

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 IgG₁ antibody molecule, it has been found that ADCs with a DAR of 8 are more prone to aggregation, resulting in shorter serum half-lives

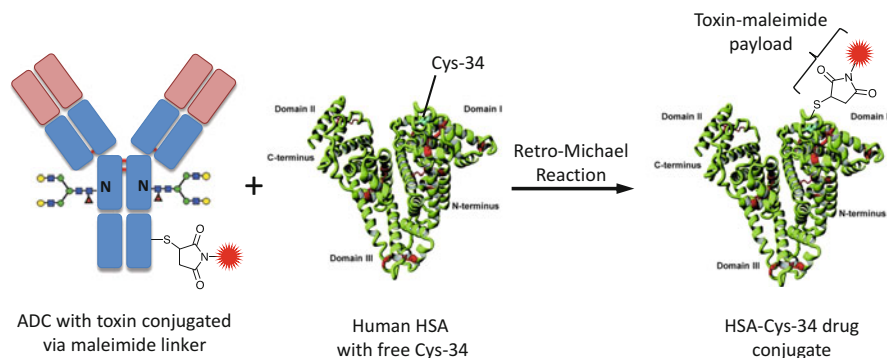


Fig. 5.1 Proposed mechanism of the transfer of payload (*red-colored, spiked ball*) conjugated to an antibody (*left*) via a maleimide linker group to HSA. In the presence of free thiols, the thioether bond of the maleimide linker can be broken via a so-called Reverse Michael Reaction, and the maleimide-payload 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 Kadcyra[®] 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 IgG₁ 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 IgG₁ antibody. This concept was developed further by mutation of the asparagine 297 to glutamine in the IgG₁-Fc portion, which is the normal attachment site for N-linked glycosylation of IgG₁ antibodies. This N297Q mutation resulted in the generation of aglycosylated IgG₁ 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

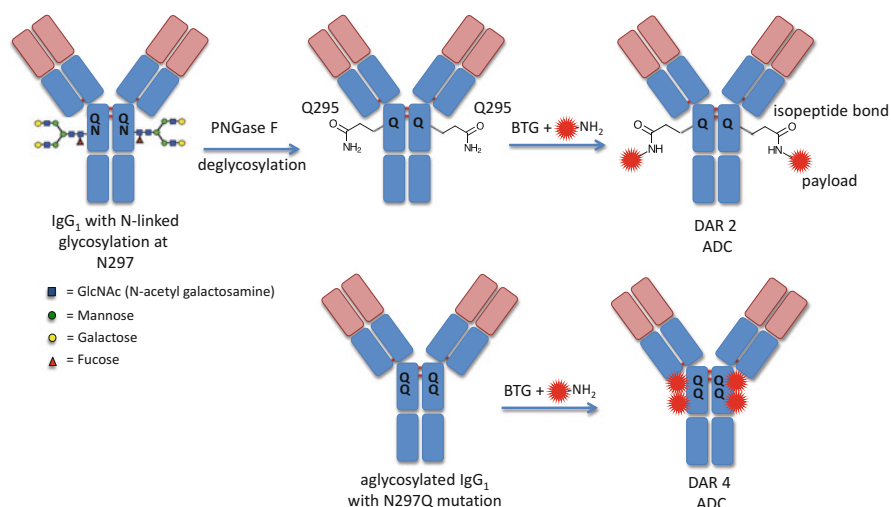


Fig. 5.2 Representation of site-specific conjugation using endogenous glutamine Q295 (*top*), or Fc mutant N297Q (*bottom*) using bacterial transglutaminase (BTG) with a payload (*red-colored spiked ball*), modified with a primary amine function. Endogenous Q295 becomes accessible for BTG conjugation 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

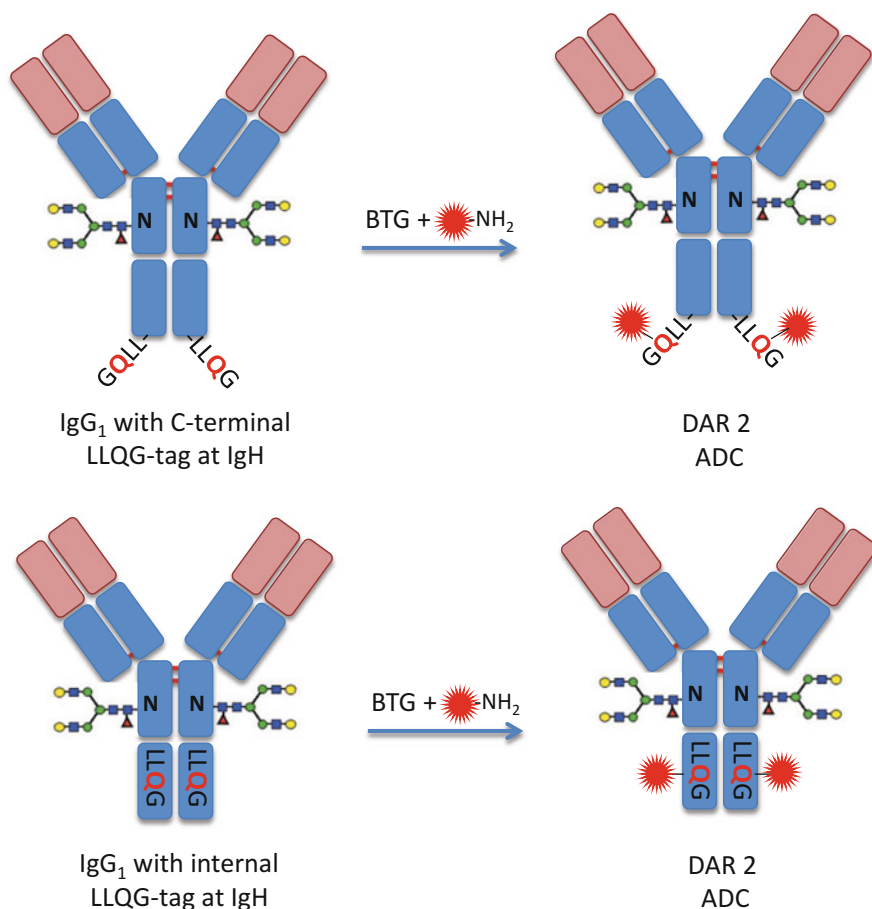


Fig. 5.3 Bacterial transglutaminase (BTG) can also recognize a glutamine (Q) amino acid as a substrate for conjugation, if it is part of a LLQG amino acid sequence that can either be appended to the C-termini of Ig chains (*top*) or placed internally in the antibody sequence (*bottom*). In each case, 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™,

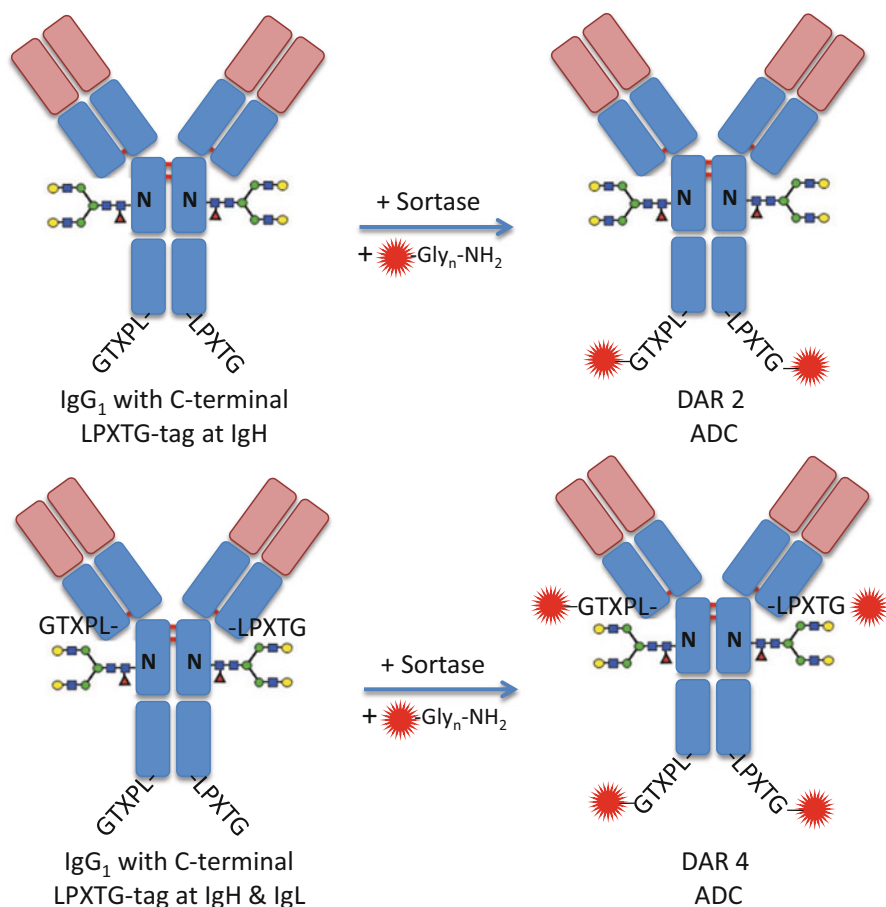


Fig. 5.4 Schematic representation of site-specific conjugation of a glycine (Gly_n)-modified payload (red-colored spiked ball; $n \geq 1$) with Sortase A enzyme from *S. aureus*. The enzyme recognizes a C-terminal penta-peptide motif, LPXTG, for the generation of either IgH chain (DAR2; top) or IgH & 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 Kadcyła[®] 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 Gly₅-maytansine payload (Gly₅-May) showed comparable anti-tumor responses in xenograft models with HER2-positive SKOV-3 ovarian carcinoma cells, when compared to Kadcyła[®] comprising an SMCC chemical linker component. In order to achieve equally efficient SMAC-Technology[™] conjugation of Gly₅-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 Gly₅-modified maytansine without any additional linker structure displayed comparable in vitro and in vivo potency as SMCC linker containing chemically conjugated Kadcyła[®], 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 Kadcyła[®] versus SMAC-Technology[™] conjugated Gly₅-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 Kadcyła[®] and incubated at 37°C for up to 7 days, whereas this transfer was not detectable with SMAC-Technology[™] conjugated Gly₅-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-Gly₅-maytansine is expected to have positive impact on the therapeutic

index of these ADCs in comparison to Kadcyła[®]. 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 Gly₅-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

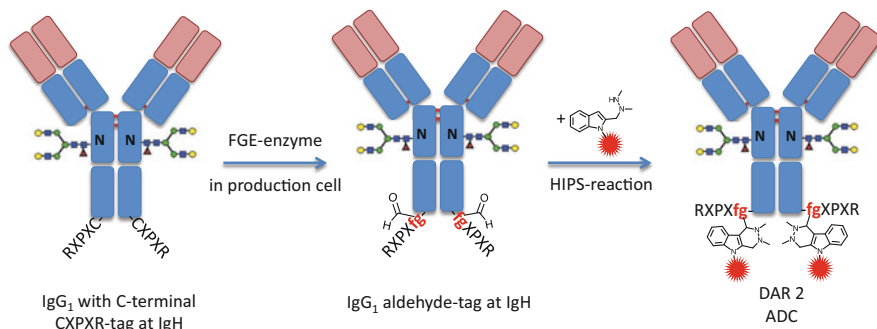


Fig. 5.5 Schematic drawing of site-specific conjugation of payloads to antibodies using the chemo-enzymatic FGE (formyl-glycine generating enzyme) approach. Antibodies for conjugation can be generated by genetic engineering to contain a consensus peptide sequence CXPXR for the FGE-enzyme, e.g., positioned 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-glycine (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 (acquired by Catalent Pharma Solutions) under the name SMARTag™.

Based on the IgG₁ 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 (*intervening proteins*) 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*)

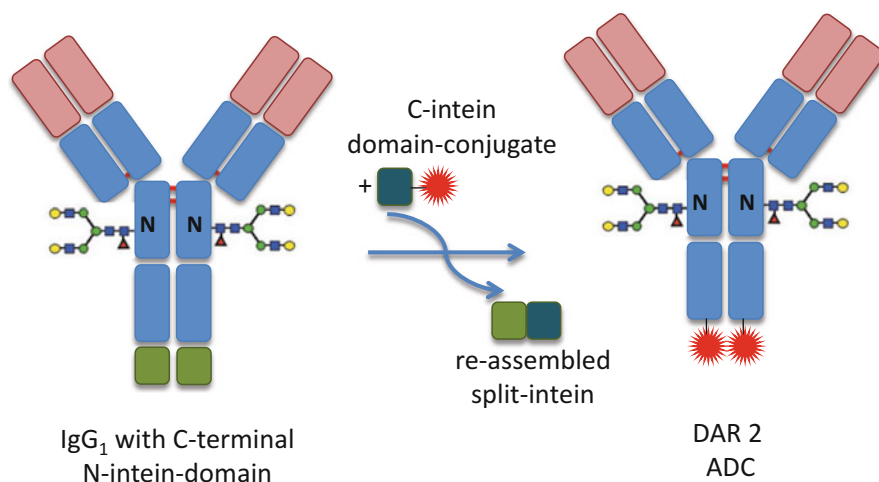


Fig. 5.6 Schematic representation of split intein-mediated site-specific conjugation of antibodies with payloads. The N-terminal split intein domain (N-intein, *light green*) can be fused to the C-termini of, e.g., IgH chains of an antibody by standard genetic engineering. If this engineered antibody 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.zipolutions.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 IgG₁ 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* β 1,4-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 β 1,4-galactosyltransferase (Boeggeman et al. 2009; Ramakrishnan and Qasba 2002). Functionalized 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 β 1,4-galactosyltransferase and α 2,6-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 post-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 tryptptide 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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