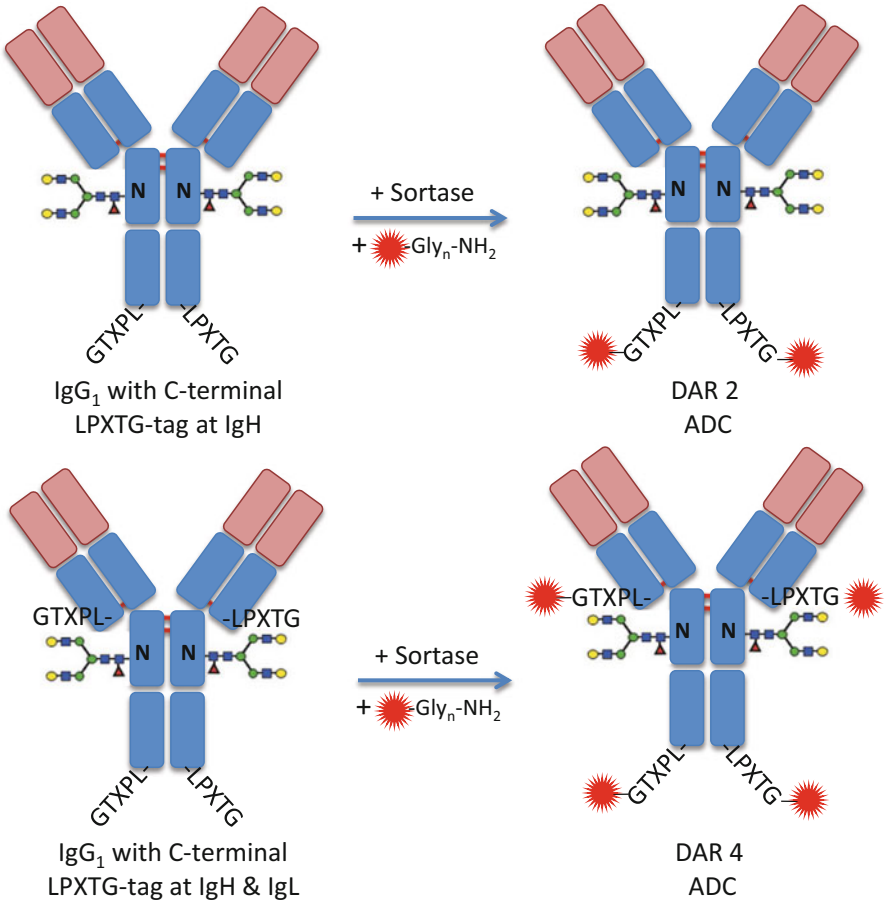


**Fig. 5.4** Schematic representation of site-specific conjugation of a glycine (Gly$_n$)-modified payload (*red-colored spiked ball*; $n \geq 1$) with Sortase A enzyme from *S. aureus*. The enzyme recognizes a C-terminal penta-peptide motif, LPXTG, for the generation of either IgH chain (DAR2; *top*) or IgH & IgL chain (DAR4; *bottom*) conjugated ADCs

and refers specifically to the site-specific conjugation of small-molecular weight payloads to the C-termini of IgH and/or IgL chains of full-length antibodies.

In the above-mentioned study, we could demonstrate that anti-HER-2 maytansine conjugates without any additional linker component that are typically part of chemically conjugated ADCs exhibited comparable specific in vitro potencies as commercial Kadcyla® on HER2-positive breast cancer cells. Indeed, addition or omission of the SMCC linker component in SMAC-Technology™-conjugated ADCs had no significant effect on the in vitro potency of the site-specifically conjugated ADCs. More importantly, it was also shown that SMAC-Technology™ conjugated ADCs with a $Gly_5$-maytansine payload ($Gly_5$-May) showed comparable anti-tumor responses in xenograft models with HER2-positive SKOV-3 ovarian carcinoma cells, when compared to Kadclya® comprising an SMCC chemical linker component. In order to achieve equally efficient SMAC-Technology™ conjugation of $Gly_5$-modified small molecular weight toxins to the C-termini of IgH and IgL chains, it became apparent that we needed to append at least five amino acids to the C-terminal cysteine of the IgL chain. This was required because the C-terminal cysteine amino acid of the IgL chains is involved in forming an intra-chain disulphide bridge to the $C_{H1}$ domain of the IgH chain in full-length antibodies. Therefore, this amino acid residue is structurally more constrained than the C-terminal amino acids of the IgH chain. In addition, the IgL chain C-terminus is positioned close to the hinge region of the IgG molecule, which could hinder access of the sortase enzyme in the "kink" of the antibody. Therefore, insertion of additional C-terminal amino acids at the C-terminus of the IgL chain increases sortase-mediated conjugations in all antibodies tested. In contrast, addition of "spacer" amino acids to the C-terminus of the IgH chain did not result in further enhancement of conjugation, which depending on toxin payload ranged between 80% and 95% efficiency. This indicates that a sortase tag, e.g., LPETG directly added to the C-terminus of the IgH chain is sterically accessible for sortase-enzyme-mediated conjugations (Fig. 5.4).

The demonstration that a $Gly_5$-modified maytansine without any additional linker structure displayed comparable in vitro and in vivo potency as SMCC linker containing chemically conjugated Kadcyla®, did not represent a significant advantage apart from the homogeneous composition of the SMAC-Technology™ conjugated ADC. Based on prior publications claiming the transfer of toxin to human serum albumin (HSA) by way of a reverse Michael reaction (Alley et al. 2008), we have analyzed whether this effect can be measured with SMCC linker containing Kadcyla® versus SMAC-Technology™ conjugated $Gly_5$-maytansine ADC. Using a sandwich-based ELISA assay with anti-HSA and an anti-maytansine antibody, we have found that indeed maytansine toxin was transferred significantly to HSA, when mixed with Kadcyla® and incubated at 37°C for up to 7 days, whereas this transfer was not detectable with SMAC-Technology™ conjugated $Gly_5$-maytansine ADC (*manuscript in preparation*).

The absence of significant transfer of maytansine toxin to HSA and the defined conjugation positions and homogeneous DAR of SMAC-Technology™ conjugated trastuzumab-$Gly_5$-maytansine is expected to have positive impact on the therapeutic

index of these ADCs in comparison to Kadcyla®. This needs to be demonstrated in additional tolerability and dose-finding studies in HER-2-positive breast cancer xenograft studies in vivo. However, all data taken together suggest that SMAC-Technology™ conjugation of $Gly_5$-modified payloads to antibodies is a viable method to generate site-specific, safe, and potent ADCs.

### 5.2.3 *Formyl-glycine Converting Enzyme (FGE) Approach*

Site-specific generation of ADCs has also been described using a two-step chemo-enzymatic strategy, consisting of an enzymatic modification of the antibody to introduce a chemical handle followed by chemical conjugation of the payload (Drake et al. 2014). In this approach, the recognition sequence CXPXR of formyl-glycine generating enzyme (FGE) is introduced at specific positions of the antibody's heavy or light chain constant regions (Fig. 5.5). FGE is then used to oxidize the cysteine side chain of the consensus sequence to a formyl-glycine, whose aldehyde group in turn serves as a chemical handle for the site-specific conjugation of small molecule payloads (Fig. 5.5). While this modification could be done with recombinant FGE, the use of FGE-expressing CHO cells for antibody production greatly facilitates the procedure (Drake et al. 2014). Once aldehyde-tagged antibody is produced, hydrazino-iso-Pictet-Spengler (HIPS) chemistry is used to conjugate payloads, yielding hydrolytically stable bio-conjugates suitable for in vivo use (Agarwal et al. 2013a, b) (Fig. 5.5). This technology is commercially

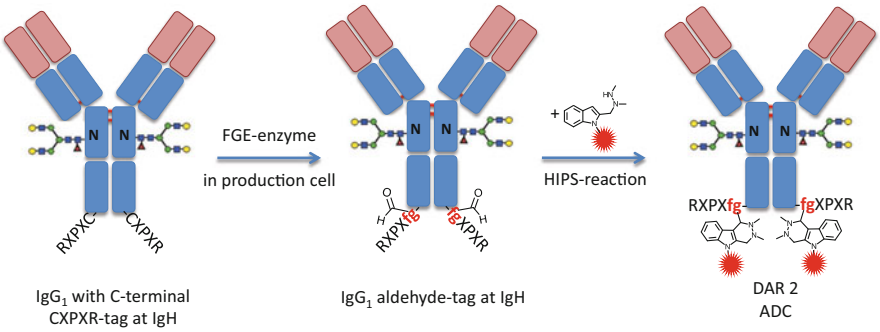


**Fig. 5.5** Schematic drawing of site-specific conjugation of payloads to antibodies using the chemo-enzymatic FGE (formy-lglycine generating enzyme) approach. Antibodies for conjugation can be generated by genetic engineering to contain a consensus peptide sequence CXPXR for the FGE-enzyme, e.g., positioned at the C-terminus of an Ig chain, as depicted here, or internally in the antibody structure (not shown here). If the antibodies are expressed in host cells overexpressing the FGE enzyme, the majority of cysteines in the CXPXR motif will be converted to formyl-gycine (abbreviated fg in *red*), which serves as a handle for a hydrazino-iso-Pictet-Spengler (HIPS) reaction with an alkyl hydrazinyl nucleophile coupled to payload (*red-colored spiked ball*). This results in the formation of a stable carbon–carbon bond between a three ring structure coupling the payload to the antibody at the position of the formylglycine

exploited by Redwood Biosciences (aquired by Catalent Pharma Solutions) under the name SMARTag™.

Based on the $IgG_1$ crystal structure, placement of the FGE recognition site was initially evaluated in eight positions of the antibody (Drake et al. 2014). Of these, one was on the light chain and seven on the heavy chain. Interestingly, the properties of the resulting antibody variants varied significantly, with two displaying a propensity for aggregation and one showing increased immunogenicity. Of the remaining five sites, three were investigated in more detail by generating ADCs using Trastuzumab as a model antibody and Maytansine as a model payload. Conjugation efficiencies varied among the sites, with two heavy chain variants (modified in $C_{H1}$ or at the C-terminus) proving easier to conjugate than the light chain variant (>90% vs. 75%). In fact, preparative hydrophobic interaction chromatography (HIC) had to be employed to generate homogenous and comparable ADC preparations. Conjugation on all three sites had a negligible effect on thermal stability, FcRn binding or immunogenicity. Likewise, all three ADC variants had similar in vitro cell killing activity that was comparable to a chemically conjugated Trastuzumab–DM1 conjugate used as a reference. In contrast, maytansine conjugates differed with respect to serum stability, pharmacokinetics and in vivo efficacy, with the C-terminal heavy chain conjugate displaying the most favorable properties. Significantly, when compared to chemically conjugated Trastuzumab-DM1, the C-terminal conjugate exhibited a substantially improved safety profile, causing no mortality in Sprague–Dawley rats even at a dose of 60 mg/kg.

Taken together, the FGE approach is an attractive method for the generation of site-specifically conjugated ADCs, with several properties superior to chemical conjugates such as Kadcyla®. However, the need for HIC purification to achieve homogeneous ADC preparations and the involvement of a two-step chemo-enzymatic conjugation makes this approach less straightforward than some of the other site-specific approaches. The flexibility of placing the FGE tag at different locations within an antibody is a clear advantage. However, the different properties of the resulting ADCs and the identification of the heavy chain C-terminus as the preferred site render this advantage academic. Given the highly encouraging data available to date, it will be exciting to see how the FGE approach translates into the clinic.

### *5.2.4 Split Inteins for Generating Site-Specifically Conjugated ADCs*

Another enzyme class that can catalyze the formation of peptide bonds are the so-called inteins (Shah and Muir 2014). Inteins (*inte*rvening prote*ins*) are naturally occurring "protein introns" that excise themselves out from larger precursor proteins by the cleavage of two peptide bonds and the formation of one novel peptide bond between the so-called exteins, flanking the intein. In analogy to RNA–

splicing, these could be considered "protein exons." The process does not require any external factor or energy source and only requires proper folding of the intein domain. Such intein domains can also occur as so-called split inteins (Aranko et al. 2014). In split inteins, the intein domain is split into two sub-domains (N-intein and C-intein) that can be appended to different proteins (exteins, i.e., the N-extein and C-extein). In this configuration, the process leads to the ligation of the two extein domains and is referred to as protein trans-splicing (PTS) (Wood and Camarero 2014). Split inteins can occur naturally or have been engineered artificially from naturally occurring inteins, and about 100 split inteins are known to date. These split inteins have been used as tools in protein engineering, because they can be used to ligate random protein domains, or to append (poly-)peptides with or without modifications to other proteins. Therefore, split inteins can also be used in a versatile manner for protein engineering, protein purification, and protein labeling (Shah and Muir 2014; Mills et al. 2014).

As a consequence, the principle can also be applied to antibodies or antibody fragments (Fig. 5.6). Möhlmann and colleagues have been the first to suggest the use of inteins and split inteins for the site-specific conjugation of payloads, including toxins to the C-termini of full-length antibodies, in order to generate site-specifically conjugated ADCs with DAR2 (Möhlmann et al. 2011b). In this study, two different intein approaches have been evaluated: First, the *Mycobacterium xenopi* (*Mxe*)

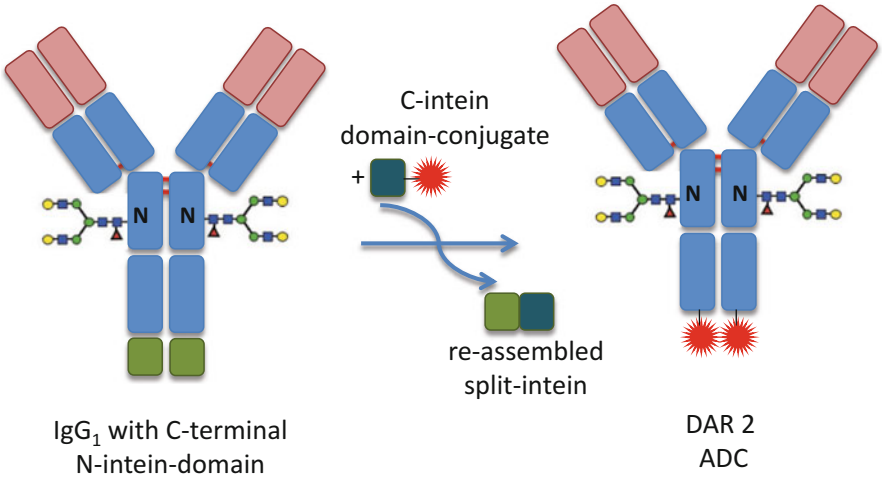


**Fig. 5.6** Schematic representation of split intein-mediated site-specific conjugation of antibodies with payloads. The N-terminal split intein domain (N-intein, *light green*) can be fused to the C-termini of, e.g., IgH chains of an antibody by standard genetic engineering. If this engineered antibody is incubated with the matched C-terminal domain of the intein (the C-intein, *dark green*), to which a payload (*spiked, red-colored ball*) has been coupled, the N- and C-intein domains of the split intein will reassemble and catalyze the formation of a peptide bond between a(n) amino acid residue(s) that is part of the payload (serving as a C-extein) and the Fc-domain of the IgG antibody (serving as an N-extein). This will lead to the formation of a site-specifically conjugated ADC with DAR2

GyrA intein by way of expressed protein ligation (EPL) and second, the *Nostoc punctiforme* (*Npu*) DnaE naturally occurring split intein that is known to efficiently catalyze PTS. While antibody intein/N-intein fusions to the C-terminus of the IgH chains resulted in antibodies with good expression yields upon transient expression in HEK293 cells, the GyrA protein showed some level of self-cleavage already, rendering the *Mxe* GyrA intein less straightforward for ADC manufacturing (Möhlmann et al. 2011b). In contrast, the *Npu* DnaE N-intein domain fused to the C-termini of the IgH chains did not result in any auto-cleaved product and could be used for PTS with a *Npu* DnaE C-intein domain, that was either functionalized with a biotin or with a larger (ca. 80 kDa) Glutathione-S-transferase (GST) protein domain. This study showed for the first time that inteins and split inteins can be used to site-specifically conjugate small molecules or proteins to the C-termini of antibodies, and the authors suggested that this could equally be applied to small molecular weight or protein toxins for the generation of ADCs and immune-conjugates. Some commercial entities, like, e.g., Proteodesign (http://www.proteodesign.es) and ZIP-Solutions (http://www.zipsolutions.es) are working on ADC manufacturing involving split intein-based approaches.

For the C-terminal modification of proteins, Volkmann and coworkers have shown that an *Ssp* GyrB S11 split intein can be split, such that it only requires an extremely short, six amino acid long C-intein peptide to regenerate a functional intein (Volkmann and Liu 2009). This split intein can be used to C-terminally label proteins with short synthetic peptides, which could be modified by small molecular weight probes (Volkmann and Liu 2009). This concept would ideally be suitable for the generation of ADCs, because no large, recombinant C-intein proteins would be required to catalyze PTS for C-terminal conjugation of payloads to antibodies. Indeed, with the *Ssp* GyrB S11 split intein, short synthetic peptides that can be modified with toxophores using standard medicinal chemistry could be used instead.

### 5.2.5 *Glycan-Remodeling Approaches to Create Handles for Site-Specific Conjugation*

Generation of site-specifically conjugated ADCs has also been described by attaching payloads to the glycan structure found on Asn-297 (N297) of $IgG_1$ antibodies. There are essentially two alternative approaches, both involving enzymatic remodeling of the glycan structure, followed by chemical conjugation of a suitably modified toxic payload. Both approaches are briefly summarized below.

The first approach involves an initial treatment of the antibody with *S. pneumonia* β1,4-galactosidase, leading to a release of all terminal galactoses and the formation of a homogenous G0 isoform of the antibody. The de-galactosylated antibody is then modified with derivatives of galactose containing a chemical handle at the C-2 position, such as ketone or azide, using a

mutant β1,4-galactosyltransferase (Boeggeman et al. 2009; Ramakrishnan and Qasba 2002). Funtionalized small molecules such as biotin or fluorescent dyes can then be conjugated to the antibody using an appropriate chemistry (Boeggeman et al. 2009). More recently, this method has been adapted to the production of ADCs, and an ADC based on the anti-HER2 mAb m860 carrying an auristatin F payload has been produced and characterized to some extent (Zhu et al. 2014). Mass spectroscopic analysis showed that the homogeneity of the conjugate was dramatically improved compared to chemical conjugates, with the majority of the antibody molecules carrying four drugs. The in vitro cell killing activity of this ADC on HER2-overexpressing SKBR3 cells was also evaluated and shown to be similar to that of T-DM1. Further, the ADC was shown to be stable in human serum over a period of up to 4 weeks. Unfortunately, no in vivo efficacy data have been presented.

A second site-specific approach based on remodeling of an antibody's glycan structure was reported recently (Zhou et al. 2014). In this approach, a mixture of β1,4-galactosyltransferase and α2,6-sialyltransferase was used to incorporate terminal sialic acid residues into the native glycan structure of an antibody. Sialic acid residues were then oxidized under mild conditions, leading to the formation of aldehyde groups that in turn served as chemical handles. Amino-oxy-functionalized cytotoxic payloads are conjugated to the antibody's modified glycan structures via oxime ligation. Several ADCs with DARs in the range of 1.6–1.9 were produced, using appropriately functionalized monomethyl auristatine A (MMAE) and dolastatin-10 payloads. Glyco-conjugated ADCs based on Trastuzumab were found to display potent in vitro cell killing activities on HER-2-overexpressing SKBR3 cells and had noticeable in vivo antitumor activities in a HER-2-overexpressing SKOV-3 xenograft model. However, when compared to the corresponding conventional ADCs prepared by thiol-maleimide chemistry, glyco-conjugated ADCs were significantly less potent. While this was attributed to an approximately two-fold lower DAR of the glyco-engineered ADCs compared to the conventional ADC used for comparison, this will have to be confirmed experimentally with ADC variants having the same DAR.

In summary, glycoengineering provides an elegant approach for the preparation of ADCs, without the need of additional antibody engineering/modification. There are currently several Biotech companies actively pursuing various aspects of glycoengineering for the manufacturing of ADCs, including Synaffix (http://www.synaffix.com/) and Glykos (http://www.glykos.fi/). It will be interesting to see, if and which place these novel glyco-ADC formats will take in the clinic.

### *5.2.6 Other Enzymatic Approaches*

Additional enzymes that recognize peptide tags as specific conjugation sites in antibodies are being explored. This includes, e.g., protein farnesyl transferase (PFT), which is an enzyme that prost-translationally adds a farnesyl group to a

C-terminal cysteine within a CAAX motif located at the C-terminus of proteins (Palsuledesai and Distefano 2015). Commercially, this approach is pursued for the development of ADCs by Korean Legochem. Phosphopantetheinyl transferase (PPTase) is another enzyme class involved in posttranslational modification of proteins at serine residues in 11- or 12-mer peptide substrates. Researchers at Novartis have screened 110 positions in trastuzumab leading to the identification of 63 positions, at which a Sfp PPTase enzyme, which recognized variants of acetyl-CoA as a substrate, could be used to conjugate auristatin payloads (Grünewald et al. 2015). Like the Pfizer-Rinat group, who found that conjugations at different positions catalyzed by BTG have a significant impact on the pharmacokinetic properties of the ADCs, this was also confirmed by the study using PPTase enzyme. A selected ADC showed tumor regression upon a single dose of 3 mg/kg in a HER2-positive xenograft model, demonstrating the potential of this enzymatic conjugation approach (Grünewald et al. 2015). Further, researchers at Eucodis have generated a mutant of the protease trypsin by directed evolution that can promote the formation of peptide bonds, rather than their hydrolysis. This enzyme was termed trypsiligase and recognizes the trypeptide sequence YRH. This approach has been used to generate a C-terminally modified anti-HER2 $F_{ab}$ fragment with smaller (fluorescein) and larger molecules (PEG) (Liebscher et al. 2014). The list of enzymes that can be used to conjugate payloads site-specifically to binding proteins and antibodies is likely to increase in the future. It will be interesting to see, which of these technologies, based on parameters like, e.g., efficiency of the enzyme, selectivity, scalability, and cost of goods, will develop into a commercially viable technology with broader application in the development of clinically applied ADCs.

## 5.3 Conclusions

The development of enzyme-based approaches for site-specific conjugation of toxin payloads to antibodies to generate potent and effective ADCs has seen a surge in recent years. All first-generation ADCs currently approved for clinical use, or which are currently in clinical evaluation, have been generated by chemical conjugation technologies. However, next-generation ADCs generated by site-specific enzymatic conjugation are finally entering into the field. Certainly two most compelling arguments for enzymatic conjugation are: First, enzymes usually work under physiologic conditions that are most optimal to preserve the structural and functional features of the tumor targeting antibody, such that the resulting ADC product will remain in a functional configuration throughout the manufacturing process. Second, the natural selectivity of enzymes can be exploited to direct the conjugation to any region or site in the antibody molecule, rather than relying on naturally existing positions for conjugation that are determined by the primary, secondary, or tertiary structure of the antibody. However, not all enzyme-based conjugation approaches offer this type of flexibility.

Because many enzymatic conjugation technologies rely on enzymes that are naturally involved in post-translational modification of proteins, often a diversity of enzymes or enzyme specificities have evolved evolutionarily. Alternatively, orthogonal enzyme specificities or optimized properties of the enzymes can be engineered by directed evolution. Such orthogonal conjugation specificities or more active enzymes are available within the same platform, allowing generation of more complex ADC molecules, e.g., site-specifically conjugated dual-toxin ADCs with two therapeutic modalities combined in a single molecule. Many enzymatic conjugation technologies that have been evaluated in the context of preclinical in vivo experiments almost exclusively show improved therapeutic indices in comparison to conventional ADC products. This is mostly due to increased stability of such ADCs in circulation/human serum that translated into higher tolerability of such ADCs.

Enzymatic conjugation technologies of course involve one additional component in the cGMP manufacturing of ADCs, namely the enzyme. While this needs to be added to the cost-of-goods (COGS) of ADC manufacturing, this may be compensated by advantages in the selectivity, efficiency, and the ease of enzymatic reactions, which do not require elaborate reagents or organic solvents that represent their own challenges in cGMP manufacturing, like, e.g., incompatibility with (cheaper) disposable bag-based reaction vessels, need for glass vessels, or more complex cleaning/disposal procedures in the manufacturing process. The promise of enzymatically conjugated, next-generation ADCs now needs to be translated into generation of clinical-grade ADC material and its validation in clinical phase studies. Based on promising data from preclinical evaluation, it is likely that we will soon see first ADC products generated by enzymatic conjugation for the treatment of cancer in patients.

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# Chapter 6
# Substance P–Saporin for the Treatment of Intractable Pain

**Hugh Nymeyer, Douglas A. Lappi, Denise Higgins, Carl E. Noe, and Arthur E. Frankel**

**Abstract** Pain is a worldwide chronic health issue, and additional therapies are needed for the management of severe pain. Substance P–Saporin (SP-SAP) covalently links a biological toxin (SAP) and an endogenous peptide (SP) that targets that toxin to the subset of neurons expressing the NK1 receptor. SP-SAP, currently in phase I clinical trials, is unique as the only targeted toxin for pain to undergo human testing. We review the history of SP-SAP and related NK1-receptor targeted toxins. We review animal data on the safety and efficacy of SP-SAP for the treatment of pain in light of the results of NK1 receptor antagonists and knockouts/knockdowns of either SP or the NK1 receptor. Finally, we discuss possible mechanisms of action of SP-SAP.

**Keywords** SP-SAP • Substance P • NK1 • Hyperalgesia • Allodynia • Pain

## 6.1 Introduction

Worldwide, one in five individuals suffer from chronic pain (Gureje et al. 1998), more in those with acute or chronic disease. Half of current and former cancer patients report persistent pain, and a third of these patients grade their pain as moderate to severe (van den Beuken-van Everdingen et al. 2007). Two-thirds of terminal cancer patients have pain, more if the cancer is of the head, neck, or pancreas (van den Beuken-van Everdingen et al. 2007).

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Despite the widespread nature of pain, pain therapies have not changed substantially in the past several decades. Pain treatment remains based upon the WHO's analgesic ladder for cancer pain (Organization 1986, 1996), which recommends a treatment progression from (step 1) nonsteroidal anti-inflammatories (NSAIDs) and acetaminophen to (step 2) weak opioids and NSAIDs and then to (step 3) strong opioids and NSAIDs. This is the basis for treatment guidelines both of cancer pain (Ripamonti et al. 2012; Caraceni et al. 2012; Auret and Schug 2013) and of most chronic non-cancer pain (Turk et al. 2011). Although other approaches to pain control do exist, most are restricted to pain having a certain specific etiology or anatomical localization.

Despite their central importance for pain management, NSAIDs and opioids carry significant and well-appreciated risks (Rainsford 1999; McNicol et al. 2003; Hansen et al. 2014). In a retrospective analysis (Meuser et al. 2001) of 593 cancer patients treated by the pain service at Cologne University Hospital, conventional pain therapy with NSAIDs and opioids resulted in impaired activity 74%, mood changes 22%, sedation 14%, constipation 23%, nausea 23%, dyspnea 16%, dysphagia 11%, and urinary problems 6%. Furthermore, the use and misuse of opioids carry a significant societal cost (Hansen et al. 2014). The nonmedical use of prescription opioids cost the United States $53.4 billion dollars in 2006 (Hansen et al. 2011), and more than 16,000 opioid overdose deaths occurred in the United States in 2010 (Centers for Disease and Prevention 2012).

Despite the significant drawbacks to NSAIDs and opioids for pain control, their effectiveness is mixed. In the same retrospective analysis from Cologne University Hospital, pain relief was deemed inadequate in 14% of patients (Meuser et al. 2001), a statistic consistent with other reports from pain management groups. In one analysis, 9% of cancer patients were reported to have inadequate pain control at last therapy (Schug et al. 1990), and in another study 12% of palliative care patients were reported to have inadequate pain control over the course of treatment (Zech et al. 1995). For neuropathic pain, efficacy is significantly less: only one in three individuals with neuropathic pain achieve a 50% or greater reduction in pain using conventional therapy (Sindrup and Jensen 1999). Clearly, new approaches to pain management are sorely needed.

In this chapter, we review the development and animal tests of SP-SAP, a promising non-opioid and non-NSAID pain therapeutic with a novel mechanism of action. SP-SAP is the first targeted pain drug to undergo human trials: it covalently links a targeting molecule (substance-P, SP), selective for spinal cord neurons that carry C-fiber nociceptive signals, and an effector molecule (saporin, SAP), an endonuclease that destroys the targeted neuron. Delivered to the intrathecal space, data suggests that SP-SAP selectively destroys pain-transmitting pathways, permanently reducing the sensation of pain.

SP-SAP has several potential advantages over most conventional pain therapies. A single intrathecal dose of SP-SAP could provide a lifetime of pain relief, because it permanently removes selected pain-transmitting neurons. Because this is a targeted therapeutic and because only a single dose is administered over a patient's lifetime, systemic effects from this therapy would be minimized and, unlike

opioids, psychological dependence would be precluded. Because SP-SAP targets pain fibers in the spinal cord, it should be effective for treating pain from multiple regions and of multiple origins. For example, it should be able to treat both neuropathic and non-neuropathic pain. Because of its targeted nature, motor and non-nociceptive sensation should be preserved. Finally, because SP is a molecule that is primarily released from C-fibers, there is evidence that SP-SAP may ameliorate chronic pain arising in C-fibers while preserving the sharp pain transmitted by A-delta fibers. This selective modification of pain is a potential advantage, because sharp pain has an important role in preventing self-injury.

Although a number of studies have investigated SP-SAP in animal models, the results of these investigations are largely unknown to the broader medical and scientific community. For this reason and because SP-SAP is now undergoing human trials, we thought it would be useful to provide a review of the background and animal testing of this drug. We begin with a brief description of substance P (SP) and its possible roles in pain transmission and pain sensitization. We describe the SP-SAP drug conjugate, its safety and efficacy in animal models of pain, and the current human trials of SP-SAP. The results of these studies are then interpreted in light of the paradoxical results of knockout and knockdown animals and antagonists to SP receptors. Finally, we review possible mechanisms of action of SP-SAP.

## 6.2 Animal and Human Tests of SP-SAP

### *6.2.1 The Substance-P and Saporin Molecules*

SP was first identified in 1931 by von Euler and Gaddum (1931) as a vasodilator and peristaltic inhibitor concentrated in brain and intestinal tissue. SP was quickly determined to be an oligopeptide (v. Euler 1936), but its sequence was not determined until 1971 (Chang et al. 1971). SP belongs to a class of tachykinin peptides sharing a C-terminal sequence motif and widely expressed throughout the body (Steinhoff et al. 2014). The native SP receptor is the neurokinin-1 receptor (NK1R), a highly conserved G-protein-coupled receptor with two isoforms, a long form and a truncated form, that are differentially expressed in different tissues (Kage et al. 1993; Mantyh et al. 1996; Baker et al. 2003; Caberlotto et al. 2003; Lai et al. 2006, 2008; Gillespie et al. 2011). High sequence homology among the tachykinins and the measured in vitro binding constants (Masu et al. 1987; Yokota et al. 1989; Shigemoto et al. 1990) suggest there is biologically relevant cross talk among the different tachykinins and their receptors.

NK1R is highly conserved across investigated species. Based on a rhodopsin homology model, relative to the human NK1R, the rat NK1R is 94.6% identical, the gerbil NK1R is 95.1% identical, the dog NK1R is 97.5% identical, the mouse NK1R is 94.6% identical, and the guinea pig NK1R is 96.6% identical (Leffler et al. 2009).

SP is also conserved across these species and activates NK1R with similarly efficiency. For example, binding assays of SP to NK1R using rabbit, guinea pig, and rat cerebral cortex were used to derived binding constants of 165 pM, 122 pM, and 106 pM, respectively (Beresford et al. 1991). Human and canine NK1 receptors are especially similar, sharing 100% homology in the transmembrane helices and differing in the extracellular loops at only three positions (Leffler et al. 2009). Similarly, functional assays show that different synthetic NK1R antagonists have similar $IC_{50}$ values in most species, especially between humans and canines (Leffler et al. 2009).

Initially, it was not recognized that SP is a neurotransmitter; however, when it was discovered that SP is more concentrated in the dorsal spinal nerve roots than the ventral nerve roots, Lembeck and Pernow suggested that SP may have an important role in the transmission of pain (Lembeck 1953; Pernow 1953). A number of subsequent discoveries supported that hypothesis. For example, in the spinal cord, SP is concentrated in unmyelinated fibers in Lissauer's tract and the dorsal horn (Hokfelt et al. 1975a, b; Takahashi and Otsuka 1975). Although SP can be found in many parts of the gray matter (laminae III, IV, and V, the lateral spinal nucleus, lamina X, and the medial edge of the dorsal horn, see Fig. 6.1), SP density is greatest in lamina I and the outer regions of lamina II (Ribeiro-da-Silva and Hökfelt 2000), areas known to be important in the transmission of pain. Furthermore, ligation of the dorsal roots depletes SP in the dorsal horn (Takahashi and Otsuka 1975), suggesting that SP is synthesized by sensory afferent fibers and transported to axon terminals in the dorsal horn, where it is stored in vesicles (Cuello et al. 1977). As expected for a pain neurotransmitter, high temperature (Go and Yaksh 1987; Duggan et al. 1987) pinch (Duggan et al. 1988), noxious chemicals (Kuraishi et al. 1985; Duggan et al. 1988) capsaicin (Go and Yaksh 1987; Takano et al. 1993), and direct C-fiber stimulation (Go and Yaksh 1987; Brodin et al. 1987; Klein et al. 1992) all induce the release of SP in the spinal cord, and this release can be blocked by opioids (Yaksh et al. 1980). Direct iontophoric application of SP slowly depolarizes neurons in the dorsal horn (Henry et al. 1975; Randić and Miletić 1977). Finally, intrathecal injections of SP evoke behaviors in animals associated with pain (Piercey et al. 1981; Hylden and Wilcox 1981; Matsumura et al. 1985).

In the dorsal gray matter, the highest fraction of NK1R+ neurons is in lamina I (Todd et al. 1998), consistent with the association of SP and pain signaling via lamina I projection fibers; however, there is a background level of NK1R throughout the gray matter, in several ventral motor nuclei, and the intermediolateral nucleus (Torrens et al. 1983; Ninkovic et al. 1984; Shults et al. 1984; Mantyh and Hunt 1985; Dietl et al. 1989; Yashpal et al. 1990). The mismatch between SP+ neurons and NK1R+ neurons, between the neurotransmitter and its receptor, was observed throughout the central nervous system (Torrens et al. 1983; Quirion et al. 1983; Viger et al. 1983). This mismatch, the absence of co-localization of NK1R at synaptic clefts, the demonstrated ability of SP and other peptides to diffuse long distances throughout the spinal cord parenchyma, and the lack of known peptide reuptake mechanisms, all led to the suggestion that SP may act primarily via volume transmission (Agnati et al. 1986) rather than purely via synaptic

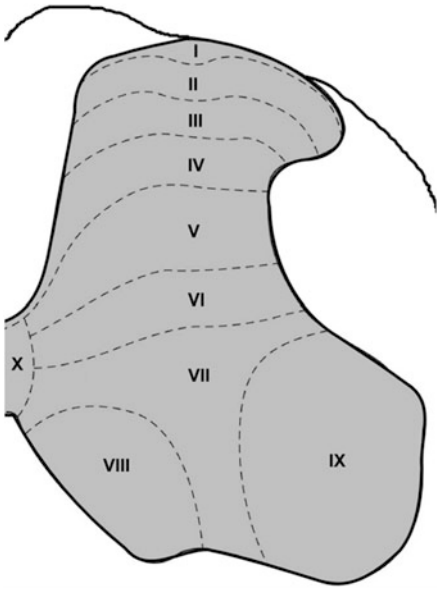


**Fig. 6.1** An illustration showing the typical shape of the grey matter on one half of a transverse section of the lumbar spinal column. Numbered regions show the Rexed laminae, regions identified by their cytoarchitecture (Rexed 1952, 1954). Lamina I is the most dorsal, and lamina X is central to the spinal cord. The majority of neurons that transmit pain and temperature sensation begin in lamina I, with a smaller number scattered throughout laminae III–V. 80% of the projection neurons in lamina I show NK1R immunoreactivity (Todd 2010). However, only a small fraction (perhaps 5%) of the lamina I neurons are projection neurons; most are interneurons (Todd 2010). SP-SAP treatment can ablate NK1R neurons throughout the grey matter, but the strongest effect is in lamina I because of the higher concentration of NK1R+ neurons and possibly because of its superficial location, proximate to the SP-SAP injection

transmission. It is now generally recognized (Kandel et al. 2000) that SP modulates pain sensation but is not *the* primary pain neurotransmitter.

SP is endocytosed when bound to many different NK1R+ cells, both cultured non-neural cells (Larsen et al. 1989; Sjodin 1992; Garland et al. 1994) and neurons (Mantyh et al. 1995a, b). After endocytosis, NK1R-bound SP is lysosomally degraded, and the NK1Rs are recycled to the cell surface (Grady et al. 1995). This feature of the SP/NK1R system provides an opportunity to selectively target and destroy NK1R+ cells by covalently attaching to SP a cellular toxin that cannot normally enter cells but that is able to survive and enter via NK1R-mediated endocytosis and lysosomal processing. This selective targeting of a particular neural cell for destruction in this way has been described as "molecular neurosurgery" (Wiley and Lappi 2003). Examples of conjugates that can kill NK1R+ cells in this manner are $DAB_{389}$SP-Gly, a conjugate of SP and diphtheria toxin (Fisher et al. 1996); SP-SAP, the focus of this article (Wiley and Lappi 1997); SSP-SAP (Wiley and Lappi 1999); BoNT/A-LC:SP (Mustafa et al. 2013), which covalently links the Botulinum neurotoxin A light chain and SP; and SP-PE35 (Saka et al. 2002), which

covalently links SP to a *Pseudomonas* exotoxin that selectively targets cholinergic and nitric oxide synthase interneurons.

The kinetics of SP-induced NK1R endocytosis is quite rapid. In cells from the rat dorsal horn, NK1R-containing endosomes have migrated to the cell interior 90–120 s after stimulation by SP with a best fit single exponential time constant of 71 s (Wang and Marvizon 2002), a result that is in close agreement with internalization of NK1 receptors in lamina I neurons of live rats stimulated by hindpaw capsaicin injections (Mantyh et al. 1995b). In the SP-SAP conjugate, internalization of SP-SAP on NK1 receptors is also rapid; however, cellular death takes several days secondary to SP-SAP either exiting the lysosomes or inducing cellular death (Mantyh et al. 1997).

SSP, also known as [$Sar^9$, $Met(O_2)^{11}$]-SP, is a modified SP peptide in which $Gly^9$ has been replaced with a sarcosine (*N*-methylglycine) residue, and $Met^{11}$ has been replaced with a sulfone group (Drapeau et al. 1987). SSP has an increased affinity for NK1R (Drapeau et al. 1987; Tousignant et al. 1989, 1990) and an increased resistance to proteolysis (Sakurada et al. 1994). A number of animal studies have utilized SSP-SAP, which functions much as SP-SAP does, but with an increased activity due to the stronger affinity of SSP for NK1 receptors that could potentially allow for more precise neural targeting (Wiley and Lappi 1999).

SAP, the cellular toxin used in SP-SAP, is a 30 kDa protein found in seeds of the soapwort plant *Saponaria officinalis* (Stirpe et al. 1983) that induces apoptosis via its *N*-glycosidase activity on the large ribosomal subunit (Stirpe 2004). It has several isoforms. Isoform three of saporin-6 (Barthelemy et al. 1993) was selected for use in SP-SAP because of its abundance and potency. A disulfide linkage from the derivatized N-terminus of SAP covalently links it to a $CYG_5$ peptide, which is connected by a peptide bond to the N-terminus of SAP (Lappi and Wiley 2000).

### *6.2.2 Selective Neuronal Cytotoxicity of SP-SAP*

SAP by itself has only a weak ability to enter cells, so it is relatively nontoxic unless covalently attached to SP: a non-covalent mixture of SP and SAP is 500 times less toxic than the conjugated SP-SAP molecule as measured by the median effective dose for inducing cell death (Wiley and Lappi 1997). Similarly, an SP receptor is necessary for lethality: SP-SAP is 500 times less toxic to a cell that does not express an NK1R than to one that does by the same measure (Wiley and Lappi 1997).

Because of this 500-fold difference in toxicity, SP-SAP can kill cells that express high levels of NK1R while leaving other cells in the vicinity undamaged. In neonatal spinal cord neurons in culture, a $10^{-7}$ M mixture of SP and SAP left no visible cellular damage, but $10^{-7}$ M SP-SAP produced widespread cell death secondary to NK1R endocytosis (Mantyh et al. 1997) with full lethality observed only after 10 days. Similar differences in toxicity between SP, isolated toxin, and SP-toxin conjugates were also observed in $DAB_{389}$SP-Gly (Fisher et al. 1996) and SSP-SAP (Wiley and Lappi 1999).

The selective cytotoxicity of SP-SAP was also demonstrated in the central nervous system (CNS). When injected in rat striatum, SP-SAP induced a dose-dependent cytotoxicity (Wiley and Lappi 1997). Similarly, when SP-SAP was injected intrathecally into rat spinal cords at the L4 level, it reduced NK1R levels in the combined laminae I/II region by 85%. More importantly, this cytotoxicity was selective. At 28 days posttreatment, cell counts of preganglionic sympathetic neurons, motor neurons, astrocytes, microglia, SP-expressing cells in the dorsal root ganglion (DRG), and SP+ neurons in laminae I/II were all unchanged—indicating that these other cells, including the SP-secreting afferent fibers, were not reduced by the SP-SAP treatment (Mantyh et al. 1997).

In all existing studies of SP-SAP, NK1R+ cell counts were shown to be strongly depleted in laminae I/II after SP-SAP treatment: 85% (Mantyh et al. 1997), 59% (Nichols et al. 1999), 68% (Khasabov et al. 2002), greater than 58% (Vierck et al. 2003), and 90% (Abe et al. 2005). Similar reductions were seen in the lamina III or combined laminae III/IV NK1R+ cell counts (Nichols et al. 1999; Khasabov et al. 2002; Vierck et al. 2003; Abe et al. 2005; Allen et al. 2006; Choi et al. 2012). In most studies, deeper laminae were less affected by SP-SAP; however, in one particular study NK1R+ cell counts were depleted by one-half to one-third in deeper laminae (laminae IV and V), but this depletion was observed only 100 days or more posttreatment (Weisshaar and Winkelstein 2014). It is unknown if these effects were a direct effect of SP-SAP or changes caused by spinal cord reorganization secondary to the interruption of circuits involving superficial NK1R+ neurons.

The reduced cytotoxicity seen in deeper lamina—particularly the ventral horn motor neurons as quantified by acetylcholine acyltransferase staining—was speculated to be due to the short (less than 15 min) half-life of SP-SAP in the CNS (Allen et al. 2006; Wiese et al. 2013) and the time required to diffuse to deeper lamina (Mantyh et al. 1997). Although NK1R is expressed throughout the body on neural and nonneural tissues (Steinhoff et al. 2014), risks of damage to cells outside the CNS are minimal because the mean half-life of SP-SAP in plasma is less than 5 min (Schaffalitzky De Muckadell et al. 1986; Allen et al. 2006). The primary concern for unintentional SP-SAP damage is in the CNS, where NK1R is expressed in the cerebral cortex, mesencephalon, brainstem nuclei, and diffusely throughout the spinal cord gray matter (Snijdelaar et al. 2000; Steinhoff et al. 2014). More specifically, motor horn cells of the ventral spinal cord contain NK1R+ neurons, which raise the possibility that SP-SAP treatment might result in paresis or paralysis (Allen et al. 2006). For example, in at least one study, high doses of SP-SAP injected into the cisterna magna did produce paraparesis and ataxia in a small number of animals (Brown and Agnello 2013). Risks of SP-SAP to areas outside the spinal cord are less of a concern in larger animals undergoing intrathecal injections, because the rostral–caudal spread of SP-SAP is limited to a region of about 5–10 cm (Allen et al. 2006).

It is difficult to predict the therapeutic index (TI) of SP-SAP—here taken to be the ratio of the concentration of SP-SAP that produces significant damage to NK1R− cells to the concentration of SP-SAP that produces significant damage to

NK1R+ cells—because the TI depends on the efficiency with which SP-SAP can enter a cell, the lethality of SAP to the cell, and the efficiency with which SP-SAP can enter NK1R- cells, all of which differ between cell types and cellular environment. In KNRK cells transfected with the NK1 receptor, the TI is about 500 (based on an $LD_{50}$ of SP-SAP of 2 nM in NK1R+ cells and an $LD_{50}$ of 1000 nM in NK1R− cells) (Wiley and Lappi 1997). In rat striatum, SP-SAP appears to have a TI of at least 100 based on direct injections of SP-SAP into the parenchyma (Wiley and Lappi 1997). In canines, using intrathecal injections of SP-SAP at the L2–L3 interspace, the TI also appears to be greater than 100, although the exact value is unknown (Allen et al. 2006). Based on the high homology between canine and human NK1 receptors, we expect the TI for humans to be similar in magnitude.

### *6.2.3 Small Animal Studies of SP-SAP and Related Compounds*

#### 6.2.3.1 Methods for Quantifying Pain in Small Animals

For SP-SAP to be useful as an analgesic drug, its safety and efficacy profile must be quantified; unfortunately, analgesic testing in animals is fraught with interpretive difficulties (Le Bars et al. 2001). First, there are numerous types of pain, and each can respond differently to different therapies. Pain can vary in its origin, intensity, character, method of transmission, and in the behaviors elicited. Second, pain is only inferred indirectly via those behaviors, and pain behaviors can be triggered and modified by other things besides pain, e.g., anxiety, aggression, conditioning, and non-pain reflexes, which can mask the pain response. Third, most animal pain models quantify changes in the nociceptive or sensory threshold rather than pain intensity, which makes them less than ideal models for clinically important pain.

Despite these limitations, methods to induce and measure pain in animals have been developed and are in wide usage (Le Bars et al. 2001). A commonly used predictive model of acute pain is the formalin test; pain is induced by injecting the dorsal surface of a rat paw with a 0.5–15% solution of formalin, and the level of pain is assessed by changes in posture with respect to the paw. The formalin test elicits a biphasic response, an early phase that occurs about 3 min after injection and a slow phase that occurs 20 min postinjection. Both early and late phases respond to opiates, but only the later phase responds to NSAIDs. Other algogenic agents, including capsaicin or complete Freund's adjuvant (CFA), have been used in place of formalin in a similar test.

The formalin test is a test of acute or tonic pain; however, injections of a chemical irritant, e.g., carrageenan, capsaicin, or CFA, into the plantar surface of a rat's paw will also induce inflammation leading to thermal hyperalgesia and mechanical allodynia. Mechanical allodynia is usually detected by the threshold force needed to induce paw withdrawal using von Frey hairs, and thermal hyperalgesia is normally assessed by the latency for paw withdrawal from a hot

surface. Variants of these three tests—the formalin test, the thermal hyperalgesia test, and the mechanical allodynia test—have been used for the study of targeted pain therapies including SP-SAP.

#### 6.2.3.2 The Effect of SP-SAP on Models of Acute Pain, Hyperalgesia, and Allodynia

A number of studies have investigated the safety and efficacy profile of intrathecal spinal injections of SP-SAP in small animals. Intrathecal injections into the lumbar region of rats produced no observable changes in body weight, alertness, or behavior within the 1 month observation period; however, this injection did reduce nocifensive behaviors in response to subsequent capsaicin injections (Mantyh et al. 1997), which included an 85% reduction in mechanical hyperalgesia, a 60% reduction in thermal hyperalgesia, and a 75% reduction in acute pain quantified by lifting and guarding of the injected paw. These behavioral changes were evident 3 days post SP-SAP injection and remained throughout the 28-day period.

A follow-up study using rats pretreated 30 days prior to sensitization showed similar analgesic effects on a wide variety of pain tests (Nichols et al. 1999). Statistically significant reductions were observed in late-phase pain behaviors induced by subcutaneous formalin injections, mechanical allodynia from subcutaneous carrageenan injections, mechanical allodynia from CFA injections, and mechanical allodynia created by nerve ligation. Results were durable for 90 days until the study end. These sorts of reductions in pain were supported by similar studies using intrathecal SP-SAP (Suzuki et al. 2002; Khasabov et al. 2002), intrathecal $DAB_{389}$SP-Gly (Benoliel et al. 1999), intra-cisternal injections of SP-SAP on oral capsaicin-induced pain (Simons et al. 2002), intra-cisternal injections of BoNT/A-LC:SP on taxol-induced thermal hyperalgesia (Mustafa et al. 2013), carrageenan-induced hyperalgesia (Choi et al. 2012), in a study of mechanical injury to the zygapophyseal joints of rats pretreated with SSP-SAP (Weisshaar and Winkelstein 2014), and in a study of opioid-induced and incision-induced hyperalgesia (Rivat et al. 2009).

#### 6.2.3.3 The Effect of SP-SAP on Operant-Conditioning Models of Pain

As in all animal pain studies, one should consider the fact that most of these studies quantified the effects of treatment via the threshold of stimulation necessary to elicit a response, e.g., the threshold mechanical force needed to elicit a paw motion. Threshold measurements might not be an accurate method of quantifying clinical pain. Furthermore, certain pain-induced behaviors such as paw licking and guarding responses in response to applied heat can be elicited in decerebrate rats and might be poor models of clinical pain (Woolf 1984; Berridge 1989).

Two studies addressed this concern directly by using an operant escape test in which a rat could voluntarily partition its time between a box with a hot floor and

another box with an unpleasant bright light. The amount of time spent in each box was quantified to determine the relative level of discomfort from the heated floor. Intrathecal lumbar injections of SP-SAP (Vierck et al. 2003) or SSP-SAP (Wiley et al. 2007) both produced significantly reductions in the time spent in the brightly lit box and increased the time spent in the box with the hot floor—supporting the observations of previous studies that treatment produced real reductions in pain sensation. A previously discussed study (Simons et al. 2002) also demonstrated an effect—consumption of capsaicin-laced water—with similar characteristics.

#### 6.2.3.4 The Effect of SP-SAP on Models of Neuropathic Pain

SP-SAP treatment was tested in a model of neuropathic pain, where it was shown to reduce the mechanical hyperalgesia induced after several weeks of ligation of the L5 and L6 spinal nerves (Nichols et al. 1999). SP-SAP treatment was also tested in a model of spinal cord injury induced by intraspinal injections of quisqualic acid, an agonist for the AMPA glutamate receptor, which induces not only pathological spinal changes resembling spinal cord injury but also a biting behavior and excessive grooming, indicative of pain (Yezierski 2006). Intrathecal SP-SAP treatment was shown to delay the onset of excessive grooming, to decrease the area of excessive grooming, and to decrease the severity of excessive grooming; these changes occurred whether treatment occurred at the time of injury or after the onset of excessive grooming (Yezierski et al. 2004).

#### 6.2.3.5 Summary of the Small Animal Studies of SP-SAP

Highlights from the various studies include the following. First, pain avoidant behaviors are produced by a diversity of neural mechanisms. Some of these behaviors are strongly affected by SP-SAP treatment and others are not. For illustration, intrathecal SP-SAP treatment produced no statistically significant reduction in the time spent in licking or guarding behaviors after standing on a hot plate (Vierck et al. 2003), and SSP-SAP showed only a weak effect at low temperatures (Wiley et al. 2007). It was suggested that licking and guarding are spinal or possibly bulbar reflexes present in decerebrate rats (Woolf 1984; Berridge 1989) and are thus less affected by SP-SAP treatment than higher-cortical responses to pain. In contrast, behavioral responses to mechanical allodynia, thermal hyperalgesia, and formalin-induced pain were all reduced by SP-SAP treatment (Suzuki et al. 2002; Khasabov et al. 2012; Khasabov and Simone 2013).

Second, intrathecal SP-SAP does not completely abolish pain transmission as much as modify pain sensitivity, hyperalgesia, and allodynia. Ablation of NK1R+ neurons affects behavioral responses to intermediate temperature challenges (42–48 °C), but responses to the highest temperatures are unaffected by SP-SAP or SSP-SAP injections, as measured behaviorally (Wiley et al. 2007) or through direct electrophysiological measurements of projection fibers (Suzuki et al. 2002).

Likewise, signaling of spinal neurons in response to 10 μg of applied capsaicin is reduced after SP-SAP treatment, but signaling is identical between SP-SAP treated rats and untreated rats when 100 μg of capsaicin is applied (Khasabov et al. 2002). These effects are consistent with data from mice missing the preprotachykinin-A protein—a protein whose cleavage products include SP—and from mice missing the NK1 receptor, both of which show a reduction in middle-intensity responses to pain but preserve normal high-intensity pain responses (Cao et al. 1998; De Felipe et al. 1998); however, see Zimmer et al. (1998) for a conflicting result.

## *6.2.4 Canine Studies of SP-SAP*

### 6.2.4.1 Safety Studies

The complexity of measuring pain using animal models, the possibility of off-target effects due to the ubiquity of the NK1 receptor, the complexities of dosing an intrathecally administered drug, and the risk of treatment-induced hyperalgesia necessitated that SP-SAP be tested on larger animals prior to human testing. Two pharmacokinetic and pharmacodynamic studies of intrathecal SP-SAP were recently carried out on canines (Allen et al. 2006; Wiese et al. 2013). Both studies used intrathecal lumbar catheters to deliver a single bolus of SP-SAP of different amounts with at least 28-day medical and behavioral observation followed by sacrificial staining of the spinal cord. Both studies observed the depletion of NK1R-expressing cells in the ventral horn with injections of 15 μg (approx. $5 \times 10^{-10}$ mol) or more of SP-SAP without statistically significant long-term medical, behavioral, or histological changes outside of the dorsal horn gray matter.

In the study by Wiese et al., four of six canines that were given the highest dose of SP-SAP (150 μg) did show unexpected neurologic dysfunction. One canine developed a crooked tail carriage, and three other canines developed pelvic limb paraparesis, lost tail tone, lost pelvic limb proprioceptive sense, and lost pelvic limb withdrawal reflexes. Two of these three dogs also acquired bladder dysfunction. Furthermore, all six canines given this dose showed a statistically significant loss of ventral horn NK1R+ staining and, in some, pathological changes in the upper spinal cord and brain. Although behavior changes were not observed in the other study by Allen et al. using injections of 45 μg or 150 μg, CNS infiltrates were observed in the 150 μg group, suggesting similar changes might have occurred there, although without frank motor dysfunction.

### 6.2.4.2 Double-Blind Randomized Controlled Efficacy Studies

Brown and Agnello (2013) recently reported the results of a double-blinded randomized controlled trial of intrathecal SP-SAP to assess efficacy in the treatment of bone pain in 70 canines divided equally between a single treatment arm and a single

control arm. Injections were given either at the L5–L6 junction for lower-limb pain or at the cisterna magna for upper-limb pain. All canine subjects were prescribed a fixed standard-of-care pain therapy at the time of randomization. Primary study endpoints were the time to unblinding, which occurred either upon canine death or upon request by the owner for a modification of pain treatment, and the total number of canines unblinded at 6 weeks post-randomization due to death or owner request. Four secondary endpoints were based upon (1) the Canine Brief Pain Inventory (BPI) estimation of pain severity (as answered by the blinded owners of the dogs), (2) the Canine BPI estimation of pain interference (similarly answered), (3) change in lameness as evaluated by an orthopedist blinded to both treatment group and visit, and (4) daytime activity counts. All secondary endpoints were evaluated 2 weeks post-randomization. Statistically significant reductions in pain in the SP-SAP treated animals were detected in both time to unblinding ($P = 0.002$) and number of canines unblinded ($P = 0.001$). Although secondary endpoints did not reach the level of statistical significance, all secondary endpoints showed improvements in the SP-SAP treated arm relative to the control arm.

Interestingly, similar motor neuron problems—hind-limb weakness and ataxia—were seen in some canines in this study, but only when injections were performed into the cisterna magna at the base of the brain and only at the higher doses—60 μg for dogs above 30 kg in weight and 40 μg for dogs 16–30 kg in weight. After the detection of paraparesis and ataxia, the remaining five canines that received cisterna magna injections received half-doses. No ataxia or plegia were seen either at the lower dose injections in the cisterna magna or in any of the lumbar injections. Furthermore, unlike previous motor dysfunction, onset was slow, occurring over 5–7 weeks. Both the lack of dysfunction in the lumbar injections and slow onset led the authors to speculate that this may be an effect of SP-SAP on higher order brain centers after SP-SAP was injected into the cisterna magna rather than direct action on the ventral horns of the spinal cord.

#### 6.2.4.3 Summary of Safety and Efficacy Studies in Canines

Based on these results, SP-SAP has been shown to produce a statistically and clinically significant reduction in pain sensation in canines consistent with the reductions in mechanical and thermal allodynia and acute pain seen in smaller animals. Second, the observed analgesic effect is correlated with reductions in the number of NK1R+ neurons in the dorsal spinal cord known to transmit nociceptive signals. Third, these effects appear to be permanent, as expected from a targeted neurotoxin. In the two canine safety studies, observations extended to 90 days (Allen et al. 2006; Wiese et al. 2013), and in the canine efficacy study (Brown and Agnello 2013), observations extended until the canines succumbed to cancer, which was in some instances several 100 days (Brown and Agnello 2013). No compensatory changes were observed in any study that would suggest the development of induced hyperalgesia after treatment. Fourth, there are potential side effects to SP-SAP due to the wide distribution of NK1R. Fortunately, SAP in isolation has

low toxicity, and substance P is rapidly hydrolyzed by endogenous peptidases; the mean half-life in the CSF is about 15 min (Wiese et al. 2013; Allen et al. 2006), and the mean half-life in plasma is less than 5 min (Schaffalitzky De Muckadell et al. 1986; Allen et al. 2006). More detailed information on efficacy for clinical relevant pain will have to wait for the results of randomized controlled trials.

### *6.2.5 Human Studies of SP-SAP*

A phase I clinical trial of SP-SAP in human subjects to assess safety is ongoing (ClinicalTrials.gov identifier NCT02036281) and due for completion by 2017. SP-SAP is being delivered intrathecally at the L5–S1 interspace in a single bolus with a maximum planned dose of 90 μg. Injections at L5–S1 are inferior to the spinal cord, minimizing the risk of spinal cord injury and reducing the risk related to cephalad spread of SP-SAP. It is expected that the spread of the SP-SAP will be limited to about 10 cm from the point of injection as was observed in the canine studies (Allen et al. 2006). The primary effectiveness assessment in this clinical trial is the change in the self-reported pain intensity, bothersomeness, and mood using a number of surveys—including the VAS or visual analog scale (Scott and Huskisson 1976) of "bothersome pain" (Patrick et al. 1995), the VAS scale of "pain intensity" (Carlsson 1983), the Oswestry Disability Index (Roland and Morris 1983), the EuroQol EQ-5D quality of life index (Rabin and Charro 2001; Oppe et al. 2007), and the Beck Depression Inventory (Beck et al. 1961; Lee and Song 1991)—as well as a daily log of analgesic use. For the purposes of dosing and toxicity evaluation, the current human trials of SP-SAP use a catheter to ensure complete injection of the drug.

## 6.3 The Relationship of SP-SAP and NK1R Antagonists

The successful application of SP-SAP as an analgesic might seem paradoxical in light of the results of NK1R antagonists, NK1R knockouts and knockdowns, and SP knockouts. It was once believed that SP is *the* primary neurotransmitter for pain signals in the spinal cord; hence, NK1R antagonists were promising candidates as opioid replacements. Unfortunately, NK1R antagonists showed only weak analgesic effects (Hill 2000).

Despite the disappointing results of NK1R antagonists for the treatment of pain, it is critically important to distinguish the effects of SP-SAP, which ablates neurons expressing NK1R, and the effects of specific antagonists to NK1R, NK1R knockouts/knockdowns, or SP knockouts. The former destroys a whole class of neurons using NK1R as a marker, but the latter merely blocks a single signaling pathway on those neurons.

Selectively blocking the NK1 receptor signal can sometimes produce the same effect as ablating NK1R-expressing neurons. For example, an intrathecal SP-SAP injection (Mantyh et al. 1997; Nichols et al. 1999; Suzuki et al. 2002), which destroys the neurons expressing NK1R, reduces pain in the formalin test. Blocking the NK1 receptor without killing the neurons—via an antagonist (Hui et al. 1996; King et al. 2005; Chu et al. 2009; Liang et al. 2011; Sahbaie et al. 2012), a knockdown (Naono-Nakayama et al. 2011), or via a knockout (De Felipe et al. 1998; Santarelli et al. 2001)—produces similar reductions in pain. Likewise, a knockout (Cao et al. 1998; Zimmer et al. 1998) of the preprotachykinin A (PPT-A) gene, from which substance P, neurokinin A, neuropeptide K, and neuropeptide γ are produced by alternate splicing and proteolytic cleavage, appears to produce similar reductions in pain in this test. *As far as the formalin test goes*, these interruptions of NK1R, SP (via the PPT-A knockout), and treatment with SP-SAP have apparently identical effects.

On the other hand, selectively blocking NK1R signaling can sometimes produce results greatly at variance with the results of ablating NK1R-expressing neurons with SP-SAP. For example, intrathecal SP-SAP injections reduce allodynia induced by nerve ligation (Nichols et al. 1999) or by CFA injections (Suzuki et al. 2002; Nichols et al. 1999), but PPT-A knockouts, from which SP is produced, show wild-type allodynia to both nerve ligation and CFA injections (Cao et al. 1998). In this pain test, ablation of the NK1R+ neuron reduces pain behavior, but selectively blocking NK1R does not.

To supply an analogy, the difference between NK1R knockouts and SP-SAP ablation is the difference between the removal of all instances of the text "NK1R" in all manuscripts (knockouts) versus the removal of all manuscripts that contain the text "NK1R" (SP-SAP ablation). Although the data on antagonists, knockouts, and knockdowns are informative, they are clearly incapable of predicting the effects of SP-SAP ablation on all types of pain.

## 6.4 Possible Mechanisms of SP-SAP Treatment

The mechanisms by which SP-SAP ablation produces analgesia are not precisely known. First, ablation may simply reduce the number of projecting pain fibers in the spine. Although fewer than half of the lamina I neurons are NK1R+ (Brown et al. 1995; Todd et al. 1998; Nichols et al. 1999; Polgar et al. 2013), approximately 80% of the lamina I neurons that project to the thalamus (Marshall et al. 1996) or brainstem (Todd et al. 2000) are NK1R+, and intrathecal SP-SAP kills the majority of NK1R+ lamina I neurons (Mantyh et al. 1997; Nichols et al. 1999; Wiese et al. 2013; Allen et al. 2006). Interestingly, spinal pain reflexes, as measured by the rat tail-flick test, appear unaffected by SP-SAP treatment (Vierck et al. 2003), which is understandable if NK1R+ projection neurons to the brain stem are ablated and local NK1R− interneurons are preserved. The reduction in the number of projection neurons via SP-SAP treatment would not unexpectedly reduce pain sensitivity and

intensity. It is less clear that this can explain the differential modulation of nocifensive responses to moderate-intensity versus high-intensity thermal stimuli (Vierck et al. 2003; Wiley et al. 2007) or the differential neuronal signaling of wide dynamic range neurons to low amounts versus high amounts of capsaicin (Khasabov et al. 2002).

Second, ablation by SP-SAP could reduce central sensitization mediated by local spinal neuronal interactions. After capsaicin sensitization, wide dynamic range (WDR) neurons in deep dorsal horn laminae fire more frequently in response to mechanical stimuli and have a lower thermal threshold for firing. Central sensitization and wind-up of WDR neurons are eliminated after SP-SAP treatment (Suzuki et al. 2002; Khasabov et al. 2002; Choi et al. 2012), an effect also seen in NK1R knockouts (Rygh et al. 2006). Finally, long-term potentiation (LTP) in WDR neurons is eliminated by SP-SAP treatment (Rygh et al. 2006). Although supraspinal circuits may participate in wind-up and LTP, intrinsic spinal connections must mediate part of these effects since they are both present in spinal cord slices that lack descending controls (Herrero et al. 2000; Ikeda et al. 2003).

Although SP is involved in peripheral sensitization and although NK1Rs are present on the presynaptic afferents in the dorsal horn (Hu et al. 1997), peripheral sensitization does not appear to be affected by intrathecal SP-SAP treatment, since such treatment does not visibly deplete SP-staining afferent fibers in the dorsal horn (Mantyh et al. 1997).

Third, intrathecal SP-SAP treatment could disrupt descending pain facilitation pathways that involve the 5-HT3 receptor. In untreated rats, blocking the 5-HT3 receptor with ondansetron prevents mechanical allodynia, thermal hyperalgesia, and late-phase formalin responses similar to SP-SAP treatment (Suzuki et al. 2002). In SP-SAP treated rats, ondansetron lacks these effects, suggesting that the descending pain facilitation pathway blocked by ondansetron involves NK1R+ neurons in lamina I (Suzuki et al. 2002). It appears that ablation of NK1R+ neurons with SP-SAP also eliminates a noradrenergic descending pain inhibitory circuit, since treatment eliminates the typical increases in WDR activity seen after the α2-adrenoreceptor is selectively blocked (Rahman et al. 2008).

It is likely that there are several mechanisms contributing to the analgesic effects of treatment, which could include a reduction in number of afferent pain fibers, reduction in central sensitization, reduction in LTP, elimination of wind-up of WDR neurons, and disruption of descending pro-nociceptive pathways.

However, there are potential benefits of SP-SAP treatment beyond the direct reduction in pain, hyperalgesia, and allodynia. Opioid tolerance (the requirement for increasing amounts of opioid to get the same biological response) and opioid-induced hyperalgesia (a paradoxical increase in pain sensitivity with opioid use) are both impediments to long-term clinical pain therapy (DuPen et al. 2007). NK1R knockout mice (King et al. 2005), PPT-A knockout mice (which lack SP, one of the products of PPT-A) (Sahbaie et al. 2012), rats treated with an NK1R antagonist (Hui et al. 1996; King et al. 2005; Chu et al. 2009; Liang et al. 2011; Sahbaie et al. 2012) and rats administered intrathecal SP-SAP (King et al. 2005; Vera-Portocarrero et al. 2007; Rivat et al. 2009) all show reduced opioid-induced

hyperalgesia, which is mediated at least in part by a 5-HT3-dependent mechanism (Hui et al. 1996; King et al. 2005; Chu et al. 2009; Liang et al. 2011; Sahbaie et al. 2012). It is reasonable to speculate that the 5-HT3/NK1R+-dependent mechanism of opioid-induced hyperalgesia is related to the 5-HT3/NK1R+ mechanism that has been implicated for reductions in late pain in the formalin test, mechanical allodynia, and thermal hyperalgesia. These results offer the promise that intrathecal SP-SAP treatment will benefit patients by increasing opioid effectiveness and reducing opioid dose and subsequent side effects. This would also be a highly valuable effect regardless of any direct effects that SP-SAP has on pain sensation.

## 6.5 Conclusions

Although pain is widespread, new pain therapies are needed that are more effective and have fewer side effects. A single intrathecal SP-SAP injection can be used to selectively ablate NK1R+ projection neurons in lamina I of the dorsal horn that carry the majority of the afferent pain signals to supraspinal centers. This "molecular neurosurgery" reduces the number of afferent pain fibers, reduces wind-up of WDR neurons, reduces LTP, reduces central sensitization, disrupts a spinal-bulbar-spinal pro-nociceptive circuit, and reduces opioid tolerance and opioid-induced hyperalgesia. In animal models, SP-SAP treatment has been shown to reduce acute pain, reduce hyperalgesia, and reduce allodynia.

Lumbar SP-SAP injections in canines produced no detectable side effects using either 15 or 45 μg doses, although some motor dysfunction and CNS infiltrates were observed with 150 μg doses (Allen et al. 2006; Wiese et al. 2013). Similarly, 60 μg doses in canines above 30 kg weight appeared safe—no motor or behavioral dysfunction was observed (Brown and Agnello 2013); however, 60 μg doses produced detectable dysfunction in a subset of canines when injected into the cisterna magna near the brain stem but not when injected into the lumbar region.

It seems unlikely that similar dysfunction would occur in human trials with intrathecal injections at the L5–S1 interspace, because of the length of the spinal cord in humans and the minimal cephalocaudal spread of SP-SAP, measured to be 10 cm from the point of injection in the canine studies (Allen et al. 2006). If cephalic spread were to be a significant problem, it most likely could be controlled through careful selection of the baricity and solvent of the SP-SAP injection. Although NK1R+ neurons exist in the ventral motor areas, these neurons do not appear to be affected by SP-SAP injections in animal models. The safety of more caudally located intrathecal injections in canines supports this assumption.

In a double-blinded test of intrathecal SP-SAP on bone cancer pain in canines (Brown and Agnello 2013), SP-SAP was shown to give statistically significant reductions in pain severity as measured by the time to request additional pain therapy (or canine death) and as measured by the total number of such requests.

A phase I clinical trial of SP-SAP in human subjects to assess safety is ongoing (ClinicalTrials.gov identifier NCT02036281) and due for completion by the first half of 2016. This is the first phase I trial of a targeted toxin for pain.

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# Chapter 7
# Recombinant Immunotoxins for Chronic Inflammatory Disease

## Targeting M1 Macrophages Through CD64

**Theo Thepen and Stefan Barth**

**Abstract** Over the last couple of decades, a demographic transition towards an aging population has occurred. This has been accompanied by an increase in chronic inflammatory disorders like atopic dermatitis, rheumatoid arthritis, and diabetic ulcers, as many of these are strongly age related. There is thus an increasing demand to develop better therapeutic strategies to manage and cure chronic diseases. In many of these diseases, the trigger for onset of the inflammation is diverse, but their chronic, non-resolving phases share many similarities like, e.g. composition of the cellular infiltrate and cytokine milieu. One of the main constituents of the cellular infiltrate are macrophages (MØs), and these have been recognised as key players in chronicity. In particular, the pro-inflammatory M1 subpopulations has been ascribed a role in persistence, whereas the M2 population is involved in tissue remodeling and resolution. The high expression of the high affinity receptor for IgG is specific for M1. In this chapter, we describe the preclinical development of immunotoxins, targeting CD64 on M1, for the treatment of the chronic phase of chronic inflammatory diseases. Although ADCs using a protein toxin as effector molecule, also known as immunotoxins, were mainly developed for life-threatening disease like cancer, the ongoing improvement on these therapeutics has broadened their potential clinical application. Preclinical data on immunotoxins targeting CD64 show that targeting M1 cells through CD64 in chronic inflammation has a beneficial effect on course and development

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of the disease and leads to resolution of the inflammation, both in vivo in preclinical animal models and ex vivo on patient-derived cells and biopsies. This efficacy, combined with the improved safety profile of the latest generation immunotoxins, qualifies these CD64-targeted agents for M1-specific therapeutic intervention in chronic inflammatory disease.

**Keywords** CD64 • Macrophages • Immunotoxin • Chronic inflammatory diseases

## 7.1 Introduction

Antibody Drug Conjugates (ADCs) and Immunotoxins (ITs) are next generation antibody therapeutics, whereby functionality is added to an adapted, antibody-based framework. In immunotoxins, the small synthetic toxins attached to the antibody in ADCs are replaced by apoptosis-inducing plant or bacterial proteins. The concept was already entertained by Paul Ehrlich (Himmelweit 1960), but it took till the development of the monoclonal antibody technology (Köhler and Milstein 1975) before the concept could be exploited and first ITs could be developed at the beginning of the 1980s (Vitetta et al. 1983). Especially at the beginning, ITs had several disadvantages, the most crucial being lateral toxicity that limited their use to life-threatening disease like cancer. However, ITs have come a long way from the first crude, chemically linked, plant- or bacterial toxin-based molecules to recombinant versions in which an antibody binding fragment is fused to a protein toxin by genetic engineering resulting in a single chain fusion protein expressed mainly in bacteria. Since the approval of trastuzumab-DM1, a Her2 antibody delivering a small molecule toxic (microtubule interacting Maytansine) payload to metastatic breast cancer cells, by the US Food and Drug Administration, step wise the side effects have strongly been diminished (Weiner 2015), broadening the application of ADCs into other diseases like chronic inflammation.

Indeed, next to different types of cancer, monoclonal therapeutic antibodies, e.g. Canakinumab (Ilaris®), Adalimumab (Humira®), Infliximab (Remicade©), Secukinumab (Cosentyx©), and Tocilizumab (Actemra®), are effectively employed in a range of chronic conditions (Reichert 2015). These antibodies neutralise the effect of cytokines like IL-1β, IL-6, and TNF-α that play a key role in inflammation and chronic disease. Interestingly, these pro-inflammatory cytokines are mainly produced by MØ, and therefore it can be assumed that these cells could also provide a good target to develop next generation ADCs and ITs, aiming at resolving chronic inflammatory conditions.

## 7.2 Macrophages as Therapeutic Target

### *7.2.1 Nomenclature*

Initially, MØs were considered cells specialised in just one function: removing ("eating") large quantities of foreign material that somehow entered the body. This is why Metchnikov coined them 'macrophages' (from Greek; makros 'large' + phagein 'eat', consequently 'big eaters') already in the nineteenth century (Metchnikoff 1892). They were hereby the first cells described of the immune system. It was only much later that they were found to be part of the mononuclear phagocyte system that originates from progenitor cells in the bone marrow. After differentiation and transport by the blood, controlled extravasation, and migration, they become resident tissue MØs. This is a naturally and continuously occurring phenomenon whereby the cells differentiate into, among others, alveolar MØs in lungs, microglial cells in the central nervous system, osteoclasts in bone marrow, Kupffer cells in the liver, and various forms of spleen MØs. This was actually the first subdivision into MØ subsets, as well as the nomenclature of the subsets (Gordon and Taylor 2005). The presence of different MØs in various tissues led to the realisation by researchers that the function of these cells was not just limited to clearing tissue from foreign material. They were involved in a much wider range of processes like tissue remodeling and were exerting many (immunological) functions. Subsequently, further characterisation led to the discovery of a range of MØ subpopulations based upon their different phenotypic and functional properties.

### *7.2.2 M1 and M2 Polarisation*

A major paradigm shift was the proposal to subdivide MØs based upon their characteristics into M1 and M2 polarised phenotypes (Mills et al. 2000). At that time, this was linked to the Th1/Th2 concept for T helper cells. M1 or classical activated MØs are induced by stimulation with, e.g. IFN-g and LPS. These M1 are pro-inflammatory and play a key role in host defense against infection. M2 on the other hand are induced by, e.g. Il-4 and Il-13 (Mantovani et al. 2004), play a more anti-inflammatory role, and are involved in tissue remodeling. These stimuli mimic the Th1-type and Th2-type response, respectively (Mills et al. 2000). This simplified concept was especially useful to understand the role that MØs play in regulation of inflammation and their relationship with other immune cells. The original authors already indicated that this might be an over-simplification and that intermediated types could exist. Indeed, analyses of transcriptomes after stimulation of human MØs showed a much more complicated mathematical model distinguishing an intricate network of different MØ populations (Xue et al. 2014). As these different populations have differential effects on the immune response, it would be beneficial to be able to target specific polarised populations for specific

therapeutic interventions (Liu et al. 2014). For cancer therapy, it could be beneficial to target distinct M2 populations as distinct subpopulations of M2 are attributed a role in tumour growth. In chronic disease, the M1 play a decisive role, as during the chronic phase of several chronic conditions, the M1 remain the predominant phenotype, which prevents resolution of the inflammation (Hristodorov et al. 2012).

## 7.3 The FcγR (CD64) for Targeting Activated MØs

Prior to the M1 versus M2 model, the distinction between activated inflammatory MØs versus resting MØs was used, which was mainly based upon activation markers (Italiani 2014). Upon entrance at the site of inflammation, MØs respond to local stimuli and become activated. This leads to up-regulation or induction of activation markers (Mosser and Edwards 2008). One distinctive feature of activated MØs is a strong expression of FcγR1, the high affinity Fc receptor (FcR) for Immunoglobulin G (IgG), also referred to as CD64. The FcγRs all belong to the Ig super family, and the IgG binding class is called FcγR. They comprise three members, from low to high affinity: FcγRIII (CD16), FcγRII (CD32), and FcγRI (CD64). FcγRI has several properties which sets it apart from the other FcγRs, like the high affinity, the ability to bind monomeric IgG, and the constitutive expression only on MØs, monocytes, and their progenitors (Van De Winkel and Capel 1993; van der Poel et al. 2011). In addition, binding of monomeric IgG is sufficient to induce internalisation and recycling of the receptor, while phagocytosis is induced upon cross-linking. Due to these characteristics, FcγRI provides a good target for immune toxin treatment aimed at MØs.

## 7.4 Activated MØs, Chronic Inflammation, and CD64

The first direct link between chronic inflammation, activated MØs, CD64, and immune regulation was reported in studies in which we performed Atopy Patch Tests on patients with Atopic Dermatitis (Thepen et al. 1996). In such tests, the allergen is applied onto the skin of patients with Atopic Dermatitis that show an allergic response to this allergen. This induces a cutaneous inflammatory response that bears many similarities, both macroscopically and microscopically to skin lesions of Atopic Dermatitis patients (Langeveld-Wildschut et al. 1996). In these studies, the ratio between Il-4 and IFNγ on the protein level was determined by immunocytochemistry. The initial allergic, predominantly Th2 response, in the acute phase, converted into a persistent Th1 response, leading to the chronic phase. This phenomenon was later corroborated by others at who analysed cellular activation at mRNA level (Werfel et al. 1996). This led to the hypothesis that the initial response to the allergen was an allergic response, induced by resident allergen specific Th2 cells in skin (de Vries et al. 1997). Due to aberrant local

regulation, this response does not resolve, but rather transforms into a chronic cutaneous inflammatory phase that is not necessarily allergen driven anymore (Thepen et al. 1996; Grewe et al. 1998). Interestingly, this Th2/Th1 switch occurs after 24 h and coincides with an influx of activated MØs that remain in skin during the chronic phase. One marker specific for this population of MØs was CD64, which is a strong marker for M1 (Kiekens et al. 2000).

## 7.5 CD64 Targeting Using Antibodies

As described above, CD64 is the high affinity receptor for IgG, with the subclass preference: IgG1 = IgG3 >IgG4, >>IgG2 and with a dissociation constant ($K_d$) between $10^{-8}$ and $10^{-9}$ M. This basically implies that in vivo, the receptor is always occupied by IgG. In order to be effective, a therapeutic agent that acts through binding of CD64, therefore has to bind an epitope outside the ligand (Fc) binding site (Fridman 1991). Although there is overlap in nomenclature and function, there are differences between FcRs in humans and mice (van der Poel et al. 2011).

Several mAbs have been generated against human CD64 recognising different epitopes. The murine mAb m22 binds with high affinity and the binding is not inhibited by human IgG1, demonstrating that this mAb binds outside of the IgG binding site of CD64 (Guyre et al. 1989). Due to their immunogenicity, murine antibodies are not suitable for repeated application in patients. Therefore, the murine 22 mAb was humanised by CDR-grafting onto human V region frameworks based on human immunoglobulins KOL $V_h$ and REI $V_\kappa$ attached to human IgG1 and κ constant domains. The resulting humanised H22 mAb displayed equivalent binding specificity, affinity, and functionality to the parental murine m22 mAb (Graziano et al. 1995). The human IgG1 Fc tail equips it with added functionality; it can now for instance, through Fc binding, cross-link CD64. However, via the Fc binding capability, the H22 mAb can now also bind the medium (CD32) and low (CD16) affinity FcγR's. This implicates a loss of specificity, especially as the ligand binding sites of both CD16 as well as CD32 are not permanently occupied, as is the case in CD64. In addition, both CD16 and CD32 bind IgG1 with higher affinity; then the other IgG subclasses and humanised H22 mAb can therefore displace already bound antibodies of the other isotypes, thereby increasing the non-specific binding of H22 even more (Ravetch and Kinet 1991).

Therapeutic antibodies exert their curative effect through different mechanisms. This includes, e.g. simply binding a target in an antagonistic fashion and thereby interfering with the function of the bound target, which may either be a receptor of a soluble mediator. In addition, functionality can be mediated by the Fc tail; e.g. via complement dependent cytotoxicity and antibody-dependent cellular cytotoxicity (Goswami et al. 2013; Chan and Carter 2010). Therapeutic antibodies represent constantly growing, successful class of pharmaceuticals, and until now around 50 therapeutic monoclonal antibodies are approved in the EU or the USA with more than 400 therapeutic monoclonal antibodies still at various

stages of clinical trials (Reichert 2015). MAbs have many advantages and disadvantages and since the invention of the monoclonal antibody technique several generations have been developed with improvements on immunogenicity, stability and efficacy (Goswami et al. 2013). Instead of just relying on the intrinsic functional properties of MAbs, researchers have engineered antibodies, by, e.g. glycoengineering, Fc amino acid mutations, and size reduction, generating bi- and multispecific formats, in order to optimise their functional properties. Another option for adding specific functionality is to couple mAbs to cellular toxins. One of the earliest strategies in this direction was the development of ITs in which a protein toxin, mostly of plant (e.g. ribosome-inactivating Ricin A) or bacterial origin (e.g. NAD-ribosylating Diphtheria toxin), was chemically linked to a full size, often murine, antibody. This line has evolved into an area of MAb research and development, designing next generation antibody therapeutics based upon a modified antibody framework. The basis of this concept is specific and targeted delivery of a therapeutically active payload to a tissue or cell type, by engineering molecules with minimal immunogenicity and lateral toxicity, yet optimal efficacy. These ADCs are considered the most auspicious group of MAb-based therapeutics.

Genetic engineering is one method by which protein toxins can be appended to a binding ligand, in most cases an antibody fragment or an alternative ligand like a receptor binding protein. In the case of mAbs, different formats can be generated to meet specific requirements. One of the smaller fragments of mAbs, still having the original binding specificity, is the single chain variable fragment (scFv) with a molecular weight of ~25–30 kDa. This can be used as a targeting unit for specific delivery of an effector molecule as part of an IT.

## 7.6 Human CD64 Expressing Transgenic Animal Models

To test the hypothesis that infiltrating activated MØs are involved in the perpetuation of chronic inflammation, a transgenic mouse was established that expressed human CD64 (Heijnen and van de Winkel 1995; Heijnen et al. 1996). Despite similar nomenclature, the function of the different Fc receptors can vary between species. Therefore, we decided to establish a human CD64 transgenic model in which the hCD64 gene (hFcɣR1A gene) is expressed under control of the native promotor and key regulatory motifs, including the IFN-ɣ response region (Heijnen et al. 1996; van der Poel et al. 2011). In addition, this model has the major advantage that it allows for testing of the final, human CD64-specific therapeutics. It avoids the need to switch from a mouse CD64-specific targeting moiety to a human CD64 specific one for the final therapeutics. It also avoids translation of the data obtained by targeting the murine CD64 to the human CD64. In addition, later a huCD64 transgenic rat was generated using the

same construct as was used in the mouse (van Vuuren et al. 2006). In both animal models, the functionality as well as the regulation of expression and activity was tested and found to be identical to that in humans (Heijnen and van de Winkel 1995).

## 7.7 The First CD64 Targeting IT: H22-Ricin-A

### *7.7.1 In Vitro Efficacy*

To the full size H22 MAb, two Ricin A (RA) toxin molecules were chemically linked and tested for cytotoxicity towards the pro-monocytic cell line U937. This H22-RA IT showed a low toxicity towards the U937 cell line. After stimulation with IFNγ, however, they became very sensitive and apoptosis could readily be induced. IFNγ induces up-regulation of CD64, but this increase in receptors alone could not explain the difference in sensitivity. Also the non-stimulated cells have an expression level that should allow for the internalisation of sufficient H22-RA IT molecules to induce apoptosis. This indicates that the activation had additional effects that increased their susceptibility towards the H22-RA IT (Thepen et al. 2000). It should be noted that at that time, the concept of M1 and M2 MØs still had to be conceived and only a distinction between resting/resident MØs and infiltrated/activated MØs was made. These initial in vitro experiments strongly supported the notion that CD64 could be used as an ADC/IT target for activated, inflammatory MØs.

### *7.7.2 In Vivo Efficacy*

#### 7.7.2.1 Chronic Cutaneous Inflammation

Having established that CD64-activated MØs play a decisive role in establishing chronicity, and non-resolution of inflammatory responses, and the fact that H22-RA IT specifically kills activated MØs in vitro, targeting of hCD64 with the H22 MAb was evaluated in the hCD64 transgenic mouse model.

Through epicutaneous application of a 5% solution of Sodium Lauryl Sulfate (SLS), chronic cutaneous inflammation was induced in the hCD64 transgenic mice. SLS was used, as this irritant also causes an inflammatory response in humans. Especially, people suffering from chronic skin conditions are far more sensitive to contact with SLS than normal individuals. The skin of people treated with SLS shows a dermal infiltrate with a strong presence of CD64 expressing MØs. This infiltrate showed a strong resemblance to that from people suffering from other chronic skin conditions, apart from the epidermal thickening, which was considerably less. The chronic conditions analysed in comparison included etiologically

completely different conditions, like Atopic Dermatitis (AD), late time-point APT skin, Polymorphic Light Eruption, and Psoriasis. In all, strongly CD64 expressing MØs were abundantly present in large infiltrates and dispersed through dermis, not in epidermis (Thepen et al. 2000).

Immunohistochemical analyses of the skin of mice treated with SLS also showed a prominent presence of hCD64 expressing MØs, as well as other immune cells like T-cells and Dendritic cells (DC), similar to the situation in skin from patients with a chronic inflammatory skin condition. At that point, all the tools to test whether elimination of activated inflammatory MØs could have a beneficial effect were available. A single intradermal injection of the H22-RA IT (10 μl, $2 \times 10^{-8}$ M, referring to the Ricin-A moiety, in saline) in skin from SLS induced hCD64 mice resulted in clearance of hCD64 expressing MØs from the inflamed skin within 24 h. This effect lasted till 96 h under continuous SLS application, after which the cells returned, reaching normal numbers after 120 h. Also other indicators for inflammation like increased skin temperature and vascular leakage were restored to normal after this period. With this in vivo experiment, it could clearly be demonstrated that elimination of activated/inflammatory MØs during chronic inflammation could be a valid treatment (Thepen et al. 2000).

#### 7.7.2.2 Rheumatoid Arthritis

In Vitro

This principle observed in cutaneous inflammation could also apply to other chronic inflammatory conditions in which MØs play an already well established role, in the inflammation, with one of the most promising of such diseases being Rheumatoid Arthritis. In this disease, inflammatory MØs present in the joints are instrumental in initiation and maintenance of the inflammation and mediate direct damage to these joints. Targeted elimination of inflammatory MØs could therefore provide a novel approach for therapeutic intervention. When studying synovial fluid MØs isolated from patients, we indeed observed strong expression of CD64 and also synovial lining MØs were strongly positive for CD64. The synovial fluid MØs were also highly sensitive to the H22-RA IT, which induced apoptosis in 84% of these cells. Peripheral blood monocytes from the same patients, although expressing CD64 stronger than healthy individuals, were only marginally affected the H22-RA IT (20% apoptosis). In healthy individuals, no or very low expression of CD64 was observed. Ex vivo treatment of synovial explants with H22-RA IT showed inhibited TNF-α and IL-1β production and decreased cartilage degrading activity (van Roon et al. 2003).

In Vivo

Rat models for arthritis are considered superior to murine models with respect to predictive value for therapeutic efficacy in rheumatoid arthritis in humans. Therefore, we have also created hCD64 transgenic rats, using the same hCD64 gene (hFcγR1A gene) as described earlier. Here the Adjuvants arthritis (AA) model was used, and a strongly CD64 expressing infiltrate in and around the joints was observed. AA is an aggressive model, which induces damage to the cartilage and bone in the animal joints. When untreated, all animals reached maximum tolerable scores and had to be killed. Animals that were treated with H22-RA IT upon the appearance of first symptoms, so after disease had already started, only showed a mild increase in score. None of these animals reached maximum scores and all animals survived. Immunohistochemical analyses showed a strong reduction in CD64 expressing MØs both in the joints as well as in surrounding tissue. Untreated animals showed massive infiltration in synovial fluid, synovium, surrounding tissue, and metacarpal bones, which resulted in strong bone erosion. These results correlated with the high disease scores the animals reached. In contrast, the treated animals showed far less infiltration in all areas and practically no damage to the metacarpal bones, again correlating with the low score these animals reached. This shows that elimination of CD64 expressing activated MØs inhibits the progress of inflammation and limits damage to joints. Combined with the data obtained from patient material, this provides strong evidence that targeting CD64 expressing activated MØs can be a valid therapeutic approach for RA. Especially as the effect on CD64 cells outside the highly inflamed joints, e.g. in peripheral blood, is limited (van Roon et al. 2003).

#### 7.7.2.3 Acute Myeloid Leukaemia

In Vitro

The initial in vitro testing of the H22-RA IT was done on the pro-monocytic cell lines U937 and HL60, both of which are actually Acute Myeloid Leukaemia (AML) derived cell lines. AML is one of the most common acute haematological malignancies, characterised by an anomalous proliferation of myeloid precursor cells. In particular, AML of the M4 and M5 sub-classification shows overexpression of CD64.

In Vivo

Zhong et al. could demonstrate that the H22-RA ADC was able to inhibit the proliferation and ability to form colonies in a dose-dependent fashion, both in cell lines as well as in cells from AML patients in vitro (Zhong et al. 2001). In a human AML model in NOD/SCID mice, they observed tumor inhibition in vivo.

Interestingly, this inhibition was only observed when H22-RA IT was combined with IFN-ɣ. This would indicate that also in the case of AML cells, which are in essence partially differentiated, early stages of MØs, the activation state of the cells is essential for sensitivity towards H22-RA IT (Zhong et al. 2001). AML is an acute form of Leukaemia and the course of the disease is highly progressive and the 5-year survival rate is on average around 25%, depending on the subtype. In diseases with such a low survival rate, relatively severe side effects of the treatment are acceptable. However, the authors recognised that the full potential of this kind of therapy could only be exploited when the H22-RA IT molecule could be altered to improve applicability.

## 7.8 From H22-RA to H22(scFv)-ETA′

There are several limitations to the use of the H22-RA IT that was used in the aforementioned experiments. Firstly, the molecule consists of two Ricin-A molecules and the full size H22 antibody. This brings the MW up to 210 kDa, which strongly impedes extravasation from blood and tissue penetration. This prolongs half-life in blood which may lead to a potential breakdown, especially as the Ricin-A molecules are linked to the Fc tail of the mAb, which prevents binding to the FcRn, which normally protects antibodies from breakdown in circulation. This would thereby negatively influence the pharmacokinetics (Challa et al. 2014). Next to this, the H22-RA IT is constructed by chemical conjugation of the Ricin-A domains to the antibody, and therefore, the site, where the Ricin-A is coupled, as well as the number of Ricin-A molecules conjugated to the H22 mAb is variable. Therefore, standardisation of the final formulation requires extensive purification and testing.

As described previously, protein toxins can also be fused to fragments of antibodies through genetic engineering thereby generating recombinant immunotoxins (rITs). In addition, further functional sequences for, e.g. intracellular routing and tissue localisation can be introduced.

Therefore, a scFv was constructed in which the VH and VL regions were derived from H22 in order to construct CD64-specific recombinant immunotoxins that can be used to target activated MØs. Previously, we constructed several scFv based ITs using a modified Pseudomonas exotoxin A variant (ETA′) as a toxin moiety (Klimka et al. 1999). For bacterial production, periplasmic expression under osmotic stress conditions in the presence of compatible solutes was established (Barth et al. 2000). We, therefore, opted to genetically link the H22(scFv) to ETA′ (Tur et al. 2003). The resulting recombinant immunotoxin has a molecular weight of 70 kDa, cf. 210 kDa for the H22-RA IT, and this IT was fully functional regarding binding specificity and toxicity. Like H22-RA IT, it killed IFN-ɣ activated HL60 and U937 cells, but not their non-activated counterparts. In addition, it efficiently killed primary cells from AML patients. Next to this H22(scFv)$_2$-ETA′, a variant comprising two H22(scFv) linked to one ETA′ was constructed (Ribbert et al. 2010). Having two binding moieties, this bivalent construct can cross-link CD64 on the cell membrane, which

improves internalisation. Indeed, when compared in parallel, the H22(scFv)$_2$-ETA′ showed higher toxicity than the H22(scFv)-ETA′, especially at low concentrations. The stability of the bivalent construct was however lower. Both rITs showed an in vivo therapeutic effect after local application in the SLS induced skin model in hCD64 transgenic mice (Ribbert et al. 2010). This effect was comparable to that of the original H22-RA IT and confirms that the therapeutic effect is mediated by the CD64 targeting and is irrespective of the toxin moiety.

### *7.8.1 Targeting of CD64 Expressing MØs in Renal Inflammation*

Ischaemia/reperfusion injury (IRI) is one of the leading causes of acute renal failure (ARF) both in native and transplanted kidneys (Perco et al. 2007). It is known that MØs play an important role in the pathogenesis of IRI (Sean Eardley and Cockwell 2005; Boros and Bromberg 2006). In a review by Wang and Harris, it was suggested that shifting the balance from M1 to M2 predominance, either by inducing M2 or eliminating M1, would be beneficial in resolving inflammation and repair in renal disease (Wang and Harris 2011). The validity of this approach was indeed confirmed using the H22(scFv)-ETA′ in an ischemia model in hCD64 transgenic rats. A single intravenous administration of the H22(scFv)-ETA′, 6 h after injury by 30 min of ischaemia in uninephrectomised rats, strongly reduced the number of MØs in renal tissue. This was accompanied by preserved renal function and morphology (Fet et al. 2012). Ischaemia/reperfusion appears during every transplantation procedure, as the transplanted organ is disconnected from circulation for a certain time. The principle here demonstrated with renal IRI may therefore also hold valid for other types of transplantation. In addition, ischaemia and accompanying damage can also be caused by vascular occlusion by, e.g. thrombi and also here balancing the M1/M2 ratio by CD64-targeted ITs can result in reduced tissue damage.

## 7.9 M1 Specificity for CD64-Targeted ITs

At this point, the ITs targeting CD64 showed a therapeutic effect in a range of chronic inflammatory diseases. As mentioned before, these are all triggered by different stimuli: SLS in the skin model, adjuvants/autoimmune antibodies in the RA model, and ischaemia in the renal inflammation. In addition, the ex vivo application to primary cells or biopsies from patients suffering from different types of chronic inflammation showed a beneficial effect in all cases, as determined by a variety of parameters. This basically confirmed the hypothesis that spurred the development of CD64 targeting ITs in the first place. Distinct subpopulations of activated MØs play a similar and essential role in the chronic phase of many

chronic diseases. This raises the question as to the exact nature of the MØ subpopulation that is affected and what the effect is on the local immune response and more notably also on the resolution of the immune response. To explore this, we tried to pinpoint the population of MØs that was killed and what the conditions were for the H22(scFv)-based rIT's to be effective.

All experiments described until now, either in vitro IFN-ɣ activated cells or MØs at sites of chronic inflammation, were affected. Key elements here are the 'activation state' of the cells and IFN-ɣ. As mentioned in the introduction on MØs, the subdivision into M1 and M2 provides a simple model to describe different subpopulations of MØs. In the polarisation into M1, IFN-ɣ activation plays an important role (Mills 2015).

We therefore started to study the effect of H22(scFv)-ETA′ on different M1 and M2 populations. We tested out different protocols and selected a set of both soluble and cell surface markers to identify M1 and M2. For M1 polarisation, stimulation with LPS and IFN-ɣ was chosen while M2 polarisation was induced by IL-4.

A first observation was that CD64 was indeed a strong surface marker for M1 together with CD14. For M2 polarisation, CD301 and CD206 were distinctive surface markers, both in mice and in humans (both on cells and in tissues). Also regarding cytokine profiles, a clear defining pattern could be established for M1 as well as M2 (Hristodorov et al. 2015).

When applying the H22(scFv)-ETA′ to either population in vitro, it became clear that only the inflammatory M1 were killed, whereas the anti-inflammatory M2 were not affected. Again, this effect was observed on both murine and human MØs. The H22(scFv) alone, without toxic payload, had no effect on either populations. In parallel to the H22(scFv)-ETA′, two other H22(scFv)-based next generation rITs were tested and yielded the same result. This shows that the M1 specificity is not determined by the nature of the toxin, as different toxin moieties all showed the same effect: specific killing of M1 polarised MØs. As we observed previously with the H22-RA IT, the mere expression of CD64 is not the only crucial factor for sensitivity towards CD64-targeted ADC. The M2 also show a basal expression of CD64, as this receptor is constitutively expressed on all cells of the monocytic lineage. The toxins that were used in the H22-based rITs are highly effective, and internalisation of a small number of molecules should suffice to drive the cell into apoptosis. Differential regulation of binding affinity after cytokine stimulation has been described (van der Poel et al. 2010), but no studies regarding differences between M1 and M2 are available. There is however data available on expression, stabilisation, and routing of CD64 or CD64 complexes. From these publications, it is apparent that Filamin A plays an important role (Beekman et al. 2008; van der Poel et al. 2010). These data on CD64-IgG interaction can possibly not directly be translated to the effects of H22 binding, either as full size IgG1 or as scFv, as this MAb binds outside the ligand binding domain.

These data all pertain to effects related to CD64-ligand (IgG) binding. They may not necessarily be translated directly to effects following CD64-H22(scFv) or even CD64-H22 binding. This is because the binding site for H22 is outside the ligand binding domain.

We, therefore, focused on events happening after binding of H22 to CD64 on the cell and especially on the breakdown of the internalised CD64–H22-payload complex. Due to their disparate function in the immune response and inflammation, there is a difference in proteolytic capacity between M1 and M2. M1 MØs play a role in the initiation of immune responses and thereby in antigen presentation. They therefore do not completely breakdown phagocytosed proteins, but rather process them into a size and form, compatible with MHC mediated presentation (Yates et al. 2007). M2 on the other hand, play a role in tissue re-modelling and repair and therefore require stronger protease activity (Balce et al. 2011). We could indeed demonstrate a difference in protease content and a direct link between low protease content in M1 and sensibility towards H22(scFv)-ETA′. When endosomal protease activity was suppressed with Calpain inhibitor, the resistance of M2 was completely reversed into sensitivity, comparable to that of M1 (Hristodorov et al. 2015). This strongly indicates that the high endosomal protease activity of M2 protects them from the effect of a H22-based IT, by completely degrading the protein. The lower protease activity of the M1 subset allows for time to escape from the endosomes and translocate to the cytosol, which is essential for the toxin to exert its toxic effect (Fig. 7.1). Together, these experiments show that the CD64-targeted ITs specifically target strongly polarised M1 subpopulation.

Originally, MØs were considered end stage differentiated cells in tissue which performed a fixed specific function. Later it was realised that the MØs were not comprising fixed populations, but they could adapt their function depending on local requirements. The M1/M2 concept implies that MØs are highly susceptible to stimuli from the micro-environment and can quickly respond to them (Sica and Mantovani 2012). We examined the phenotypic and functional plasticity of MØs and their sensitivity towards H22(scFv)-ETA′ by polarising them into either direction and subsequently re-polarising them in the opposite direction. Firstly, this re-polarisation was indeed feasible, and more interestingly, the sensitivity towards H22(scFv)-ETA′ did change accordingly, which has important implications for the potential of CD64-targeted therapy. Both the H22-RA IT as well as the H22(scFv)-ETA′ IT showed a potent therapeutic effect in vivo in chronic cutaneous inflammation (Thepen et al. 2000; Ribbert et al. 2010). We repeated these experiments, but this time focused on the M1/M2 ratios prior to, during, and after treatment. The results clearly showed a predominance of M1 in skin after induction of inflammation. After treatment, the vast majority of M1 is eliminated, and this is accompanied by a slight increase in M2. As a consequence, this caused the M1/M2 ratio to drop dramatically and shows that treatment with H22(scFv)-ETA′ completely reverses the local environment from pro-inflammatory M1 into anti-inflammatory, M2. This change subsequently led to the resolution of the chronic inflammation. It should be noted that in this model, the inflammatory trigger, SLS, is continuously being applied.

There is a wide variety of chronic cutaneous conditions with different aetiology. When we analysed these for the presence of CD64 expressing MØs, all were associated with high numbers of CD64 expressing M1 cells (Kiekens et al. 2000). The clinical relevance of CD64-targeted therapy was already indicated by co-culturing human synovial explants with H22-RA IT (van Roon et al. 2003).

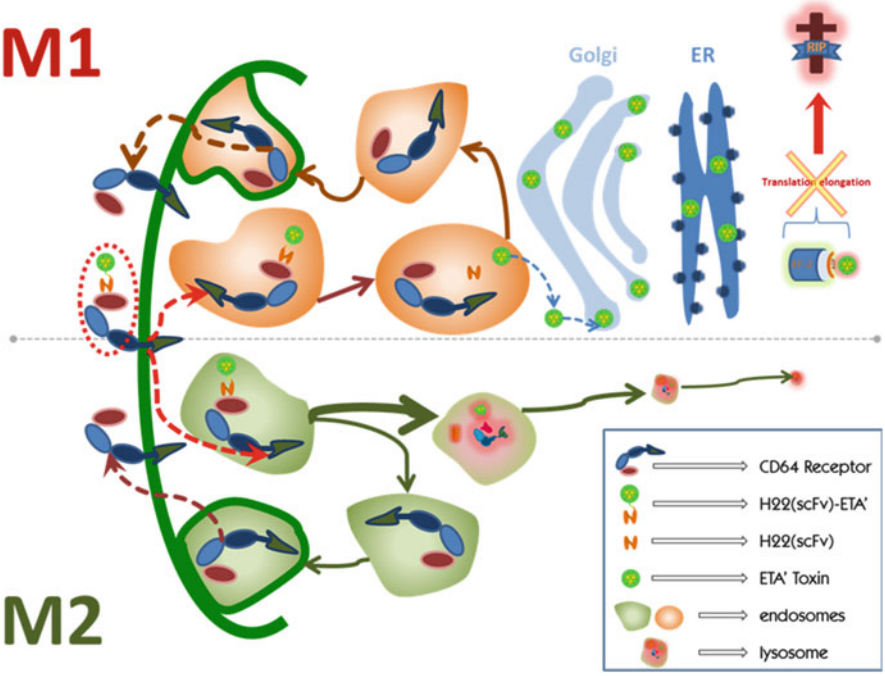


**Fig. 7.1** M1 specificity of CD64-targeted therapeutics. After binding of the H22-toxin IT to CD64, internalisation occurs and the complex is routed into endosomes. In M2, the content is digested in lysosomes, and a small proportion of the receptor is recycled towards the membrane. In M1 endosomes, partial digestion and processing takes place. Also here a proportion of the receptor is recycled, but part of the content, including the toxin, is routed towards Golgi and ER, where it exerts the toxic effect leading to apoptosis

These experiments, however, did not yet look at M1 specific effects directly, but rather at indirect parameters, showing beneficial effects. Co-culture of skin biopsies, obtained from lesional skin from patients with Atopic Dermatitis or from diabetes type II patients with chronic wounds with H22(scFv)-ETA′ readily showed a swift decline in numbers of M1 from these tissues, without negatively affecting the M2 population, which showed a slight tendency to increase. The latter can be of particular importance, as in this system, an isolated biopsy in culture, an influx of 'new' cells like monocytes, is not possible. It was further shown that polarised MØs show plasticity, which implies they can convert from M1 into M2 and vice versa (Hristodorov et al. 2015). Also in the supernatants from these cultures, we detected a significant change in cytokine profile. Pro-inflammatory IL-6 was decreased, whereas IL-10 levels were strongly increased. This again unambiguously points to a change in local milieu from pro-inflammatory to anti-inflammatory MØs, instigated by the targeted elimination of M1 by the CD64 targeting ITs. In combination with the in vitro and in vivo data, this validates the clinical potential of therapies, aimed at M1, through targeting CD64, to counteract chronic inflammation.

## 7.10 Hypothesis for the Mechanism and Applicability of M1 Targeting for Therapy

The idea to use MØs as therapeutic target in chronic inflammation has been pursued for several decades. Over these years, the advances in technology and understanding of the role of MØs have been significant. The first attempts to target MØs were based on their localisation and functionality, like phagocytic capacity. At present it is acknowledged that there is an increasing demand for targeting specific subpopulations of MØs. This is inspired by improved insight into the function MØs perform in regulation and outcome of immune responses and in particular the notion that specific subpopulations play a key role in aberrant responses that lead to pathology. Which population needs to be targeted greatly depends on the specific pathology of the disease. In chronic disease, there is a failure to resolve the inflammatory response, irrespective of the original trigger. To induce resolution of the chronic phase of inflammatory responses, a range of anti-inflammatory drugs has been developed. Especially, Glucocorticoids were the quintessential drug for the majority of chronic inflammatory diseases, supplemented by Non Steroid Anti-Inflammatory Drugs (NSAID). Steroids interfere with the transcription of pro-inflammatory genes (Coutinho and Chapman 2011). Interestingly, M1 are important producers of these pro-inflammatory cytokines during inflammation (Mantovani et al. 2004; Mills 2015).

The most successful therapeutic antibodies used in chronic inflammation are actually targeting/neutralising pro-inflammatory cytokines or their receptors. Adalimumab (Humira®) and Infliximab (Remicade®) block TNF-α, Canakinumab (Ilaris®) blocks IL-1β, Siltuximab (Sylvant®) blocks IL-6, and Atlizumab (Actemra) antagonistically blocks the binding of IL6 to the IL-6 receptor. Targeting M1 would simultaneously inhibit the complete range of pro-inflammatory cytokines and would thereby be more effective than targeting the individual cytokines separately. As the total amount of foreign therapeutic IT/antibody to be administered would be lower, also the side effects are expected to be much lower. The pro-inflammatory effects of M1 in chronic inflammation is very likely not limited to the production of mediators but also cell–cell contact can may be of importance. Next to this, M1 can produce a range of substances that can cause direct tissue damage (Mosser and Edwards 2008). Therefore, elimination of the subpopulation situated at the far end of the M1 spectrum could be more effective in breaking the local inflammatory cycle, than targeting the individual mediators.

The data obtained till now with CD64-targeted ITs indicate that it is feasible to reduce a chronic inflammatory reaction by these types of targeted therapies.

The original subdivision into M1 and M2 was linked to the Th1 Th2 paradigm, and it was suggested that M1 or M2 dominance would induce either a Th1 or Th2 response. Recently, several publications have pointed towards the interaction between M1 and Th1, or more specific Th17 cells, which are important in chronic inflammatory conditions. In a murine model for MS, there a direct link between impaired demyelination, Th17 cells infiltration in the central nervous system and

peripheral M1 was demonstrated (Baxi et al. 2015). In pulmonary inflammation, allergic asthma is associated with Th2 cells and here M2 are the predominant MØ subtype. Pulmonary inflammation caused by exposure to farm dust extracts, however, induces Th1/Th17-mediated lung inflammation and in this case M1 polarised MØs were more prevalent (Robbe et al. 2015). In Rheumatic disease, M1 and Th17 cells form an interactive circuit promoting inflammatory condition and damage (Li et al. 2013). In this paper, and also in a number of recent papers studying the role of M1 in chronicity of a number of chronic diseases, the findings suggest that specific targeting of the M1 would have great therapeutic potential.

From the data described above, it is evident that CD64 targeting eliminates the strongly M1 polarised population. This is evidenced by the dependency on IFN-ɣ stimulation of the target cells for the ITs to be effective in vitro. Only after stimulation with optimal amounts of IFN-ɣ, high killing rates could be achieved (Thepen et al. 2000; Zhong et al. 2001; Tur et al. 2003; Ribbert et al. 2010; Hristodorov et al. 2015).

As mentioned before, the subdivision into M1 and M2 is an oversimplification of the real-life situation at a site of inflammation. Polarisation of all MØs into M1 can only be accomplished under artificial conditions. Due to the plasticity of MØ subpopulations, the individual MØ will in vivo adapt continuously to their environment and will adopt intermediate phenotypes. Indeed it was found that both in vivo and ex vivo, the percentage of M1 that is eliminated is frequently lower than that of M1 polarised cells in vitro. This again points to the fact that the targeting of CD64 by ADCs or ITs mainly eliminates the extreme M1 polarised cells. The observed increase in M2 could point to an M2 polarisation of newly infiltrating ‘M0’ due to the absence of M1 cells. It is intriguing that in the ex vivo H22 ADC treated biopsies also a tendency towards increased M2 polarisation is observed. This combined with the plasticity of the MØ suggests that the remaining M1 cells which were not at the far extreme M1 end of the spectrum escape elimination. These cells then have the capability to shift into the M2 direction once these extreme M1 are eliminated (Hristodorov et al. 2015). This would fit with the spectrum model of MØ polarisation as posed by Xue et al. (2014,) and it would also explain why the targeting of CD64 by ADCs is so effective in abrogating the inflammatory response and in several models show resolution of chronic inflammation.

## 7.11 Conclusions

Together, the recent data increasingly point to a key role for M1 in (dys-)regulation of the immune response in chronic inflammatory diseases. This makes this population of immune cells prime targets for therapeutic intervention. As we put forward in this chapter, the targeting of CD64 on the M1 by ITs results in a significant and specific reduction of exactly this population. Most preclinical data so far has been obtained using the H22 (scFv)-ETA′ as this construct is fully characterised,

**Table 7.1** Levels of preclinical testing of H22(scFv)-ETA′

| Model | | Disease | | | | | |
|---|---|---|---|---|---|---|---|
| | | Atopic dermatitis | Arthritis | Chronic wounds | Multiple sclerosis | Leishmania | Myeloid leukaemia |
| In vitro | | X | X | X | X | X | X |
| hCD64 Transgenic animals | Mouse | X | X | X | X | X | |
| | Rat | | X | | X | | |
| Murine xenograft tumour model | | | | | | | X |
| Ex vivo human biopsies | | X | X | X | | X | X |

developed, and optimised, both in functionality as well as production (Table 7.1). We are presently, however, developing a range of CD64 targeting, H22(scFv) based, next generation rITs that avoid the disadvantages of the H22(scFv)-ETA′ construct. Exchanging the bacterial toxin ETA′ for different human apoptosis-inducing cytolytic proteins (hCFP), including Granzyme B and Angiogenin (Berges et al. 2014), already resulted in hCFPs with indeed comparable efficacy to the ITs comprising ETA′. These constructs are completely human and thereby lack the immunogenicity of the ETA′ constructs and can be administered multiple times over a prolonged period of time in humans. Combined with the demonstrated efficacy, this would qualify such CD64 targeting drugs candidates for clinical testing in a range of chronic inflammatory conditions.

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# Chapter 8
# BL22: A Milestone in Targeting CD22

**Robert J. Kreitman, David J.P. FitzGerald, and Ira Pastan**

**Abstract** BL22 is a recombinant immunotoxin, a chimeric protein composed of an Fv fragment of the monoclonal antibody (MAb) RFB4, and a truncated portion of *Pseudomonas* exotoxin. The antibody fragment that binds to the target cell is internalized along with the toxin, and the toxin fragment is transported to the cytosol where it catalytically kills the cell. Preclinical development showed significant cytotoxicity and antitumor activity toward CD22+ leukemia and lymphoma cells. Phases 1 and 2 testing showed high response rates in relapsed and refractory hairy cell leukemia (HCL), with complete remission rates of 47–61% depending on the design of the trial. We did not encounter chemotherapy-type toxicities, which is understandable due to its mechanism of action. However, we did observe a completely reversible hemolytic uremic syndrome (HUS) which did not require plasmapheresis for complete resolution. BL22 has since undergone further engineering to increase binding affinity to CD22. The improved molecule, moxetumomab pasudotox, is currently undergoing pivotal phase 3 testing in HCL. BL22 was thus a milestone in surface-targeted therapy producing a promising new treatment option for CD22+ chemotherapy-relapsed leukemia.

**Keywords** CD22 • Immunotoxin • Hairy cell leukemia

## 8.1 Introduction

### *8.1.1 Protein Toxins*

Organisms which produce protein toxins have evolved to produce toxins that kill cells catalytically and hence at very low concentrations. Toxins which have been shown to kill cells with great potency include the plant toxins, ricin, abrin, and modeccin (Endo et al. 1987; Eiklid et al. 1980), and the bacterial toxins, diphtheria

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toxin (DT) (Yamaizumi et al. 1978) and *Pseudomonas* exotoxin (PE). Plant toxins are referred to as ribosomal-inactivating agents since they prevent the association of elongation factor-1 and factor-2 (EF-1 and EF-2) with the 60s ribosomal subunit by removing the base of adenine4324 in the 28s ribosomal RNA (Endo et al. 1987; Zamboni et al. 1989). Plant toxins referred to as holotoxins contain a binding domain that is disulfide bonded to a catalytic domain and also include viscumin, derived from the mistletoe plant (Stirpe et al. 1982). Some plant toxins referred to as hemotoxins consist of just a catalytic domain alone, such as BRIP (Bernhard et al. 1994), gelonin (Zamboni et al. 1989), momordin (Porro et al. 1993), pokeweed antiviral protein (Uckun et al. 1999), saporin (Flavell et al. 1998), and trichosanthin (Bolognesi et al. 1992). Differing from plant toxins, the bacterial toxins PE and DT enzymatically ADP-ribosylate elongation factor-2 (EF-2), thereby inhibiting protein synthesis (Carroll and Collier 1987; Van Ness et al. 1980). While plant cells naturally produce plant toxins composed of binding and enzymatic domains connected by a disulfide bond, prokaryotic bacterial cells produce toxins as single chains (Kreitman 1997). This actually makes it much easier to produce recombinant immunotoxins from bacterial toxins. Bacterial toxins are produced with a binding domain at one end and a catalytic domain at the other. Between these two domains is a domain which has a proteolytic cleavage site so that the toxin can be separated from the binding domain and translocated to the cytosol without the ligand. The binding domain of DT is at the carboxyl-terminus and the catalytic domain at the amino terminus, while in PE the orientation is opposite. To make recombinant immunotoxins, a recombinant antibody (Fv) replaces the native binding domain of the toxin. Compared to the older immunotoxin chemical conjugates which contained a whole antibody chemically conjugated to the toxin, recombinant immunotoxins are smaller, more homogenous in terms of the toxin-antibody junction, and are much more easily produced since they may be directly purified from *E. coli*.

### *8.1.2 Mechanism of PE Cell Intoxication*

Whole PE is a 613 amino acid protein containing three functional domains (Hwang et al. 1987; Allured et al. 1986). As shown in Fig. 8.1, domain Ia (amino acids 1–252) is the binding domain, domain II (amino acids 253–364) contains the proteolytic cleavage site allowing translocation of the bacterial toxin fragment into the cytoplasm, and domain III (amino acids 400–613) has catalytic activity which ADP-ribosylates EF-2 once in the cytosol. It was originally thought that domain II was necessary for translocation into the cytosol, but in fact most of it can be removed without loss of cytotoxicity for most cell lines (Weldon et al. 2009, 2015). Domain Ib (amino acids 365–399) has no known function, and amino acids 365–380 may be removed without loss of cytotoxic activity (Siegall et al. 1989;

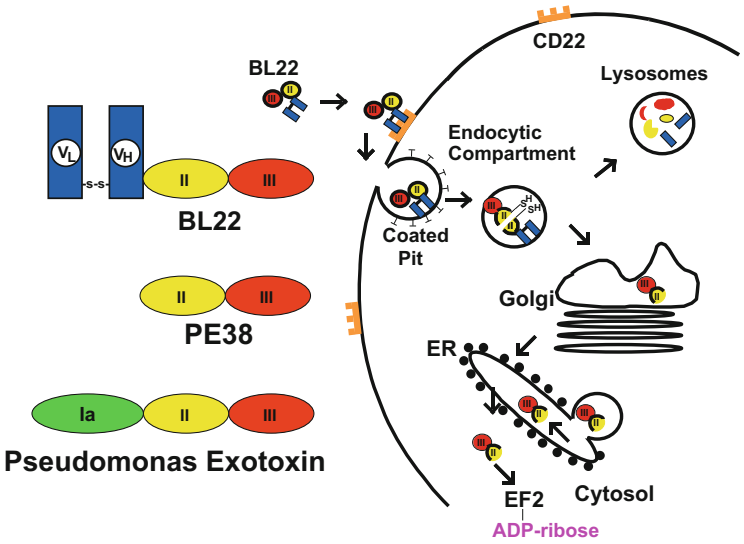


**Fig. 8.1** Schematic diagram of BL22 and mechanism of intoxication

Kreitman et al. 1992). The steps involved in intoxication by PE, several of which are depicted in Fig. 8.1, include:

1. The carboxy-terminal residue lysine-613 is removed by a carboxypeptidase in the plasma and conditioned cell culture medium (Hessler and Kreitman 1997).
2. Domain Ia binds to the alpha-2 macroglobulin receptor on normal animal cells.
3. The toxin is internalized into endosomes which traffic to the transreticular Golgi (Kounnas et al. 1992).
4. Furin proteolytically cleaves domain II between amino acids 279 and 280 (Chiron et al. 1994; Fryling et al. 1992; Ogata et al. 1992).
5. The disulfide bond between cysteines 265 and 287, which up to that point joins the two cleavage fragments, is reduced.
6. The terminal amino acids 609–612 (REDL) bind to the KDEL receptor, a sorting receptor which normally functions to keep endoplasmic reticulum (ER)-resident proteins from being secreted (Kreitman and Pastan 1995).
7. The 37 kDa carboxy-terminal fragment of PE is transported from the transreticular Golgi to the endoplasmic reticulum (ER) (Kreitman and Pastan 1995; Chaudhary et al. 1990).
8. The 37 kDa fragment of PE translocates into the cytosol (Theuer et al. 1993a, 1994).
9. The diphthamide residue in EF-2 is inactivated by the ADP-ribosylating activity encoded within amino acids 400–602 of PE (Carroll and Collier 1987), including residues His440 and Glu553 (Carroll and Collier 1987; Li et al. 1995, 1996; Han and Galloway 1995).

ADP-ribosylation is believed to occur by the following steps:

(a) His440 of PE binds to nicotinamide adenine dinucleotide (NAD) by forming a hydrogen bonding with the adenosine monophosphate (AMP) ribose O2′ group.
(b) Glu553 of PE, via its carboxyl group, hydrogen bonds with Tyr481and Glu546, which result in Tyr481 binding NAD by a ring stacking interaction. The diphthamide residue in EF-2 is a posttranslationally modified histidine residue, which is essential for the development of most eukaryotes (Webb et al. 2008).
(c) The adenosine diphosphate (ADP) ribose nucleotide subunit of NAD+ is transferred to the NE2 atom of the diphthamide-containing imidazole ring (Jorgensen et al. 2006).
(d) ADP ribosylation inhibits EF2 activity, arrests protein synthesis lowering Mcl-1 levels, and stimulates apoptosis (Brinkmann et al. 1995; Keppler-Hafkemeyer et al. 2000; Decker et al. 2004) .

### *8.1.3 Toxin Fragments for the Production of Recombinant Immunotoxins*

The original truncated form of PE that had a deletion of domain I and could not bind to normal cells was called PE40; it is missing amino acids 1–252 that comprise the binding domain (Hwang et al. 1987; Kondo et al. 1988). Since amino acids 365–380 of domain 1b were found to be unnecessary for cytotoxicity and these amino acids contain a disulfide bond potentially complicating the refolding process during production, this region was removed. The resulting 38 kDa truncated toxin PE38 had cytotoxicity comparable to PE40 (Siegall et al. 1989; Kreitman et al. 1993a), as well as similar or better production yields.

### *8.1.4 Types of Fv Fragments Used in Recombinant Immunotoxins*

Single-chain Fv fragments were originally used to make recombinant immunotoxins (Kreitman et al. 1993a; Chaudhary et al. 1989; Brinkmann et al. 1991). These recombinant immunotoxins were composed of the variable heavy and light domains connected via the flexible $(G_4S)_3$ linker. To increase stability, disulfide-stabilized recombinant immunotoxins were produced, containing an engineered disulfide bond within the framework section of the variable domains. To accomplish this, two residues, one from $V_L$ and one from $V_H$, which were predicted to be ~5.5 angstroms apart, were each mutated to cysteine. Due to its rigid structure, the use of a disulfide bond instead of a peptide linker increased stability for several recombinant immunotoxins (Brinkmann et al. 1993; Reiter et al. 1994a, b) and also improved the yields upon renaturation. The latter was probably because

the disulfide bond prevents the variable domains from transiently separating, thus limiting interactions between variable domains of neighboring molecules. The variable domains are joined by a disulfide bond, so the toxin can be linked to either $V_L$ or $V_H$. Since $V_H$ is less stable than $V_L$, the toxin is fused to $V_H$. Consequently, any $V_H$-toxin fusion molecule not disulfide bonded to VL will aggregate, precipitate out of solution, and not co-purify with the slightly larger recombinant immunotoxin. Nevertheless, the disulfide-stabilized immunotoxins are still considered recombinant because the disulfide bond in the Fv forms during redox renaturation; chemical conjugation is not needed. In recombinant immunotoxins, target cells are intoxicated in a manner similar to whole toxins, except that the binding domain is an Fv fragment rather than a binding domain of a toxin.

### *8.1.5 Production of Recombinant Immunotoxin from* E. coli

To produce recombinant immunotoxins in *E. coli*, the truncated toxin gene from *Pseudomonas aeruginosa* which naturally produces PE is used to make plasmids expressing the recombinant immunotoxin containing the ligand at the amino terminus of PE. The DNA fragment is placed into a plasmid containing the T7 promoter (Studier and Moffatt 1986), and the plasmid is transformed into *E. coli* cells containing a repressed RNA polymerase gene. Repression is released in late log phase, by adding the lactose analog isopropyl-b-D-thiogalactoside (IPTG), and the T7 RNA polymerase produced leads to plasmid expression and recombinant immunotoxin translation. Initially recombinant immunotoxins were purified from the periplasm (Chaudhary et al. 1988) or harvested from *E. coli*. cytoplasm (Bendel et al. 1997). However, higher yields and purer preparations of immunotoxin can be produced from insoluble inclusion bodies (Buchner et al. 1992; Kreitman and Pastan 1993, 1997, 2000). This latter approach is preferred because the insoluble inclusion body protein can be washed extensively with detergent to remove endotoxin and other contaminants. The inclusion body protein is then dissolved, denatured, and reduced using 7 M guanidine containing a reducing agent like dithioerythritol. The reduced and denatured protein is then renatured by rapid dilution into a redox buffer, permitting native pairing of disulfide bonds. To lower ionic strength prior to ion exchange chromatography, dialysis, ultrafiltration, or dilution is used. Ultrafiltration can result in high yields through precipitation of the aggregates present, which may be easily removed. The resulting monomeric protein is then further purified by anion exchange and sizing chromatography. Using this approach, 5–20% of the total recombinant inclusion body protein can generally be obtained as a purified product. Recombinant immunotoxins have also been produced in a *Pichia pastoris* strain containing mutant EF2 to prevent intoxication of the eukaryotic cells (Woo et al. 2002, 2008), as well as other eukaryotic systems. However, prokaryotic expression has been optimized for industrial scale purification resulting in yields higher than those achievable in the lab, and with higher purity.

### 8.1.6 CD22 Targeting by Immunotoxins

CD22 contains three immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and is a member of the siglec family. CD22 functions as a B-cell inhibitory receptor which associates with the B-cell receptor, inhibiting cell activation by recruiting an inhibitory tyrosine phosphatase called SHP-1 (Crocker 2002). Older-generation immunotoxin chemical conjugates containing ricin A-chain (Amlot et al. 1993; Sausville et al. 1995; Senderowicz et al. 1997; Messmann et al. 2000) were originally used to target CD22 on leukemias and lymphomas. Significant antitumor activity was seen, albeit with dose-limiting capillary leak syndrome. Though not tested in patients, chemical conjugates were made with truncated PE and either of the two anti-CD22 MAbs, RFB4 or LL2 (Kreitman et al. 1993b; Theuer et al. 1993b; Mansfield et al. 1997a). RFB4 as a single-chain Fv fragment was stable enough to produce a functionally active recombinant immunotoxin RFB4(Fv)-PE38 (Mansfield et al. 1997a). However, for increased stability and yield of production, RFB4(Fv)-PE38 was converted to a disulfide-stabilized immunotoxin RFB4(dsFv)-PE38, by mutating R44C in VH and G100C in VL each to cysteine and by removing the peptide linker (Mansfield et al. 1997b). The resulting molecule was called BL22 (Fig. 8.1).

## 8.2 Introduction to Development of BL22

### 8.2.1 Preclinical Development of BL22

In preclinical studies in vitro, BL22 was shown to be cytotoxic toward CD22+ cell lines and was stable at 37 °C (Mansfield et al. 1997b). Partly due to the disulfide-stabilized construction, it could be produced in large scale and with low endotoxin content. In vivo studies showed that BL22 induced CRs in all mice bearing subcutaneous solid Burkitt's lymphoma xenografts at doses of 275 μg/kg intravenously (i.v.) every other day for 3 doses (QOD × 3) by bolus injection or 100 μg/kg/d × 7 days by intraperitoneal (i.p.) continuous infusion (Kreitman et al. 1999). However, targeted toxicity of BL22 could not be studied in mice, since murine CD22 does not bind RFB4 or BL22. Therefore, to investigate the possible toxicity of BL22 through CD22-mediated targeting of normal tissues, BL22 was administered to monkeys, which unlike mice express CD22 capable of binding RFB4 and BL22. Only mild (grade 1) laboratory abnormalities were observed at doses of 100 or 500 μg/kg i.v. QOD × 3 (Kreitman et al. 1999). The half-life of BL22 in monkeys was 30 min, similar to its half-life in mice. Knowing that leukemic cells from patients express lower levels of CD22 than cell lines, we also tested BL22 ex vivo against primary malignant cells from patients with B-cell malignancies. At total of 28 patients with acute and chronic lymphocytic leukemias (ALL and CLL), prolymphocytic leukemia (PLL), and large cell (DLBCL), mantle cell (MCL), and

follicular lymphomas (FL) were studied (Kreitman et al. 2000a). The IC50s, defined as the concentrations necessary for 50% inhibition of protein synthesis, were in the range of 3–10 ng/ml in 5 patients (1 FL, 3 CLL, 1 ALL), 10–50 ng/ml in 7 patients (6 CLL, 1 MCL), and 50–1000 ng/ml in 12 patients (9 CLL, 1 DLBCL, 1 PLL, and 1 MCL). Cytotoxicity was associated with cell death by direct viability assay. Significant cytotoxicity could be documented with malignant cells having CD22 sites/cell as low as 350/cell. Cytotoxicity of BL22 was most potent on hairy cell leukemia (HCL) cells. Using protocols similar to those previously published (Kreitman and Pastan 2000, 1993), BL22 was made under good laboratory practice (GLP) in large scale for clinical testing. Initial lots were produced by the Monoclonal Antibody and Recombinant Protein Facility (Frederick, MD), and later lots were produced by the Advanced Bioscience Laboratories (ABL, Kensington, MD) .

### *8.2.2 Phase 1 Testing of BL22*

In 46 patients with B-cell hematologic malignancies, a total of 265 cycles of BL22 were administered at dose levels of 3, 6, 10, 20, 30, 40, and 50 μg/kg QOD × 3 (Kreitman et al. 2001, 2005). Patients received 1–33 cycles. Most (Han and Galloway 1995) of the 46 patients had HCL, while 11 had CLL and 4 had NHL. In view of the high number of HCL patients treated, it is appropriate at this point to briefly review HCL as a disease, including its standard therapy, in order to better appreciate the activity of BL22 in phases 1 and 2 testing.

## 8.3 Introduction to HCL

### *8.3.1 Classic and Variant HCL: Definitions and Diagnosis*

The successful diagnosis of HCL requires assessment of immunophenotype, usually by both flow cytometry of bone marrow and/or peripheral blood and immunohistochemistry (IHC) of the bone marrow biopsy. The bone marrow shows collections of malignant lymphocytes containing abundant cytoplasm. IHC shows expression of CD22, tartrate-resistant acid phosphatase (TRAP) (Sharpe and Bethel 2006; Matutes 2006), annexin 1A (Anxa1) (Falini et al. 2004), and the V600E mutation of BRAF (Tiacci et al. 2011). Flow cytometry identifies leukemic cells with sizes in the range of lymphocytes and monocytes, with bright positivity for CD20, CD22, and CD11c, and with positivity for CD19, CD103, and CD123 (Sharpe and Bethel 2006; Matutes 2006; Shao et al. 2012; Venkataraman et al. 2011; Jasper et al. 2011). In classic HCL, CD25 is expressed, sometimes brightly. In 2008, hairy cell leukemia variant (HCLv) was considered by the World Health

Organization (WHO) as a disease (or group of disorders) separate from classic HCL, in lacking CD25, TRAP, and/or Anxa1 (Swerdlow et al. 2008; Matutes et al. 2003; Cawley et al. 1980; Robak 2011). Clinically, HCLv patients generally have leukemic lymphocytosis rather than pancytopenia, worse splenomegaly, lymphadenopathy, and respond poorly to therapy, even first line (Matutes et al. 2003; Polliack 2002). HCLv cells are wild type for BRAF, lacking the V600E mutation (Tiacci et al. 2012; Xi et al. 2012). In 2009, we described a new variant overlapping immunophenotypically between HCL and HCLv, which expresses the IGHV4–34 type of immunoglobulin rearrangement and has >98% homology to its germline sequence, defined as unmutated (Arons et al. 2009). Patients with IGHV4–34+ HCL have lymphocytosis, lymphadenopathy, and rapidly enlarging spleens like HCLv, despite HCL immunophenotype (Arons et al. 2009). While their BRAF is wild type, some patients with HCLv or IGHV4–34+ HCL have other mutations, located those in the MAP kinase pathway within the MEK gene (Waterfall et al. 2014).

### *8.3.2 Indications for HCL Treatment*

For HCL, cytopenias considered to require treatment for HCL include neutrophils less than 1–1.5/nl, hemoglobin less than 10–11 g/dl, or platelets under 100/nl (Kreitman et al. 2001; Saven et al. 1998; Grever et al. 1995; Chadha et al. 2005). Some trials include patients with symptomatic splenomegaly, malignant lymphocytosis over 5–20/nl, enlarging malignant adenopathy, or frequent infections. Patients after splenectomy are at a significant disadvantage for qualifying for clinical trials, since they have normalized blood counts, progressive increase in marrow infiltration, and cytopenias only when bone marrow infiltration is very far advanced. Before undergoing retreatment of recently treated patients, they must be studied to determine whether cytopenias are due to chemotherapy toxicity or progressive HCL. One of the most important pitfalls we encounter is when the bone marrow biopsy is positive for HCL 1–3 months after purine analog treatment and cytopenias are present due to nonspecific toxicity and the spleen may still be enlarged. Patients should not be retreated at this point, because without further treatment, the marrow will often become negative, the normal blood counts will resolve, and the spleen will decrease, all consistent with complete remission (CR). Patients being treated for relapsed/refractory HCL should optimally wait at least 6 months after their last purine analog therapy when it is clear that cytopenias and HCL measurable in the blood by flow cytometry are progressing.

### *8.3.3 Standard Treatment of HCL*

The purine analogs, usually cladribine but also pentostatin, are the cornerstones of standard primary treatment for HCL, with 70–90% CR rates and treatment-free

intervals up to a median of 16 years (Saven et al. 1998; Grever et al. 1995; Piro et al. 1990; Spiers et al. 1987; Goodman et al. 2003a; Else et al. 2009; Flinn et al. 2000). Cladribine is more commonly used than pentostatin since it is given as a single 5–7-day course, whereas pentostatin is given every other week for 3–12 months. Either way, the median time to relapse after first-line purine analog treatment is about 16 years, using mainly blood counts as follow-up (Else et al. 2009). When bone marrow biopsy was used to follow patients and determine relapse, median time to relapse from CR was less than 5 years, in patients with good long-term follow-up, namely, age $\leq$ 40 at diagnosis (Rosenberg et al. 2014). Many of these patients did not require retreatment at the time of relapse, since the blood counts were not low enough to need retreatment. Long-term, disease-free survival curves have not shown a plateau consistent with a cured population (Else et al. 2009; Sawada et al. 1998; Goodman et al. 2003b), so it is not clear what fraction of patients, if any, are actually cured after purine analog therapy. In fact, 9 of 19 patients in CR for a median of 16 years, out of the original 358-patient group treated at Scripps Clinic, were determined to be still free of HCL by flow cytometry and IHC studies. Thus, at least some may be cured, but it appears to be a minority, and for most HCL patients, relapse is common and this has resulted in an accumulation of relapsed patients requiring additional treatment options.

## 8.4 BL22 Phase 1 Testing

### *8.4.1 Phase 1 Testing of BL22: Patient Characteristics*

The 31 HCL patients were previously treated with 1–6 (median 3) courses of purine analog, usually cladribine (1–3 courses, median 2 courses). All 31 patients had at least one course of cladribine, and 10 had prior pentostatin in addition. One of the 11 CLL patients had cladribine and one of the NHL patients had prior pentostatin (Kreitman et al. 2005). The four patients with NHL also had prior CHOP, transplant, radiation, rituximab, CVP, fludarabine, and a variety of other regimens. The 11 with CLL also had prior fludarabine, chlorambucil, CHOP, flavopiridol, VP16, ESHAP, Campath, rituximab, and other agents. Of the 31 with HCL, 3 had HCLv, and the other 28 had classic HCL. The 42 patients with HCL, HCLv, or CLL had a median of 0.53 $\times$ 109/l circulating leukemic cells, with a range of 0–600 $\times$ $10^9$/l. Patients eligible for the study had to need for treatment with either cytopenias (neutrophils $<$1000/mm$^3$, hemoglobin $<$10 g/dl, platelets $<$100,000/mm$^3$), malignant lymphocytosis (circulating HCL cells $>$20,000/mm$^3$), or tumor-related symptoms such as symptomatic splenomegaly or repeated infections.

### 8.4.2 Phase 1 Dose Levels Administered

Three patients each received 3, 6, 10, and 20 μg/kg every other day for three doses (QOD × 3). At total of 19 patients were enrolled at 30 μg/kg QOD × 3, 12 were enrolled at 40 μg/kg QOD × 3, and 3 were enrolled at 50 μg/kg QOD × 3. Patients were allowed to receive higher or lower doses for repeat cycles (retreatment) depending on toxicity concerns of the protocol. Overall, 265 cycles were given to the 46 patients, including 142 retreatment cycles administered on protocol and 77 cycles given off protocol by special exemption.

### 8.4.3 Phase 1 Toxicity of BL22

None of the 46 patients on the phase 1 trial died on study. The most common dose-limiting toxicity (DLT) was a complication called hemolytic uremic syndrome (HUS), which involves deposition of platelets and fibrin in the glomerular endothelium of the kidney and microangiopathic hemolytic anemia due to red blood cells becoming broken while traversing the intravascular fibrin strands, thrombocytopenia, and renal insufficiency (Moake 2002). The first case of HUS observed was in an NHL patient during the initial cycle 1. Four HCL patients had HUS during cycles 2 or 3 (2 each). Complete recovery from HUS in the NHL patient could not be assessed due to rapidly progressive NHL and refusal of salvage chemotherapy once being taken off protocol. In contrast, all 4 HCL patients completely recovered from HUS and received 6–12 days of plasmapheresis. Renal function in these patients remained normal after 39–57 (median 46) months of follow-up from study entry, at the time of publication, and none of the patients had recurrent or progressive renal insufficiency during 15 years of follow-up since then. Moreover, these HCL patients achieved complete clearance of HCL cells from the blood and bone marrow when their preexisting hematologic abnormalities and HUS-related abnormalities resolved. The only other DLT observed on this trial was one case of systemic (though not pulmonary) capillary leak syndrome (CLS) during the second cycle, associated with high fever and bone pain, suggesting cytokine release. This event completely resolved after 3 days. Because the HUS during cycle 2 at 50 μg/kg QOD × 3 was the most severe case of the HCL patients, with creatinine rising to 7.5, and because another patient at this dose level (#27) had a grade 1 creatinine elevation during cycle 2, no additional patients were enrolled at 50 μg/kg QOD × 3. Of the 12 patients enrolled at 40 μg/kg QOD × 3, 2 had reversible HUS, and this dose level was considered the maximum tolerated dose (MTD).

### 8.4.4 Response Achieved with BL22 During Phase 1 Testing

In the 31 HCL patients, BL22 was significantly active with 19 (62%) CRs and 6 (19%) PRs and an overall response rate (ORR) of 81% (Kreitman et al. 2001, 2005). Of the three patients with the poor-prognosis variant HCLv, which is poorly responsive to first-line cladribine therapy (Matutes et al. 2001), all three had CR with BL22. A majority (Carroll and Collier 1987) of the 19 CRs were documented by restaging after cycle 1, and the other 8 patients required 2–14 cycles before CR could be documented. CR with minimal residual disease (MRD) was defined as small collections of CD20+ (or TRAP+) cells by immunohistochemistry of the bone marrow biopsy. Tallman et al. previously reported that CR with MRD in the bone marrow biopsy by immunohistochemistry may be associated with earlier relapse than CR without MRD (Tallman et al. 1999). Of the 19 CRs, 18 (95%) were without MRD (Kreitman et al. 2005). Though not required for response by protocol, cytopenias improved in all responders and completely resolved in all CRs, in two out of six PRs, and in one out of four patients with marginal response (MR). Circulating malignant cell counts rapidly dropped in CRs, 90% by 2 days and 99% by 1 week. Spleen size also decreased in as little as 1 week. The lack of a major response was seen in patients with low doses (3–6 μg/kg QOD × 3) and in two patients with massive (>20 cm) abdominal lymph nodes. We observed five CRs and two PRs despite high levels of neutralizing antibodies, indicating that repeated doses of BL22 titrated out the neutralizing antibodies. CR was achieved in 12 (86%) of 14 patients enrolled at dose levels of at least 40 μg/kg QOD × 3, compared to 7 (41%) of 17 HCL patients enrolled at lower dose levels, consistent with a dose response (chi2 = 6.4, $p = 0.011$). CR rate after cycle 1 also correlated with dose level ($p = 0.03$).

### 8.4.5 Rapidity and Durability of Response in Phase 1 Testing

Of the 19 HCL patients who achieved CR, the median CR duration at the time of publication was 33 (range 5–61) months, and 8 remained in CR at a median of 39 (range 21–61) months of follow-up. Of the 11 who achieved CR after 1 cycle of BL22, there were 3 patients who relapsed, compared to 6 relapses out of 8 patients who achieved CR with >1 cycle (chi2 = 4.23, $p = 0.04$). Thus, the patients who responded more quickly to BL22 had more durable responses. Hematologic recovery, or hematologic remission (HR), was achieved by 22 (73%) out of 30 patients with preexisting cytopenias, defined as neutrophils, platelets, and hemoglobin at least 1500/μl, 100,000/μl, and 11 g/dl. The median HR duration was 30 months, and at the time of publication, 10 patients (45%) remained in HR at a median 38 months of follow-up.

### 8.4.6 BL22 Phase 1 Response in Patients with CLL and NHL

Unlike HCL, CRs were not achieved in CLL and NHL, although only 4 CLL and 2 NHL patients were enrolled at the upper dose levels, 30–50 μg/kg QOD × 3. Marginal responses were achieved in three CLL patients, one of which had >99.9% eradication of circulating CLL cells and near resolution of cytopenias and received maintenance BL22 for almost 5 years. However, less than 50% response/reduction in an abdominal mass meant that this patient could not be considered a major responder.

### 8.4.7 Immunogenicity of BL22 During Phase 1 Testing

To determine the immunogenicity of BL22, we detected neutralizing antibodies using a bioassay (cytotoxicity assay), similar to that used previously for LMB-2 (Kreitman et al. 2000b). In this assay, serum from patients after treatment with BL22 is incubated in 9:1 (serum to BL22) ratio with purified BL22 for 15 min at 37 °C and the serum to BL22 mixtures diluted and incubated with CD22+ lymphoma cells to determine cytotoxicity. This assay determined that 11 of 46 patients made neutralizing antibodies after 1–8 cycles of BL22 at dose levels of 20–50 μg/kg QOD × 3. No patients with CLL or NHL produced neutralizing antibodies. Most patients with HCL could receive many cycles of BL22 without generating neutralizing antibodies. High levels of neutralizing antibodies were detected in three out of seven patients at 20 μg/kg QOD × 3 versus zero out of five patients at 50 μg/kg QOD × 3. Thus, in HCL, the production of neutralizing antibodies to BL22 was not dose related. Immunogenicity was neither related to prior splenectomy (chi2 = 0.02, $p > 0.05$) nor to preexisting CD4 counts (Wilcoxon, $p = 0.48$, $n = 13$). It is possible that preexisting antibodies to BL22, i.e., from prior infection with *Pseudomonas aeruginosa*, were a major factor associated with neutralizing antibody production, but fortunately most patients could receive multiple cycles without complete neutralization.

### 8.4.8 Pharmacokinetics of BL22 in Phase 1 Testing

To determine plasma concentrations of BL22, plasma was diluted and the dilutions incubated with Raji cells, and biologically active drug was quantified by cytotoxicity assay. Peak levels were determined and found to be dose related. At 30–50 μg/kg QOD × 3 of BL22, median half-lives were about 3 h. Areas under the curve (AUC) significantly correlated with the dose level, but within each dose level, AUC was highly variable, attributed to a CD22 sink effect. This sink effect was due to the high CD22 expression on HCL cells, quantified by flow cytometry at a median of

~44,000 sites/cell. With response, tumor burden decreased, particularly after achievement of CR, and both peak levels and AUCs increased. The volumes of distribution decreased to the level of the plasma volume. This observation that drug exposure was higher on retreatment compared to initial cycles was consistent with the observation that HUS in HCL occurred only during retreatment, not during initial therapy. This also provided the rationale for lowering the dose level for retreatment cycles to avoid toxicity, which was done in the last six patients enrolled. When the relationship between PK parameters, tumor burden, and response was examined, it was determined that HCL patients with CR after 1 cycle of BL22 had significantly higher AUCs ($p = 0.01$) and lower concentrations of circulating HCL cells ($p = 0.007$) compared with HCL patients who did not achieve CR to cycle 1. We concluded that patients with lower tumor burden can achieve higher AUCs due to the lower CD22 sink and may have a higher chance to achieve CR in cycle 1. Being able to achieve CR early might be particularly important when immunogenicity occurs early.

### *8.4.9 Continued Development: Conclusions of BL22 Phase 1 Testing and Planning Phase 2*

Results of phase 1 testing of BL22 supported its efficacy in relapsed/refractory HCL, and its safety profile, despite reversible HUS, supported continued clinical development. BL22 was the first agent since purine analogs which was found to achieve CR in the majority of patients with HCL and, at the time, the only agent able to achieve high CR rate in relapsed/refractory HCL. Its success in chemoresistant patients was clearly related to the fact that CD22 is highly conserved at high density on HCL cells, even in patients who are purine analog resistant. Important lessons from the phase 1 trial of BL22 in HCL included:

1. The cycle 1 MTD was 40 μg/kg QOD × 3, with only 2 of 12 patients at that dose level having reversible HUS.
2. The CR rate was dependent on the dose used for cycle 1; the CR rate was 86% with at least 40 μg/kg QOD × 3 compared to 41% at lower doses ($p = 0.01$).
3. CR was often documented after cycle 1, and even when achieved later, it is possible that the retreatment was not needed for CR and that the patient would have achieved CR slowly without retreatment.
4. HUS was completely reversible and only occurred with retreatment.

Using these phase 1 lessons/parameters, our strategy in phase 2 testing of BL22 was to treat all cladribine-resistant HCL patients with 40 μg/kg QOD × 3, wait for sufficient time (8 weeks) to determine if patients would respond, and retreat only those patients who could not achieve hematologic remission (HR) after cycle 1. With this strategy, we could determine the effect of a single cycle of BL22,

which we believed to be very safe, and we could also determine the response rate with retreatment in those cases where patients really needed retreatment.

## 8.5 BL22 Phase 2 Testing

### *8.5.1 Patients and Trial Design Used for Phase 2 Testing of BL22*

A total of 36 patients were enrolled on protocol. To be eligible, patients had to have CD22+ HCL by flow cytometry and prior treatment with cladribine. Resistance had to be documented with <2-year duration of CR/PR after the first course or <4-year duration of CR/PR to a second or later course of cladribine. Patients had to have disease requiring treatment and not have high levels of neutralizing antibodies or recent treatment with chemotherapy within 4 weeks or monoclonal antibody therapy within 3 months prior to enrollment. Patients received BL22 over 30 min QOD × 3. The cycle 1 dose was 40 μg/kg QOD × 3, which was the MTD for the phase 1 trial. Patients with at least a hematologic remission (HR) by 8 weeks, defined as ANC, platelets, and Hgb of at least 1.5, 100, and 11, respectively, were observed and not retreated until they relapsed with blood counts low enough to meet initial eligibility criteria. Patients with either high levels of neutralizing antibodies (>75% neutralization of BL22 at 1000 ng/ml) or progression of disease (PD) were not eligible to be retreated. The retreatment dose level was 30 μg/kg QOD × 3, lower than cycle 1, and the retreatment cycle interval was at least 26 days. Retreatment was repeated until two cycles after CR without MRD was documented, or four cycles after CR with MRD was documented. CR was defined as the absence of disease by morphological (nonimmunologic) examination of blood and bone marrow, the absence of disease on CT including the absence of palpable spleen, and resolution of cytopenias to the HR level. MRD was defined as HCL clumps of 15–25 CD20+ cells visible in the bone marrow biopsy by immunohistochemistry, or HCL in the blood by flow cytometry. Partial response (PR) was defined as decrease in tumor burden by at least 50% and improvement in cytopenias either to the level required for HR, or at least 50% improvement in baseline ANC, Hgb, and platelets. Progressive disease (PD) was defined by an at least 25% increase in disease measurements radiographically, or at least a 50% increase in HCL cell count. Stable disease (SD) was defined when the response achieved was neither CR, PR, nor PD.

### 8.5.2 Patient Characteristics During Phase 2 Testing of BL22 in HCL

A total of 36 patients were enrolled. Patients were prospectively stratified with respect to cladribine resistance, as to whether there was less than 1 year CR/PR to the last course (26 patients), or 1- to 4-year CR/PR to the last course (nine patients). A third stratified group, containing only one patient, was established for patients with uncontrolled infection and no response to prior purine analog and rituximab. There were 31 males and 5 females. Ages were 43–75 (median 56). The concentration of circulating HCL cells was 0.3–74,000 cells/μl, median 61/μl. Of the 36 HCL patients, 3 had the poor prognosis variant HCLv which was CD25 negative. Otherwise, all patients had classic HCL, which by flow cytometry was positive for CD11c, CD19, CD20, CD22, CD25, CD103, and surface immunoglobulin.

### 8.5.3 Response to BL22 in Phase 2 Testing

The 36 patients received a total of 131 cycles of BL22. Of these patients, 16 (44%) received only 1 cycle and 20 (56%) received 2–13 total cycles. At the 8-week time point, treatment was held for eight (22%) patients for CR and four (11%) patients for HR. Six (17%) patients were not retreated, due to immunogenicity in two (6%), progressive disease in two (6%), infection in one (3%), and toxicity in one (3%). Of the four with HR after cycle 1, three (75%) relapsed and were retreated. Only one of eight with CR after cycle 1 relapsed, but this patient stayed in HR and was thus not retreated at the time of publication. Overall, including retreatment, responses amounted to 16 (44%) CRs, 6 (17%) HRs, and 3 (8%) PRs, for an overall response rate of 69%.

### 8.5.4 Phase 2 BL22 Efficacy with Respect to Clinical Features

To determine if response was related to the degree of purine analog resistance, patients were prospectively stratified as to whether they had <1- or 1–4-year duration of response to their last course of cladribine. As shown in Table 8.1, there was no significant relationship ($p > 0.05$) in these stratifications in either CR or overall response rates (ORR). However, as shown in Table 8.2, the ORR was 95% with the spleen present and <200 mm in caudal-cranial diameter, compared to 20% in patients with massive spleens (>200 mm) or prior splenectomy. A very large spleen could present a severe barrier to tumor penetration and may also constitute a CD22 sink for decreasing the plasma level of BL22. Patients with prior splenectomy may have tighter packing of HCL cells in the bone marrow and thus take longer to respond to BL22. Alternatively, patients with either massive spleens or splenectomy may have more advanced disease and be more resistant to therapy.

**Table 8.1** BL22 response is not related to CdA resistance

| Stratified groups | *n* | CR | ORR |
|---|---|---|---|
| <1-year CR/PR to last cladribine | 25 | 44% | 64% |
| 1–4-year CR/PR to last cladribine | 10 | 60% | 100% |
| Uncontrolled infection | 1 | 0 | 0 |
| Total | 36 | 47% | 72% |

**Table 8.2** Overall response is related to spleen status

| | *n* | ORR (%) | |
|---|---|---|---|
| Spleen <200 mm | 22 | 95 | |
| Spleen >200 mm | 5 | 20 | ($p = 0.001$) |
| Prior splenectomy | 9 | 33 | ($p = 0.0007$) |
| Total | 36 | 69 | |

($p$ values show Fisher's exact test vs. spleen <200 mm)

### 8.5.5 Patterns of Response to BL22

Despite a very large tumor burden, response, and even CR, is possible with BL22. The first patient on the trial (BH01), with spleen size 250 mm, had a CR after six cycles of BL22 and then had one consolidation cycle. This patient has remained in CR over 10 years after treatment with BL22. Patient BH03 had spleen <200 mm, had CR after five cycles, and then had two consolidation cycles. This patient also has not relapsed and, like the other patient, has not even had MRD reappear after >10 years of follow-up. Out of 16 patients who achieved CR, half required only 1 cycle while the other half required 3–10 (median 6) cycles for CR. Three patients had CR with MRD as best response, including two with CR after one cycle. One patient had CR with MRD after cycle 4 but achieved CR without MRD after consolidation with BL22. Of the 16 CRs, 4 (25%) relapsed, including 1 of 8 patients receiving one cycle and 3 of 8 receiving multiple cycles. Of the eight who received multiple cycles, the three who relapsed had received 0–1 consolidation cycles, while the five who remained in CR received 2–4 consolidation cycles each. Thus in those patients requiring >1 cycle of BL22 for CR, consolidation with <2 cycles is associated with relapse ($p = 0.018$, Fisher exact).

### 8.5.6 Rapid B-Cell Recovery After BL22

To determine how soon normal B cells would recover after treatment with BL22, which might kill normal B cells since they express CD22, patients were followed with flow cytometry to quantify normal B-cell subsets. Figure 8.2a shows normal B cells (percent of total lymphocytes) after the last cycle of BL22 in the six patients who had no detectable HCL cells during at least the last retreatment cycle of BL22 (30 μg/kg QOD × 3). In all patients, normal B cells were detectable prior to day 80, and within the first year after starting the last cycle, normal B cells recovered to

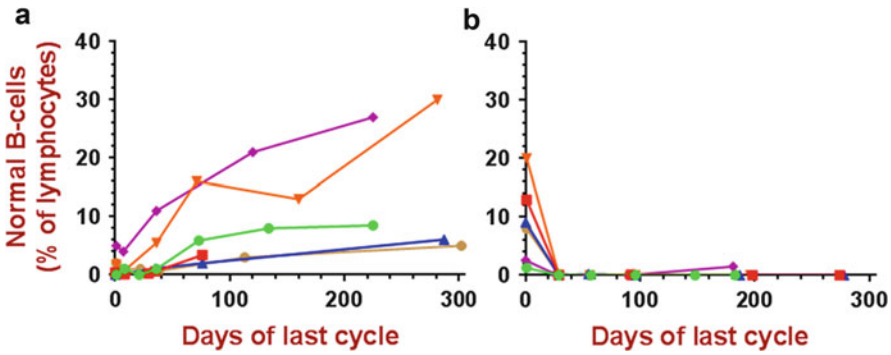


**Fig. 8.2** Normal B-cell recovery after immunotherapy. (**a**) BL22 and (**b**) rituximab

3.4–30% (median 7.3%) of total lymphocytes. To compare B-cell recovery after BL22 to that after rituximab, patients on a different trial were followed by flow cytometry after starting 8 weekly doses of rituximab 375 mg/m$^2$, starting at least 6 months after their first course of cladribine therapy for previously untreated HCL. As shown in Fig. 8.2b, normal B cells did not significantly recover even 6–9 months after starting rituximab. This may be explained by the several-week half-life of rituximab and persistent plasma levels for over 6 months after the last dose. Thus BL22 not only avoids chemotherapy-like toxicities including killing of normal T cells but also avoids long-term depletion of normal B cells as noted with rituximab.

### *8.5.7 Toxicity of BL22 During Phase 2 Testing*

Toxicity was carefully monitored to determine whether selective retreatment during phase 2 would lower the risk of HUS and other toxicities. Cycle 1 at 40 μg/kg QOD × 3 and retreatment at 30 μg/kg QOD × 3 gave very similar toxicity profiles, with the most common toxicities being low-grade hypoalbuminemia, AST/ALT elevations, edema, myalgias, proteinuria, fatigue, nausea, and fever. On protocol, 2 (6%) out of 36 patients had grade 3 reversible HUS not requiring plasmapheresis. One had HUS after cycle 2, which was administered 11 weeks after cycle 1, and the other had HUS after cycle 1. Both of these patients had prior splenectomy. A third patient who achieved CR had grade 1 HUS during cycle 3, including thrombocytopenia, hemolytic anemia, and LDH elevation but no creatinine elevation. This patient was retreated with BL22 at 10 rather than 30 μg/kg QOD × 3 for cycles 4–7, without evidence of HUS. To determine if this patient's risk of HUS had resolved and to return the patient to CR after she relapsed with low doses of BL22, she was treated off protocol by special exemption and received 20 μg/kg QOD × 3 for cycle 8. She then had grade 3 HUS which completely resolved. All grade 3–4 adverse events were reversible and were either non-dose limiting (i.e., ALT, AST, fever) or associated with HUS.

### 8.5.8 Methods Used to Prevent HUS During BL22 Phase 2 Testing

To prevent HUS and renal toxicity, prophylactic i.v. fluid was given before and after each dose and also continuous normal saline 1–2 l/day (40–80 cc/h) given via portable pump. Prophylactic normal saline did not prevent HUS but possibly decreased the degree of renal toxicity. It was noted that platelet counts in HCL were relatively high (128–412) prior to cycles of BL22 associated with HUS during phase 1, because of recent hematologic remission to BL22. To prevent HUS during phase 2, beginning with patient BH17, aspirin 81 mg/day was administered during cycles 1–3, days 1–8, when the pre-cycle platelet count was >100. A total of six, two, and one patient received aspirin beginning with cycles 1, 2, and 3, respectively, and none of these patients had HUS. Patient BH36 could not receive aspirin prior to cycle 1 due to a platelet count of 38 and had HUS with cycle 1. Two patients were receiving enoxaparin during BL22 for prophylaxis of DVT, and neither patient had HUS from BL22. Although it could not be determined whether aspirin or other anticoagulants can prevent HUS from BL22, it is possible that antiplatelet agents or heparins alone or in combination during the week of treatment might reduce or eliminate the risk of HUS without increasing the risk of bleeding.

### 8.5.9 Pharmacokinetics of BL22 During Phase 2 Testing

The half-lives of BL22 were 5–228 min ($n = 36$, median 157 min) during cycle 1, 93–199 min ($n = 15$, median 153 min) during cycle 2, and 95–199 ($n = 15$, median 131) min during cycle 3. To study the correlation between level of HCL cells, i.e., tumor burden, and plasma level of drug, patients were treated with BL22 and plasma was obtained at defined time points after a 30 min infusion. Tumor burden was assessed by circulating HCL cells and spleen size. Dilutions of plasma were incubated ex vivo with the CD22+ cell line Raji and plasma levels determined using a standard curve made from cytotoxicity of pure BL22. Patients who had spleens (enlarged or normal) during cycle 1 at 40 μg/kg QOD × 3 had AUCs inversely proportional to either log of the HCL count determined by flow cytometry ($p = 0.0001$) or spleen size ($p = 0.007$) determined by maximum caudal-cranial dimension. For patients who had spleens during retreatment at 30 μg/kg QOD × 3, AUCs were also inversely proportional to either log of HCL count ($p = 0.045$) or spleen size ($p < 0.0001$). In asplenic patients, AUC was inversely proportional to log of HCL count during both cycle 1 and retreatment. In patients who had HUS, AUCs were not low but also not unusually high compared to other patients without toxicity. We could conclude that exposure to BL22 cytotoxic activity in the plasma was inversely proportional to CD22+ tumor burden, measured either by spleen size or by circulating HCL cell count.

### 8.5.10 Conclusions Based on Phase 2 Testing of BL22 in HCL Patients

The phase 2 trial of BL22 in HCL confirmed high efficacy in patients with HCL. As shown in Table 8.3, the response rates were slightly lower than those on phase 1, although not significantly. We expected lower response rates because of the study design, since during phase 2, not all patients were allowed to undergo retreatment. In fact, patients who had obtained an HR, the counts needed for CR but a positive bone marrow, were also prevented from retreatment. Although the average dose/cycle was statistically similar in phase 2 compared to phase 1, the average number of cycles/patient in the phase 2 trial was less than half of that observed on the phase 1 trial, and this was associated with lower response rates. Immunogenicity was decreased significantly from 35 to 11% ($p = 0.021$), indicating that limiting exposure limited anti-drug antibodies. Our main goal for limiting retreatment, however, was to decrease the rate of HUS, and as shown in Table 8.3, the rate of DLT from HUS decreased by >50%. There was no statistical significance to the difference in HUS rates, however, due to the low number of events. It was certainly clear from the phase 2 experience that HUS from BL22 need not be treated with plasmapheresis, since all patients resolved without this procedure, with fluids both orally and i.v. We could not determine whether prophylactic aspirin lowered the risk of HUS, and its evaluation was complicated by having to stop it, when the platelet count was low. It is possible that other agents including heparin derivatives, platelet inhibitors, and anticoagulants alone and/or in combination could be useful for short-term prevention of HUS with BL22. We did not see HUS appear for the first time in HCL patients receiving BL22 after cycle 4, possibly because by then, patients would already have demonstrated whether they were susceptible to HUS. Alternatively, there could be actual reduction in a risk by then, for example, if a certain cell type secreting cytokines was killed. A subsequent, recently completed study showed that

**Table 8.3** Comparison of phases 1 and 2 trials of BL22 in HCL

| | Phase 1 | Phase 2 | *p* Value |
|---|---|---|---|
| HCL patients enrolled | 31 | 36 | |
| Male/females | 25/6 | 31/5 | $p > 0.5$ |
| HCL/HCLv | 28/3 | 33/3 | $p > 0.5$ |
| Dose levels on protocol | 2, 10, 20, 25, 30, 40, 50 | 30, 40 | |
| Cycles administered | 265 | 131 | |
| Average dose/cycle | 29 | 33 | |
| Average cycles/patient | 8.6 | 3.6 | |
| CR rate | 61% | 44% | $p = 0.22$ |
| PR rate | 19% | 25% | $p = 0.75$ |
| ORR | 81% | 69% | $p = 0.40$ |
| HUS dose-limiting toxicity | 13% | 6% | $p = 0.40$ |
| Immunogenicity (% patients) | 35% | 11% | $p = 0.021$ |

*p* values represent Fisher's exact comparisons

the HLA-type HLA-DRB1*11 was not only overrepresented in HCL patients compared to the normal population but also that HLA-DRB1*11 was particularly overrepresented in HCL patients who had HUS from BL22 (Arons et al. 2014). These studies suggested that HUS may be in some way immune mediated, possibly from binding of a molecule to the B-cell receptor of HCL cells in an HLA-restricted manner. Alternatively, it is possible that ADAMTS13, the enzyme which normally cleaves ultralarge multimers of von Willebrand's factor and prevents HUS, may bind to BL22 in an HLA-restricted manner and become inactivated. These hypotheses are currently under evaluation.

## 8.6 Conclusions

### *8.6.1 Current Status for CD22 Targeting of HCL by Anti-CD22 Recombinant Immunotoxin*

Results from the BL22 trials in HCL showed promising activity in relapsed/refractory disease. A low but significant rate of HUS was observed, but since it was reversible without major intervention like plasmapheresis and since BL22 avoided chemotherapy toxicity, its safety profile supported continued clinical development. However, while the phases 1 and 2 testing was underway, a new molecule was developed in which mutations were made in the complementarity-determining region 3 (CDR3) of the heavy chain, which increased CD22 binding by 14-fold (Salvatore et al. 2002). This molecule, first called HA22 for its high affinity to CD22, then CAT-3888, and finally moxetumomab pasudotox, showed higher cytotoxicity than BL22 ex vivo toward HCL cells and especially toward CLL. Its increased CD22 binding, due mainly to lower off-rate, was considered advantageous in potentially increasing the number of bound immunotoxin molecules internalized rather than disassociating from the target cell. A molecule with more selective CD22 binding was also considered potentially advantageous from a toxicity standpoint. Moxetumomab pasudotox had favorable antitumor activity as well as cytotoxicity (Alderson et al. 2009) and in May of 2007 began multicenter phase 1 testing. Early results from clinical testing indicated a high CR rate, no dose-limiting toxicity, and only two cases of grade 2 (moderate) HUS out of 28 patients treated (Kreitman et al. 2012). Moxetumomab pasudotox is now undergoing multicenter pivotal testing in relapsed/refractory HCL, and further development of its parental agent BL22 has ended. However, BL22 remains an important milestone in the anti-CD22 targeting of HCL and other B-cell disorders, which will hopefully lead to an established and approved treatment for patients in the near future.

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