Antibody drug conjugates for tumor targeting – novel conjugation chemistries and the promise of non-IgG binding proteins

Hannes Merten[†], Fabian Brandl[†,‡], Andreas Plückthun*[,†], and Uwe Zangemeister-Wittke*[,†,‡]

[†]Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

[‡]Institute of Pharmacology, University of Bern, Inselspital INO-F, CH-3010 Bern, Switzerland

Corresponding authors:

*Andreas Plückthun, Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, E-mail: plueckthun@bioc.uzh.ch, Phone: +41-44-635-5570, Fax: +41-44-635-5712

*Uwe Zangemeister-Wittke, Department of Biochemistry, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland and Institute of Pharmacology, University of Bern, Inselspital INO-F, 3010 Bern, Switzerland. E-mail: uwe.zangemeister@pki.unibe.ch, Phone: +41-31-632 3290, Fax: +41-31-632 4992

1

Abstract

Antibody-drug conjugates (ADCs) have emerged as a promising class of anti-cancer agents, combining the specificity of antibodies for tumor targeting and the destructive potential of highly potent drugs as payload. An essential component of these immunoconjugates is a bifunctional linker capable of reacting with the antibody and the payload to assemble a functional entity. Linker design is fundamental, as it must provide high stability in the circulation to prevent premature drug release, but be capable of releasing the active drug inside the target cell upon receptor-mediated endocytosis. Although ADCs have demonstrated an increased therapeutic window, compared to conventional chemotherapy in recent clinical trials, therapeutic success rates are still far from optimal. To explore other regimes of half-life variation and drug conjugation stoichiometries, it is necessary to investigate additional binding proteins which offer access to a wide range of formats, all with molecularly defined drug conjugation. Here, we delineate recent progress with site-specific and biorthogonal conjugation chemistries, and discuss alternative, biophysically more stable protein scaffolds like Designed Ankyrin Repeat Proteins (DARPins), which may provide such additional engineering opportunities for drug conjugates with improved pharmacological performance.

1. Introduction

The fundamental problem of all chemotherapeutic agents is their small therapeutic window. While most compounds in use today preferentially attack proliferating cells, classical chemotherapy causes side effects which limit the tolerated doses below the clinically effective ones. To increase the therapeutic window, dose-limiting toxicity must be reduced while at the same time effectiveness must be maintained. Antibody-drug conjugates (ADCs) combine the unique specificity of antibodies for tumor-associated antigens to discriminate between normal and malignant cells, and the cytotoxic effect of a small molecule drug as payload [1-3].

Nonetheless, far from being a magic bullet, ADCs normally will have several liabilities as well: on-target, by the target antigen being expressed in healthy tissues, and off-target, on the one hand by an unspecific uptake of the whole conjugate by healthy cells, and on the other, by the drug coming off the antibody. Additionally, the high specificity offers an escape mechanism to the tumor cells by down-regulating the relevant surface antigen [4]. These general considerations emphasize the importance of a judicious choice of the target antigen for minimizing on-target toxicity, while molecular engineering optimizing half-lives and general molecular properties is needed for minimizing off-target toxicity of the conjugate. Finally, linker chemistries must be considered to optimize toxin release after cellular uptake, but not prematurely.

The first cytotoxic agents used for antibody payloading were vinblastine, doxorubicin, methotrexate, and melphalan. In the most promising of these first-generation ADCs, doxorubicin was attached to the anti-Lewis Y antibody BR96, using an acid-labile hydrazone bond. Despite impressive effects in tumor xenograft models [5], BR96-doxorubicin, however, demonstrated very low antitumor activity and tolerability in patients [6, 7]. Binding of the antibody to normal epithelial tissues, low drug potency, and insufficient stability of the conjugate were made responsible for the clinical failure of this first-generation ADC. In the meantime, the spectrum of tumor-selective antigens has increased and more potent anti-cancer agents with cellular IC_{50} values in the pM range have become available for antibody payloading [2].

Another important element in ADC design is the choice of the linker which connects the chemically different targeting molecules to a functional chemical entity and maintains the distinct biological functions of the components. To provide high potency and tolerability, the linker must be stable to prevent premature drug release in the circulation but capable of releasing it inside the cell upon receptor-mediated endocytosis, such that the (usually)

hydrophobic drug can cross the endo-lysosomal membrane into the cytosol. Currently, two categories of linkers, cleavable and non-cleavable, are used, which address these requirements by different mechanisms [2]. The next crucial step is then to attach the linker-drug constructs to specific reactive sites in the antibody.

With common antibodies, conjugation is possible at lysines, cysteines or N-glycans. The conjugation at lysines, considering the large number in an IgG, will be stochastic and result in heterogeneous synthetic products characterized by variable drug-antibody ratios (DARs), at least over a certain range. The conjugation at cysteines can either be to disulfides (typically at the hinge), which need to be reduced first, or to genetically introduced cysteines [8]. While reaction conditions have been optimized to obtain desired DARs, pharmacological performance may well depend not only on the DAR, but also on the precise location of the drug on the binding protein [9].

Recent biotechnological advances have provided a multitude of protein modification strategies to better fix the number and location of payload attachment in ADCs [8, 10]. Also, a number of enzymatic conjugation strategies have been reported that allow the attachment of the drug to a tag introduced to the antibody [11]. In addition, alternative non-IgG formats have become available, which can be engineered to a variety of half-lives, expressed at very high yields and better tolerate the engineering procedures required for well-defined DARs and optimal efficacy [12, 13]. Here, we delineate recent progress in ADC design and the perspectives for engineering novel tailor-made drug-conjugated binding protein formats with desired pharmacological properties.

2. Clinical benefit for tumor-targeting ADCs

Owing to a better understanding of the pharmacological determinants of tumor targeting and recent progress in protein engineering, bioconjugation chemistries and the generation of highly potent payloads, the ADC pipeline is rapidly growing with currently more than 100 entities in clinical trials. For oncology indications, however, clinical results are still far from exciting and many challenges remain to help this technology establish its place in cancer therapy (www.clinicaltrials.gov).

ADCs approved by the US Food and Drug Administration (FDA) or currently in advanced clinical trials are shown in Table 1. So far, only three ADCs were approved by the FDA. They employ either the tubulin polymerization inhibitors monomethyl auristatin E and F (MMAE, MMAF), derivatives of maytansine or the DNA-strand breaking calicheamicin

(Table 1). These payloads outperform the potency of classical chemotherapeutic drugs by a factor of 1000 [2]. As the first, gemtuzumab ozogamicin (Mylotarg[®]), which consists of an anti-CD33 antibody linked to calicheamicin, was granted accelerated approval in 2000 for the treatment of relapsed acute myeloid leukemia (AML) [14]. It was withdrawn from the market 10 years later, due to excessive toxicity and lack of anti-tumor activity in a post-approval clinical trial with newly diagnosed AML [15]. Since then, several randomized studies have been completed which nevertheless demonstrate efficacy of this ADC for the same indication using a modified dosing regimen [16]. It may well be that gemtuzumab ozogamicin will soon find reapproval for this disease. However, this history impressively shows how narrow the therapeutic window in ADCs really can be, and that clinical reality can be very different from seemingly straightforward concepts.

The next approved candidate was brentuximab vedotin (Adcetris[®]), an anti-CD30-MMAE immunoconjugate, which obtained accelerated FDA approval for the treatment of Hodgkin's lymphoma and anaplastic large cell lymphoma in 2011 [17]. In 2013 finally, ado-trastuzumab emtansine (T-DM1/Kadcyla[®]), an ADC combining an anti-HER2-antibody with the maytansinoid DM1, obtained market approval for the treatment of HER2-positive metastatic breast cancer (Table 1) [18]. However, the recently reported failure of Kadcyla[®] to meet the primary endpoint of a phase III study in breast cancer patients (NCT01120184; BO22589) denotes an unpleasant setback in the field. A deeper understanding of the intrinsic properties of the distinct functional components is key to more successful ADC design in the future and to improve the perspectives of the whole ADC concept for cancer therapy.

3. Linker chemistries

One critical component of the ADC entity is the linker tethering the cytotoxin to the antibody. To achieve a maximal therapeutic window, it must be stable in the circulation, yet allow efficient drug release inside the cell upon receptor-mediated endocytosis. Therefore, it must exploit the differences between the extra- and intracellular environment, respect the properties of the target antigen for internalization and must be compatible with the chemistry of the payload. Linker design, hence, does not follow predefined guidelines and needs to be determined empirically for each ADC [2, 3].

Currently used linkers in ADCs are either cleavable or non-cleavable [1]. Payloads not tolerating substitutions without loss of activity require cleavable linkage which depends on intracellular processing such as acidic hydrolysis or enzymatic cleavage in the endo/lysosome,

leading to release and thus activation of the drug. This allows bystander killing of non-targeted tumor cells when hydrophobic drugs are used, which is particularly advantageous if antigens are heterogeneously expressed in tumors [19]. Acid-labile hydrazone linkers were among the first cleavable linkers used in ADCs. Upon internalization, the acidic pH in endosomes (pH 5.0-6.5) and lysosomes (pH 4.5-5.0) leads to hydrolysis of the hydrazone and subsequent release of the drug [2, 20]. The serum stability of hydrazone linkers, however, is poor [21, 22]. The low clinical efficacy and the safety concerns of gemtuzumab ozogamicin were mainly attributed to this linker instability and premature drug release in the circulation [5, 16, 23]. Cleavable disulfide linkers, in turn, exploit the high concentration of thiols, such as glutathione, in the cytoplasm, which reduce a disulfide bond in the linker to liberate the payload [1, 24]. To decrease reduction by reactive thiols in the serum, the stability of disulfides can be increased by introducing methyl groups flanking the disulfide bond to generate a sterically protected disulfide linker [25]. Although several disulfide-linked ADCs are currently under clinical investigation (Table 1), more stable cleavable linkers are engineered in the form of lysosomal protease substrates, e.g. containing a valine-citrulline dipeptide bond, which is specifically cleaved by cathepsin B [26-28].

Non-cleavable linkers, on the other hand, avoid premature drug release in the circulation by design [1]. Instead, the ADC must be proteolytically degraded in lysosomes and the payload is released as an active metabolite along with the amino acid it was conjugated to, i.e., usually a cysteine or lysine [19, 29]. Although the charge of a lysine-N^{ε}-coupled drug metabolite may hamper endo-lysosomal escape, it prevents externalization and bystander killing [29, 30]. This will, on the one hand, lead to enhanced specificity, but on the other hand, limit the action of the drug to the very cell that has taken up the ADC.

Furthermore, as the linker forms part of the active drug, it can be designed to add additional valuable functional properties to the drug, like prevention of MDR1 efflux and thus drug resistance, by making it more hydrophilic, e.g., in the form of a PEG4Mal linker [31].

4. Payloads

Natural products and synthetic chemistry provide a plethora of cytotoxins of which only few have been found suitable for use in ADCs [32, 33]. First, the drug's target must be crucial for cell viability to achieve highest cytotoxicity even at the low concentrations which can be realistically achieved upon internalization. Ideally, the number of drug molecules required to kill a cell are well below the number which is delivered by ADCs, that depends on

the DAR and the number of ADCs taken up by a cell in a clinical setting. Data using radiolabeled humanized antibodies suggests that localization in tumors does not exceed 0.05% of the injected dose per gram tissue [2, 34]. This, in turn, leads to the unavoidable conclusion that the vast majority of the ADC does not end up in tumor cells.

Drug activation depends on the linker chemistry and occurs after cleavage or by lysosomal degradation [1]. To show selectivity upon antigen-specific uptake, the payloads' target must be located inside the tumor cell. Many highly potent natural toxins act on the surface of cells by blocking ion channels, disturbing blood clotting etc. and are thus unsuitable, as cell specificity would not be achievable by a conjugated antibody. On top of that, the payload's molecular properties are important, too. It must be small in size to reduce immunogenicity, soluble under aqueous conjugation conditions to prevent aggregation, and compatible with the linker chemistry [35].

Consequently, as mentioned above, all ADCs approved or under late clinical investigation are payloaded with new generation drugs dominated by the DNA-strand breaking calicheamicins [25, 36, 37], duocarmycins [38], the tubulin-targeting maytansines [39, 40] and auristatins [17, 41]. The mechanisms of action and the chemistry of the coupling and release reactions of these warheads have been described in detail elsewhere [1, 42, 43]. Table 1 summarizes representative examples of clinically advanced ADCs using these highly potent drugs as payloads.

5. Conjugation chemistries

5.1. Site-selective conjugation and the drug to antibody ratio

Conjugation site-selectivity and stoichiometrically controlled conjugation of cytotoxic payloads to antibodies and other non-IgG binding proteins is crucial for tumor targeting. So far, all chemistries used for linker-drug conjugation to antibodies in late clinical trials have been based on solvent accessible cysteine and lysine residues through either thiol-maleimide coupling (e.g. brentuximab vedotin) [17] or amine NHS-ester coupling (e.g. ado-trastuzumab emtansine) [44], respectively (Table 1 and Table 2). As IgGs carry a variable number of lysine side-chains, this yields heterogeneous products with variable DARs and inter-batch variations. For instance, a typical modification of an IgG with an NHS-ester yields an average DAR of 0-8. Thus, the 40-86 lysine residues on the antibody surface are modified to a variable degree, resulting in a sample with potentially 4.5 million different molecules in terms of regioisomerism and drug load [45, 46]. If thiol-maleimide coupling is used instead, cysteine

conjugation occurs after mild reduction of the four inter-chain disulfide bonds in the hinge region of an IgG_1. Although conjugation is limited to these eight exposed sulfhydryl groups, as clinically used IgG_1 typically do not have Cys residues in the CDRs, and thus a higher coupling specificity compared to lysines can be achieved, it can still yield more than hundred different ADC species [46, 47], depending on the degree of loading and considering regioisomers. It must be noted that both DAR and conjugation sites are important for ADC function, as they influence the molecular properties through the exposed drug moiety. Whereas a drug load of 2 seems to provide the best pharmacological performance [47], the site of drug conjugation determines linkage stability and thus the ADC's tolerability and efficacy [9].

As a consequence of the limited site-selectivity of drug conjugation with conventional linkage strategies, even the clinically approved ADCs represent mixtures of regioisomers with different DARs, each of the molecular species showing a different cytotoxicity and safety profile [47, 48]. Unconjugated antibodies, which can be hardly separated from the preparations, block potential binding sites on the tumor surface and thus lower ADC efficacy, whereas drug conjugates with high DAR show increased off-target toxicity and are prone to aggregation, which narrows the therapeutic window [49]. This delineates that current ADC design is still far from optimal. To engineer ADCs for higher efficacy and tolerability, site-selective conjugation is mandatory to better control the number and attachment site of payloads.

Recently, Pillow et al. [48] reported a markedly improved homogeneity of trastuzumab-DM1 generated by engineering cysteine residues at defined sites in the antibody. Since the exposed cysteines are derivatized with cysteine or glutathione in the secretory pathway of the host cell, a reduction/oxidation process is required (THIOMAB technology), which reforms the hinge and C_L-C_H disulfides [50]. The better defined DAR of 2 to 4 and a novel linker technique for DM1 increased the efficacy and safety of the resulting ADC [48]. This must be seen in the context of recent findings that in terms of efficacy, less payload is more [47].

Another technique to site-specifically conjugate payloads to antibodies is the use of N-glycans for attachment. Major limitations of this technique, however, are the difficulty to obtain homogeneous glycan structures and to avoid unwanted oxidations of the proteins resulting from chemical modifications. Furthermore, the glycan structure is important for desired antibody functions like ADCC and, when modified, can generate new highly immunogenic epitopes [8].

5.2 Bioorthogonal conjugation

In view of the many downsides associated with uncontrolled payloading, engineering a unique, orthogonal functional group into proteins, which then can be coupled to a specific reaction partner in a bioorthogonal manner, is appealing also for antibody drug conjugation. In this constellation, two components, which are fully complementary and, additionally, orthogonal to nature's functional groups on protein (thiols, alcohols or amines), react to form a chemical bond. These components must be stable and small, whereas the reaction must be fast, and yet conditions must be so mild that the reaction can be conducted with proteins [51]. The concept of orthogonal chemistry aims to avoid unspecific side-reactions and allows stoichiometrically defined modifications. Here, we briefly discuss the various biorthogonal conjugation chemistries with potential for ADC generation. The use of enzymes, either for the conjugation reaction itself or the incorporation of unique functional groups into proteins, also represents a valid concept for drug conjugation, the benefits and drawbacks of which are described elsewhere [8, 11, 51].

The first truly bioorthogonal reaction for protein modification was reported in 2000 using the Staudinger ligation. Derived from the original Staudinger reaction, a reaction between a phosphine and an azide leading to an amine by subsequent hydrolysis, was modified to form a stable amide bond linkage [52], by installing an electrophilic trap at the phosphine component. A significant drawback of this ligation reaction is the oxygen sensitivity of the phosphine reagents and the slow reaction rate constant, and thus it is not well suited for conjugation of drugs to protein and not used nowadays.

5.3 Click chemistries for bioconjugation

Another approach to biorthogonal protein modification exploits the 1, 3-diploar cycloaddition for linking biological components. In this Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) an azide reacts with a terminal alkyne to form a stable triazole (Table 2) [53, 54]. The higher reaction rate and the stability of the reactants has made this reaction appealing and widely used for bioconjugation [55]. Its dependence on cytotoxic copper, however, limits the use of such products in biological systems. A copper-free alternative is the strain-promoted azide-alkyne cycloaddition (SPAAC), also denoted click chemistry, which uses ring-strained cyclooctynes instead of terminal alkynes (Table 2) [56]. To improve the moderate reaction rates, various derivatives such as fluorinated [57], benzoannulated [58, 59] and cyclopropanated [60] cyclooctynes, have been synthesized. Noteworthy are the cyclopropanated variant bicyclo[6.1.0]nonyne (BCN) and the benzoannulated derivative dibenzocyclooctyne

(DBCO), also denoted as aza-dibenzocyclooctyne (DIBAC). Both reactants improve reaction rates 100-fold over cyclooctyne. Altogether, DBCO was found to best combine high stability and efficiency with high hydrophilicity and fast reaction kinetics for the classical SPAAC reaction.

For protein-drug conjugation, it would be most appealing if the site of drug conjugation in the protein can be freely chosen. This is the case with non-natural amino acids containing azides as they can replace methionines. The aminoacyl tRNA synthase for methionine in *E. coli* accepts the methionine-surrogate azidohomoalanine (Aha), and thus in methionine-auxotrophic *E. coli* strains, after simple Met depletion of the culture, Aha can be introduced without any other changes in the *E. coli* strain [61]. However, this requires that the protein can be produced in *E. coli* and thus this strategy favors alternative non-IgG scaffolds.

Nevertheless, despite the use of rate-optimized cyclooctynes like DBCO and BCN, the described reactions are comparably slow (0.1 - 0.3 $M^{-1}s^{-1}$), and unspecific coupling to irrelevant reaction partners present on cell surfaces cannot be excluded and limit the orthogonality of the reaction [59, 60, 62]. On the other hand, as bioconjugation partners of ADCs are usually very pure, such side reactions are unlikely to occur and the stability of the components used for cycloaddition remains as an advantage.

The rate constant of SPAAC with BCN can be highly accelerated using electron-poor, aromatic azide components instead of Aha [63]. In another study, Borrmann et al. [64] reported even more improved rates of cycloaddition by several orders of magnitude using quinones instead of azides as reaction partner for BCN. Moreover, this reaction can be temporally controlled by oxidation of inert catechols to the reactive quinone. To make these reactions applicable to proteins, they must first be charged with aromatic azides, BCN or quinones in a stoichiometrically defined, site-specific manner. This no longer possible with a simple *met⁻* strain of *E. coli*, but requires amber-suppression technology for site-specific incorporation of BCN-lysine or other non-natural amino acids carrying functional groups like quinones or norbonenes [65-72]. So far, this technology was only used for imaging agents, as it shows unfavorably low expression yields [66].

Also in our hands, amber suppression protein variants showed comparatively low expression and also inhomogeneity, since the efficiency of suppression varies with the amber site. This requires engineering for purification and thus limits the freedom of molecular design (unpublished data). Although this may in principle be overcome using sequence optimization [73] and adapted *E. coli* strains [74], the problem remains that, compared to azide-

containing non-natural amino acids like Aha, non-natural amino acids like BCN-lysine are rather expensive, and, to our knowledge, bacterial strains and vectors carrying tRNA/RS pairs for the incorporation of specific non-natural amino acids have not been made commercially available.

Using the amber suppression technology to site specifically incorporate *para*-acetylphenylalanine (*p*AF) in antibodies and to conjugate the drug payload via oxime ligation (Table 2) [10, 75, 76], new biologicals could be generated. This technology, when applied to trastuzumab emtansine, yields a defined DAR of 2, and the authors believe that the antitumor activity and stability is increased compared to drug conjugates produced with the THIOMAB method. A possible explanation might be that a retro-Michael addition, leading to the transfer of drugs conjugated to cysteines to free thiols in blood serum components, may have partially inactivated the ADC [76-78]. However, the authors do not provide convincing data showing that their own preparation of the THIOMAB antibody had a DAR of 2, as it was published by Junutula *et al.* [50]. Furthermore the *p*AF incorporation still suffers from low expression yields, which can only be overcome by extensive cell line engineering, and the oxime ligation exhibits low reaction rates, leading to reaction times up to 48 h and is performed at pH 4-4.5, which is not tolerated by all proteins.

5.4 The inverse-electron-demand Diels-Alder reaction for bioconjugation

The highest rate constants of bioconjugation can be achieved by using the inverse-electron-demand Diels-Alder reaction of tetrazines and strained alkenes for bioconjugation [79]. As it has been reported only recently, this results from a decreased closed-shell electron-electron repulsion between the reaction partners [80]. Nothing beats rate constants of up to 10^6 $M^{-1}s^{-1}$ of *s*-tetrazines with trans-cyclooctene (TCO)-derivatives (Table 2). Since TCO and tetrazines can be incorporated in proteins using amber-suppression, numerous applications for imaging purposes were reported [72, 81, 82]. A downside lies in the diminished stability of the most reactive tetrazines in physiological buffers, and, as mentioned above, broad use of the amber-suppression-technique is still limited by the high costs of the reagents, low expression yields and restrictions in where the modification site can be introduced. Nonetheless, if these obstacles can be overcome, the value of this protein payloading technique for ADC generation would be immense.

6. Binding proteins – antibodies and alternative non-IgG formats

The key function of the antibody in ADCs is binding to its target antigen, which is ideally expressed on the surface of tumor cells in high copy numbers and absent or at least low in normal vital tissues. Upon binding, the attached payload is delivered into the cell by receptor-mediated endocytosis, detached from the antibody by a variety of mechanisms, and transferred to the cytosol, where it acts on its target. An intrinsic property of the full-IgG format is its binding to Fc receptors on immune effector cells, which may further boost the ADC's cell killing activity. The clinical benefit of this additional effector function, however, remains to be demonstrated [49]. In addition, binding to the FcRn receptor increases the circulation half-life and, hence, tumor localization. However, the comparison of a diabody-ADC with an IgG-ADC with similar DARs showed only a 3-fold decreased antitumor activity of the diabody-ADC, despite a 30-times lower level of exposure [83]. The improved tumor penetration of smaller conjugates may at least partly compensate the shorter serum half-life and thus help explain this finding [49].

From these data it can be concluded that, by engineering half-life, size, avidity or other properties of different binding protein formats over a wide range, which is not possible with classical IgGs, the best combination of high activity in the relevant (tumor) tissue and low off-target toxicity can be found. Another important advantage of smaller protein scaffolds, especially when they can be produced in *E. coli*, lies in the broader options for well-defined drug conjugation, which may be a downside of the classical IgG format.

Fab- and scFv-ADCs can be engineered for drug conjugation, and indeed, with such size-minimized IgG binding proteins, DARs of 1 have been achieved and several IgG-fragments are currently under preclinical evaluation [49]. Axup *et al.* [84] recently generated a highly potent Fab-auristatin using amber suppression for the incorporation of *p*AF and oxime ligation for drug conjugation. Novel biorthogonal chemistries like click chemistry or the inverse-electron-demand Diels-Alder reaction, however, have not yet been applied to antibody fragments. Hence, non-IgG derived binding proteins allowing the application of new bioorthogonal chemistries for fast and site-specific drug conjugation with high yields and permitting the screening of a wide range of pharmacological and molecular properties, are desired.

As an alternative to antibodies and their fragments, various non-IgG binding proteins with improved biophysical properties have been generated for tumor targeting. Their robust nature allows expression in large quantities, they can be engineered to various formats, from

mono-, bi- to tetravalent, and with a range of half-life extension features, and they can better tolerate the introduction of reactive groups for site-specific and bioorthogonal conjugation [12, 85]. These properties introduced in the design also make these formats compatible with the novel bioorthogonal conjugation chemistries described above. Despite this advantage for bioconjugate engineering, so far only DARPins have been used for drug conjugation [86]. The potential of bioorthogonally assembled peptide-drug conjugates for tumor targeting is described elsewhere [87].

DARPins have been introduced recently as a new class of binding proteins which are superior to antibodies for various biomedical applications [13]. Besides showing very high expression yields in *E. coli*, DARPins are much more stable than antibodies and have favorable biophysical properties which increase their freedom of engineering as they e.g. carry no cysteine and no essential methionine [88]. Moreover, DARPins of very high affinity can be readily generated using well-established selection techniques such as ribosome display and phage display as previously demonstrated [13]. We recently described the successful generation and preclinical testing of DARPins selected for high stability and affinity, directed against well-internalizing cell surface antigens on solid tumors, which are available for tailor-made drug conjugation [89-91].

DARPins provide a valuable platform also for manufacturing bioconjugates with various chemistries. First, the lack of native cysteines enables site-directed introduction of these residues, e.g. at the C-terminus for specific maleimide-based conjugation of effector functions, including the cytotoxic payloads known from classical ADCs (Figure 1). Of particular interest for drug conjugation of DARPins is also the lack of conserved methionines in the protein scaffold. This allows for the facile incorporation of Aha and the expression of azide-DARPins using methionine-depleted bacterial cultures [61, 85], which can be done in very high yield and at large scales. The only Met present in the original design, besides the initiator codon, is in the N-capping repeat, which can be exchanged to leucine without affecting the biophysical properties of the protein and is already replaced in some DARPins through the random mutagenesis in ribosome display [85]. Additionally, DARPins are usually expressed with an N-terminal MRGSH$_6$ tag, from which the methionine is not cleaved [92], and this is also true when an amino acid analog is introduced [93]. Conversely, if the second amino acid is small (e.g., Ala, Gly or Ser), the analog will be cleaved off, allowing the introduction of a unique methionine surrogate such as Aha at desired positions elsewhere in the chain (Figure 1) [85]. The possibility to engineer dual functionality (azide and thiol) into DARPins enables the

design of tailor-made drug conjugates with desired pharmacological properties, e.g. by site-specific payloading with a cytotoxin like MMAF and additional coupling of a half-life extension module like serum albumin [86]. Such a conjugate demonstrated nanomolar potency *in vitro* and a hundred-fold increased serum half-life. This is the first evidence that alternative binding proteins like DARPins are very compatible with the advanced bioorthogonal conjugation chemistries and can be engineered further to optimize the pharmacological properties of the targeting molecules. Importantly, not only the half-life can be engineered but also bispecific binding proteins can be easily designed from such scaffolds, allowing the optimization of drug conjugate performance for tumor targeting [94].

7. Conclusion and perspective

ADCs combine the specificity of antibodies for tumor-associated antigens to discriminate between normal and malignant cells, and the cytotoxic effect of novel drug agents as payload. The choice of the linker design is crucial for potency and tolerability as it determines stability of the ADC and the site where the drug is released and activated. Despite intensive research in these fields and a growing number of candidates in clinical trials, so far only two ADCs have been granted approval for oncology indications, indicating that there is still ample space for improvements. Clearly, in the parameter space between choice of target, optimal half-life, maximal target-tissue toxicity and minimal systemic toxicity, optima are not so easily found, as all parameters are mutually dependent on each other. Nonetheless, for every molecule, defined conjugation and regiochemistry are important components. For whole antibodies, engineered cysteine sites (THIOMAB technology) and the incorporation of *p*AF with amber suppression technology are two methodologies addressing this problem, each with its own strengths and weaknesses.

For site-specific, stoichiometrically defined and fast drug conjugation, proteins can now be engineered to contain non-native functional groups to perform novel bioorthogonal reactions. Such alternative binding proteins, e.g. DARPins, are very compatible with the novel biorthogonal conjugation chemistries for payloading and can be engineered for variable half-life, avidity and bispecificity. They hold promise to establish a new generation of functionally improved drug conjugates for cancer therapy as a broad range of desired pharmacological and biophysical functions can be readily introduced into these binding proteins.
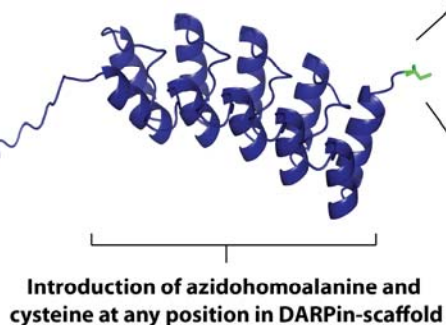
Acknowledgements

Abbreviations

ADC: antibody-drug conjugate; Aha: azidohomoalanine; BCN: bicyclo[6.1.0]nonyne; DAR: drug-antibody ratio; DARPin: Designed Ankyrin Repeat Protein; DBCO: dibenzocyclooctyne; MMAE: monomethyl auristatin E; NHS: N-hydroxysuccinimide; *p*AF: para-acetylphenylalanine; SPAAC: strain-promoted azide-alkyne cycloaddition; TCO: trans-cyclooctene
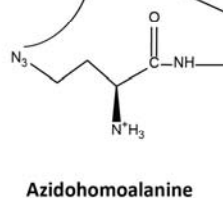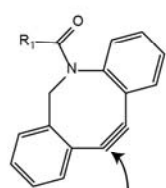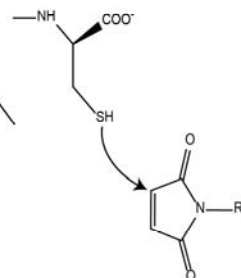
Figure 1: Double functionalized DARPin (blue) for bioconjugation of effector modules (R1 and R2) at defined positions in the DARPin scaffold.

A unique azidohomoalanine (pink) and cysteine (green) can be introduced at any position for bioconjugation by click or thiol chemistry, respectively, for producing stoichiometrically defined DARPin-cytotoxin bioconjugates. The position of azidohomoalanine and cysteine can be moved to any desired position in the DARPin scaffold, and they are shown at the termini merely for clarity. Figure adapted from [85].
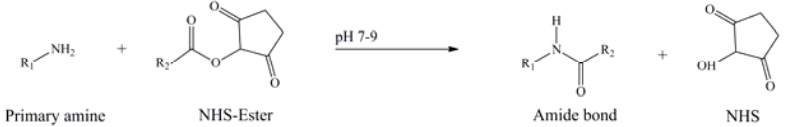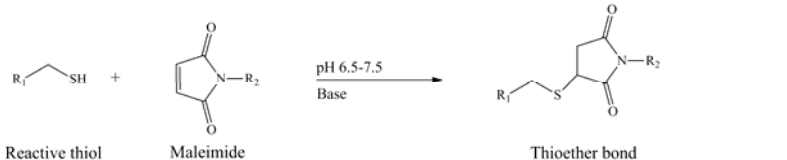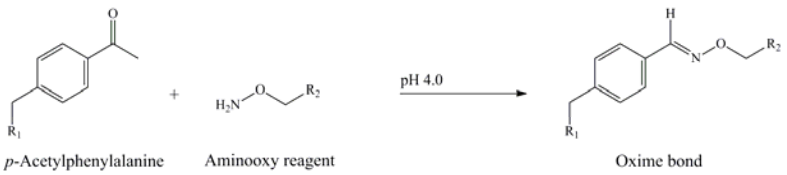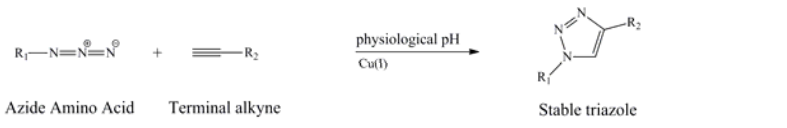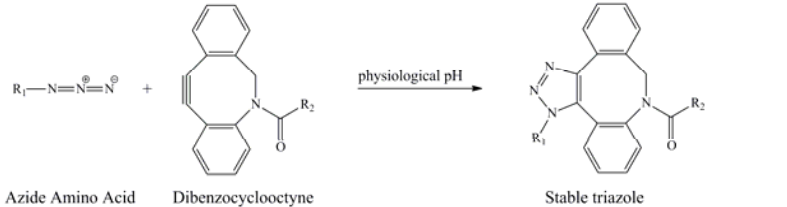
ADC	Drug	Linker	Coupling reaction	Antigen	Lead indication
Approved					
Brentuximab vedotin (Adcetris®)	MMAE	Valine-citrulline	Cys + maleimide	CD30	HL, ALCL
Ado-trastuzumab emantsine (Kadcyla®)	DM1	Thioether	Lys + NHS (SMCC)	Her2	Breast cancer
Phase III					
Gemtuzumab ozogamicin	Calicheamicin	Hydrazone	Lys + AcBut	CD33	AML
Inotuzumab ozogamicin	Calicheamicin	Hydrazone	Lys + AcBut	CD22	ALL, NHL
Brentuximab vedotin (Adcetris®)	MMAE	Valine-citrulline	Cys + maleimide	CD30	HL, T-cell lymphoma
Ado-trastuzumab emantsine (Kadcyla®)	DM1	Thioether	Lys + NHS (SMCC)	Her2	Gastric cancer, Breast cancer
Phase II					
Glembatumumab vedotin	MMAE	Valine-citrulline	Cys + maleimide	gpNMB	Breast cancer, Melanoma
Pinatuzumab vedotin	MMAE	Valine-citrulline	Cys + maleimide	CD22	NHL
Polatuzumab vedotin	MMAE	Valine-citrulline	Cys + maleimide	CD79b	B-cell lymphoma
SAR3419	DM4	Disulfide	Lys+ NHS (SPDP)	CD19	B-cell lymphoma
Lorvotuzumab mertansine	DM1	Disulfide	Lys + NHS (SPP)	CD56	[a]SCLC

Table 1: ADCs FDA-approved or currently in advanced clinical trials (www.clinicaltrials.gov).
AcBut, 4-(4'-acetylphenoxy) butanoic acid; ALCL, Anaplastic Large Cell Lymphoma; ALL, Acute Lymphoblastic Leukemia; AML, Acute Myelogenous Leukemia; HL, Hodgkin's Lymphoma; NHL, Non-Hodgkin Lymphoma; NHS, N-hydroxysuccinimide; SCLC, Small Cell Lung Cancer; SMCC, succinimidyl-4-(N-maleimidomethly)cyclohexane-1-carboxylate; SPDP, succinimidyl 3-(2-pyridyldithio)propionate; SPP, N-succinimidyl 4-(2-pyridyldithio)pentanoate
[a]Halted

Reaction Chemistry	Reaction scheme	Reaction Rate	Conjugation Site in Protein	Incorporation Method
Amine-NHS		fast	Lysine, terminal amine	Natural
Thiol-Maleimide Michael Addition		fast	Cysteine	Natural
Oxime Ligation		slow	p-Acetylphenylalanine	Amber suppression
Cycloaddition CuAAC		slow	Azidohomoalanine	Methionine - auxotrophic E. coli
Cycloaddition SPAAC		slow	Azidohomoalanine	Methionine - auxotrophic E. coli

Inverse-electron-demand Diels-Alder reaction			very fast	trans-Cyclooctene-Lysine	Amber suppression
	trans-Cyclooctene	+ Tetrazine reagent → (physiological pH, -N₂) Subsituted cyclohexene			
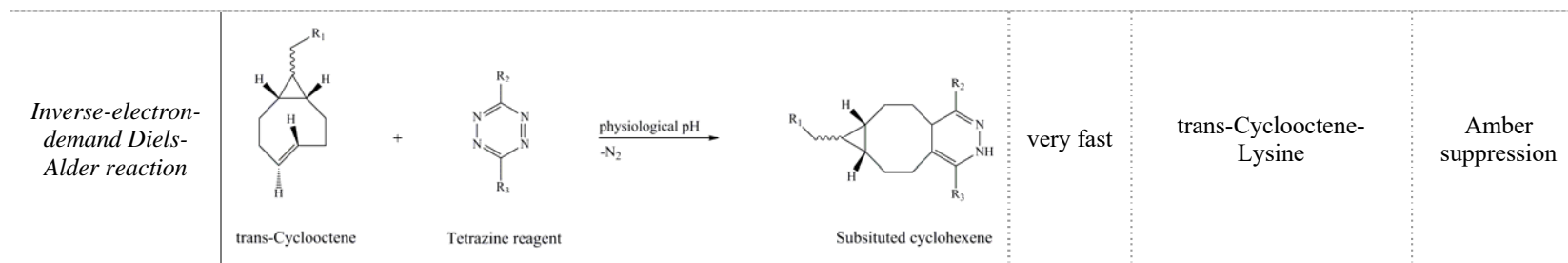
Table 2: Conjugation Chemistries for ADCs.

R_1: protein, e.g., antibody, R_2: drug or linker. Please note that many reactions can be performed with reverse location of functional groups e.g. tetrazine-charged proteins. Here, we list the most common techniques. For reaction details see [75, 95].

References

(1)   Ducry, L., and Stump, B. (2010) Antibody-drug conjugates: linking cytotoxic payloads to monoclonal antibodies. *Bioconjug. Chem. 21*, 5-13.

(2)   Chari, R. V., Miller, M. L., and Widdison, W. C. (2014) Antibody-drug conjugates: an emerging concept in cancer therapy. *Angew. Chem. Int. Ed. Engl. 53*, 3796-3827.

(3)   Carter, P. J., and Senter, P. D. (2008) Antibody-drug conjugates for cancer therapy. *Cancer J. 14*, 154-169.

(4)   Mittendorf, E. A., Wu, Y., Scaltriti, M., Meric-Bernstam, F., Hunt, K. K., Dawood, S., Esteva, F. J., Buzdar, A. U., Chen, H., Eksambi, S., Hortobagyi, G. N., Baselga, J., and Gonzalez-Angulo, A. M. (2009) Loss of HER2 amplification following trastuzumab-based neoadjuvant systemic therapy and survival outcomes. *Clin. Cancer Res. 15*, 7381-7388.

(5)   Trail, P. A., Willner, D., Lasch, S. J., Henderson, A. J., Hofstead, S., Casazza, A. M., Firestone, R. A., Hellstrom, I., and Hellstrom, K. E. (1993) Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. *Science 261*, 212-215.

(6)   Tolcher, A. W., Sugarman, S., Gelmon, K. A., Cohen, R., Saleh, M., Isaacs, C., Young, L., Healey, D., Onetto, N., and Slichenmyer, W. (1999) Randomized phase II study of BR96-doxorubicin conjugate in patients with metastatic breast cancer. *J. Clin. Oncol. 17*, 478-484.

(7)   Ajani, J. A., Kelsen, D. P., Haller, D., Hargraves, K., and Healey, D. (2000) A multi-institutional phase II study of BMS-182248-01 (BR96-doxorubicin conjugate) administered every 21 days in patients with advanced gastric adenocarcinoma. *Cancer J. 6*, 78-81.

(8)   Agarwal, P., and Bertozzi, C. R. (2015) Site-specific antibody-drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. *Bioconjug. Chem. 26*, 176-192.

(9)   Shen, B. Q., Xu, K., Liu, L., Raab, H., Bhakta, S., Kenrick, M., Parsons-Reponte, K. L., Tien, J., Yu, S. F., Mai, E., Li, D., Tibbitts, J., Baudys, J., Saad, O. M., Scales, S. J., McDonald, P. J., Hass, P. E., Eigenbrot, C., Nguyen, T., Solis, W. A., Fuji, R. N., Flagella, K. M., Patel, D., Spencer, S. D., Khawli, L. A., Ebens, A., Wong, W. L., Vandlen, R., Kaur, S., Sliwkowski, M. X., Scheller, R. H., Polakis, P., and Junutula, J. R. (2012) Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat. Biotechnol. 30*, 184-189.

(10)   Tian, F., Lu, Y., Manibusan, A., Sellers, A., Tran, H., Sun, Y., Phuong, T., Barnett, R., Hehli, B., Song, F., DeGuzman, M. J., Ensari, S., Pinkstaff, J. K., Sullivan, L. M., Biroc, S. L., Cho, H., Schultz, P. G., DiJoseph, J., Dougher, M., Ma, D., Dushin, R., Leal, M., Tchistiakova, L., Feyfant, E., Gerber, H. P., and Sapra, P. (2014) A general approach to site-specific antibody drug conjugates. *Proc. Natl. Acad. Sci. U. S. A. 111*, 1766-1771.

(11)   Rashidian, M., Dozier, J. K., and Distefano, M. D. (2013) Enzymatic labeling of proteins: techniques and approaches. *Bioconjug. Chem. 24*, 1277-1294.

(12)   Weidle, U. H., Auer, J., Brinkmann, U., Georges, G., and Tiefenthaler, G. (2013) The emerging role of new protein scaffold-based agents for treatment of cancer. *Cancer Genomics Proteomics 10*, 155-168.

(13)   Plückthun, A. (2015) Designed ankyrin repeat proteins (DARPins): binding proteins for research, diagnostics, and therapy. *Annu. Rev. Pharmacol. Toxicol. 55*, 489-511.

(14) Bross, P. F., Beitz, J., Chen, G., Chen, X. H., Duffy, E., Kieffer, L., Roy, S., Sridhara, R., Rahman, A., Williams, G., and Pazdur, R. (2001) Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clin. Cancer Res. 7*, 1490-1496.

(15) Petersdorf, S. H., Kopecky, K. J., Slovak, M., Willman, C., Nevill, T., Brandwein, J., Larson, R. A., Erba, H. P., Stiff, P. J., Stuart, R. K., Walter, R. B., Tallman, M. S., Stenke, L., and Appelbaum, F. R. (2013) A phase 3 study of gemtuzumab ozogamicin during induction and postconsolidation therapy in younger patients with acute myeloid leukemia. *Blood 121*, 4854-4860.

(16) Rowe, J. M., and Lowenberg, B. (2013) Gemtuzumab ozogamicin in acute myeloid leukemia: a remarkable saga about an active drug. *Blood 121*, 4838-4841.

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

(18) Lambert, J. M., and Chari, R. V. (2014) Ado-trastuzumab Emtansine (T-DM1): an antibody-drug conjugate (ADC) for HER2-positive breast cancer. *J. Med. Chem. 57*, 6949-6964.

(19) Kovtun, Y. V., Audette, C. A., Ye, Y., Xie, H., Ruberti, M. F., Phinney, S. J., Leece, B. A., Chittenden, T., Blattler, W. A., and Goldmacher, V. S. (2006) Antibody-drug conjugates designed to eradicate tumors with homogeneous and heterogeneous expression of the target antigen. *Cancer Res. 66*, 3214-3221.

(20) Doronina, S. O., Toki, B. E., Torgov, M. Y., Mendelsohn, B. A., Cerveny, C. G., Chace, D. F., DeBlanc, R. L., Gearing, R. P., Bovee, T. D., Siegall, C. B., Francisco, J. A., Wahl, A. F., Meyer, D. L., and Senter, P. D. (2003) Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol. 21*, 778-784.

(21) Hamann, P. R., Hinman, L. M., Hollander, I., Beyer, C. F., Lindh, D., Holcomb, R., Hallett, W., Tsou, H. R., Upeslacis, J., Shochat, D., Mountain, A., Flowers, D. A., and Bernstein, I. (2002) Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug. Chem. 13*, 47-58.

(22) Sanderson, R. J., Hering, M. A., James, S. F., Sun, M. M., Doronina, S. O., Siadak, A. W., Senter, P. D., and Wahl, A. F. (2005) In vivo drug-linker stability of an anti-CD30 dipeptide-linked auristatin immunoconjugate. *Clin. Cancer Res. 11*, 843-852.

(23) van Der Velden, V. H., te Marvelde, J. G., Hoogeveen, P. G., Bernstein, I. D., Houtsmuller, A. B., Berger, M. S., and van Dongen, J. J. (2001) Targeting of the CD33-calicheamicin immunoconjugate Mylotarg (CMA-676) in acute myeloid leukemia: in vivo and in vitro saturation and internalization by leukemic and normal myeloid cells. *Blood 97*, 3197-3204.

(24) Saito, G., Swanson, J. A., and Lee, K. D. (2003) Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities. *Adv Drug Deliv Rev 55*, 199-215.

(25) Kellogg, B. A., Garrett, L., Kovtun, Y., Lai, K. C., Leece, B., Miller, M., Payne, G., Steeves, R., Whiteman, K. R., Widdison, W., Xie, H., Singh, R., Chari, R. V., Lambert, J. M., and Lutz, R. J. (2011) Disulfide-linked antibody-maytansinoid conjugates: optimization of in vivo activity by varying the steric hindrance at carbon atoms adjacent to the disulfide linkage. *Bioconjug. Chem. 22*, 717-727.

(26) Dubowchik, G. M., and Firestone, R. A. (1998) Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin. *Bioorg. Med. Chem. Lett. 8*, 3341-3346.

(27) Dubowchik, G. M., Mosure, K., Knipe, J. O., and Firestone, R. A. (1998) Cathepsin B-sensitive dipeptide prodrugs. 2. Models of anticancer drugs paclitaxel (Taxol), mitomycin C and doxorubicin. *Bioorg. Med. Chem. Lett. 8*, 3347-3352.

(28) Doronina, S. O., Bovee, T. D., Meyer, D. W., Miyamoto, J. B., Anderson, M. E., Morris-Tilden, C. A., and Senter, P. D. (2008) Novel peptide linkers for highly potent antibody-auristatin conjugate. *Bioconjug. Chem. 19*, 1960-1963.

(29) Erickson, H. K., Widdison, W. C., Mayo, M. F., Whiteman, K., Audette, C., Wilhelm, S. D., and Singh, R. (2010) Tumor delivery and in vivo processing of disulfide-linked and thioether-linked antibody-maytansinoid conjugates. *Bioconjug. Chem. 21*, 84-92.

(30) Erickson, H. K., Park, P. U., Widdison, W. C., Kovtun, Y. V., Garrett, L. M., Hoffman, K., Lutz, R. J., Goldmacher, V. S., and Blattler, W. A. (2006) Antibody-maytansinoid conjugates are activated in targeted cancer cells by lysosomal degradation and linker-dependent intracellular processing. *Cancer Res. 66*, 4426-4433.

(31) Kovtun, Y. V., Audette, C. A., Mayo, M. F., Jones, G. E., Doherty, H., Maloney, E. K., Erickson, H. K., Sun, X., Wilhelm, S., Ab, O., Lai, K. C., Widdison, W. C., Kellogg, B., Johnson, H., Pinkas, J., Lutz, R. J., Singh, R., Goldmacher, V. S., and Chari, R. V. (2010) Antibody-maytansinoid conjugates designed to bypass multidrug resistance. *Cancer Res. 70*, 2528-2537.

(32) Mann, J. (2002) Natural products in cancer chemotherapy: past, present and future. *Nat. Rev. Cancer 2*, 143-148.

(33) Cragg, G. M., Grothaus, P. G., and Newman, D. J. (2009) Impact of natural products on developing new anti-cancer agents. *Chem. Rev. 109*, 3012-3043.

(34) O'Donoghue, J. A., Smith-Jones, P. M., Humm, J. L., Ruan, S., Pryma, D. A., Jungbluth, A. A., Divgi, C. R., Carrasquillo, J. A., Pandit-Taskar, N., Fong, Y., Strong, V. E., Kemeny, N. E., Old, L. J., and Larson, S. M. (2011) 124I-huA33 antibody uptake is driven by A33 antigen concentration in tissues from colorectal cancer patients imaged by immuno-PET. *J. Nucl. Med. 52*, 1878-1885.

(35) Singh, R., and Erickson, H. K. (2009) Antibody-cytotoxic agent conjugates: preparation and characterization. *Methods Mol. Biol. 525*, 445-467.

(36) Kantarjian, H., Thomas, D., Jorgensen, J., Kebriaei, P., Jabbour, E., Rytting, M., York, S., Ravandi, F., Garris, R., Kwari, M., Faderl, S., Cortes, J., Champlin, R., and O'Brien, S. (2013) Results of inotuzumab ozogamicin, a CD22 monoclonal antibody, in refractory and relapsed acute lymphocytic leukemia. *Cancer 119*, 2728-2736.

(37) Ogura, M., Hatake, K., Ando, K., Tobinai, K., Tokushige, K., Ono, C., Ishibashi, T., and Vandendries, E. (2012) Phase I study of anti-CD22 immunoconjugate inotuzumab ozogamicin plus rituximab in relapsed/refractory B-cell non-Hodgkin lymphoma. *Cancer Sci. 103*, 933-938.

(38) Thevanayagam, L., Bell, A., Chakraborty, I., Sufi, B., Gangwar, S., Zang, A., Rangan, V., Rao, C., Wang, Z., Pan, C., Chong, C., Cardarelli, P., Deshpande, S., and Srinivasan, M. (2013) Novel detection of DNA-alkylated adducts of antibody-drug conjugates with potentially unique preclinical and biomarker applications. *Bioanalysis 5*, 1073-1081.

(39) LoRusso, P. M., Weiss, D., Guardino, E., Girish, S., and Sliwkowski, M. X. (2011) Trastuzumab emtansine: a unique antibody-drug conjugate in development for human epidermal growth factor receptor 2-positive cancer. *Clin. Cancer Res. 17*, 6437-6447.

(40) Ribrag, V., Dupuis, J., Tilly, H., Morschhauser, F., Laine, F., Houot, R., Haioun, C., Copie, C., Varga, A., Lambert, J., Hatteville, L., Ziti-Ljajic, S., Caron, A., Payrard, S., and Coiffier, B. (2014) A dose-escalation study of SAR3419, an anti-CD19 antibody maytansinoid conjugate, administered by intravenous infusion once weekly in patients with relapsed/refractory B-cell non-Hodgkin lymphoma. *Clin. Cancer Res. 20*, 213-220.

(41) Bendell, J., Saleh, M., Rose, A. A., Siegel, P. M., Hart, L., Sirpal, S., Jones, S., Green, J., Crowley, E., Simantov, R., Keler, T., Davis, T., and Vahdat, L. (2014) Phase I/II study of the antibody-drug conjugate glembatumumab vedotin in patients with locally advanced or metastatic breast cancer. *J. Clin. Oncol. 32*, 3619-3625.

(42) Ricart, A. D., and Tolcher, A. W. (2007) Technology insight: cytotoxic drug immunoconjugates for cancer therapy. *Nat. Clin. Pract. Oncol. 4*, 245-255.

(43) Iyer, U., and Kadambi, V. J. (2011) Antibody drug conjugates - Trojan horses in the war on cancer. *J. Pharmacol. Toxicol. Methods 64*, 207-212.

(44) Burris, H. A., 3rd, Tibbitts, J., Holden, S. N., Sliwkowski, M. X., and Lewis Phillips, G. D. (2011) Trastuzumab emtansine (T-DM1): a novel agent for targeting HER2+ breast cancer. *Clin. Breast Cancer 11*, 275-282.

(45) Wang, L., Amphlett, G., Blattler, W. A., Lambert, J. M., and Zhang, W. (2005) Structural characterization of the maytansinoid-monoclonal antibody immunoconjugate, huN901-DM1, by mass spectrometry. *Protein Sci. 14*, 2436-2446.

(46) Panowksi, S., Bhakta, S., Raab, H., Polakis, P., and Junutula, J. R. (2014) Site-specific antibody drug conjugates for cancer therapy. *mAbs 6*, 34-45.

(47) Hamblett, K. J., Senter, P. D., Chace, D. F., Sun, M. M., Lenox, J., Cerveny, C. G., Kissler, K. M., Bernhardt, S. X., Kopcha, A. K., Zabinski, R. F., Meyer, D. L., and Francisco, J. A. (2004) Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin. Cancer Res. 10*, 7063-7070.

(48) Pillow, T. H., Tien, J., Parsons-Reponte, K. L., Bhakta, S., Li, H., Staben, L. R., Li, G., Chuh, J., Fourie-O'Donohue, A., Darwish, M., Yip, V., Liu, L., Leipold, D. D., Su, D., Wu, E., Spencer, S. D., Shen, B. Q., Xu, K., Kozak, K. R., Raab, H., Vandlen, R., Lewis Phillips, G. D., Scheller, R. H., Polakis, P., Sliwkowski, M. X., Flygare, J. A., and Junutula, J. R. (2014) Site-specific trastuzumab maytansinoid antibody-drug conjugates with improved therapeutic activity through linker and antibody engineering. *J. Med. Chem. 57*, 7890-7899.

(49) Deonarain, M. P., Yahioglu, G., Stamati, I., and Marklew, J. (2015) Emerging formats for next-generation antibody drug conjugates. *Expert Opin. Drug Discov. 10*, 1-19.

(50) Junutula, J. R., Raab, H., Clark, S., Bhakta, S., Leipold, D. D., Weir, S., Chen, Y., Simpson, M., Tsai, S. P., Dennis, M. S., Lu, Y., Meng, Y. G., Ng, C., Yang, J., Lee, C. C., Duenas, E., Gorrell, J., Katta, V., Kim, A., McDorman, K., Flagella, K., Venook, R., Ross, S., Spencer, S. D., Lee Wong, W., Lowman, H. B., Vandlen, R., Sliwkowski, M. X., Scheller, R. H., Polakis, P., and Mallet, W. (2008) Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat. Biotechnol. 26*, 925-932.

(51) van Berkel, S. S., and van Delft, F. L. (2013) Metal-free bioconjugation reactions. *Drug Discov. Today Technol. 10*, 45-51.

(52) Saxon, E., and Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction. *Science 287*, 2007-2010.

(53)   Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem. Int. Ed. Engl. 41*, 2596-2599.

(54)   Tornoe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057-3064.

(55)   Meldal, M., and Tornoe, C. W. (2008) Cu-catalyzed azide-alkyne cycloaddition. *Chem. Rev. 108*, 2952-3015.

(56)   Agard, N. J., Prescher, J. A., and Bertozzi, C. R. (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc. 126*, 15046-15047.

(57)   Baskin, J. M., Prescher, J. A., Laughlin, S. T., Agard, N. J., Chang, P. V., Miller, I. A., Lo, A., Codelli, J. A., and Bertozzi, C. R. (2007) Copper-free click chemistry for dynamic in vivo imaging. *Proc. Natl. Acad. Sci. U. S. A. 104*, 16793-16797.

(58)   Ning, X., Guo, J., Wolfert, M. A., and Boons, G. J. (2008) Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast huisgen cycloadditions. *Angew. Chem. Int. Ed. Engl. 47*, 2253-2255.

(59)   Debets, M. F., van Berkel, S. S., Schoffelen, S., Rutjes, F. P., van Hest, J. C., and van Delft, F. L. (2010) Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3+2) cycloaddition. *Chem. Commun. (Camb.) 46*, 97-99.

(60)   Dommerholt, J., Schmidt, S., Temming, R., Hendriks, L. J., Rutjes, F. P., van Hest, J. C., Lefeber, D. J., Friedl, P., and van Delft, F. L. (2010) Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional imaging of living cells. *Angew. Chem. Int. Ed. Engl. 49*, 9422-9425.

(61)   Kiick, K. L., Saxon, E., Tirrell, D. A., and Bertozzi, C. R. (2002) Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *Proc. Natl. Acad. Sci. U. S. A. 99*, 19-24.

(62)   van der Linden, W. A., Li, N., Hoogendoorn, S., Ruben, M., Verdoes, M., Guo, J., Boons, G. J., van der Marel, G. A., Florea, B. I., and Overkleeft, H. S. (2012) Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: a comparative study. *Bioorg. Med. Chem. 20*, 662-666.

(63)   Dommerholt, J., van Rooijen, O., Borrmann, A., Guerra, C. F., Bickelhaupt, F. M., and van Delft, F. L. (2014) Highly accelerated inverse electron-demand cycloaddition of electron-deficient azides with aliphatic cyclooctynes. *Nat. Commun. 5*, 5378.

(64)   Borrmann, A., Fatunsin, O., Dommerholt, J., Jonker, A. M., Lowik, D. W., van Hest, J. C., and van Delft, F. L. (2014) Strain-promoted oxidation-controlled cyclooctyne-1,2-quinone cycloaddition (SPOCQ) for fast and activatable protein conjugation. *Bioconjug. Chem.*

(65)   Wang, L., Brock, A., Herberich, B., and Schultz, P. G. (2001) Expanding the genetic code of Escherichia coli. *Science 292*, 498-500.

(66)   Borrmann, A., Milles, S., Plass, T., Dommerholt, J., Verkade, J. M., Wiessler, M., Schultz, C., van Hest, J. C., van Delft, F. L., and Lemke, E. A. (2012) Genetic encoding of a bicyclo[6.1.0]nonyne-charged amino acid enables fast cellular protein imaging by metal-free ligation. *ChemBioChem 13*, 2094-2099.

(67)   Lang, K., Davis, L., Wallace, S., Mahesh, M., Cox, D. J., Blackman, M. L., Fox, J. M., and Chin, J. W. (2012) Genetic Encoding of bicyclononynes and trans-cyclooctenes for

site-specific protein labeling in vitro and in live mammalian cells via rapid fluorogenic Diels-Alder reactions. *J. Am. Chem. Soc. 134*, 10317-10320.

(68) Plass, T., Milles, S., Koehler, C., Schultz, C., and Lemke, E. A. (2011) Genetically encoded copper-free click chemistry. *Angew. Chem. Int. Ed. Engl. 50*, 3878-3881.

(69) Alfonta, L., Zhang, Z., Uryu, S., Loo, J. A., and Schultz, P. G. (2003) Site-specific incorporation of a redox-active amino acid into proteins. *J. Am. Chem. Soc. 125*, 14662-14663.

(70) Lang, K., Davis, L., Torres-Kolbus, J., Chou, C., Deiters, A., and Chin, J. W. (2012) Genetically encoded norbornene directs site-specific cellular protein labelling via a rapid bioorthogonal reaction. *Nat. Chem. 4*, 298-304.

(71) Kaya, E., Vrabel, M., Deiml, C., Prill, S., Fluxa, V. S., and Carell, T. (2012) A genetically encoded norbornene amino acid for the mild and selective modification of proteins in a copper-free click reaction. *Angew. Chem. Int. Ed. Engl. 51*, 4466-4469.

(72) Plass, T., Milles, S., Koehler, C., Szymanski, J., Mueller, R., Wiessler, M., Schultz, C., and Lemke, E. A. (2012) Amino acids for Diels-Alder reactions in living cells. *Angew. Chem. Int. Ed. Engl. 51*, 4166-4170.

(73) Pott, M., Schmidt, M. J., and Summerer, D. (2014) Evolved sequence contexts for highly efficient amber suppression with noncanonical amino acids. *ACS Chem. Biol. 9*, 2815-2822.

(74) Johnson, D. B., Xu, J., Shen, Z., Takimoto, J. K., Schultz, M. D., Schmitz, R. J., Xiang, Z., Ecker, J. R., Briggs, S. P., and Wang, L. (2011) RF1 knockout allows ribosomal incorporation of unnatural amino acids at multiple sites. *Nat. Chem. Biol. 7*, 779-786.

(75) Kularatne, S. A., Deshmukh, V., Ma, J., Tardif, V., Lim, R. K., Pugh, H. M., Sun, Y., Manibusan, A., Sellers, A. J., Barnett, R. S., Srinagesh, S., Forsyth, J. S., Hassenpflug, W., Tian, F., Javahishvili, T., Felding-Habermann, B., Lawson, B. R., Kazane, S. A., and Schultz, P. G. (2014) A CXCR4-targeted site-specific antibody-drug conjugate. *Angew. Chem. Int. Ed. Engl. 53*, 11863-11867.

(76) Jackson, D., Atkinson, J., Guevara, C. I., Zhang, C., Kery, V., Moon, S. J., Virata, C., Yang, P., Lowe, C., Pinkstaff, J., Cho, H., Knudsen, N., Manibusan, A., Tian, F., Sun, Y., Lu, Y., Sellers, A., Jia, X. C., Joseph, I., Anand, B., Morrison, K., Pereira, D. S., and Stover, D. (2014) In vitro and in vivo evaluation of cysteine and site specific conjugated herceptin antibody-drug conjugates. *PLoS One 9*, e83865.

(77) Fontaine, S. D., Reid, R., Robinson, L., Ashley, G. W., and Santi, D. V. (2015) Long-term stabilization of maleimide-thiol conjugates. *Bioconjug. Chem. 26*, 145-152.

(78) Lyon, R. P., Setter, J. R., Bovee, T. D., Doronina, S. O., Hunter, J. H., Anderson, M. E., Balasubramanian, C. L., Duniho, S. M., Leiske, C. I., Li, F., and Senter, P. D. (2014) Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates. *Nat. Biotechnol.*

(79) Blackman, M. L., Royzen, M., and Fox, J. M. (2008) Tetrazine ligation: fast bioconjugation based on inverse-electron-demand Diels-Alder reactivity. *J. Am. Chem. Soc. 130*, 13518-13519.

(80) Talbot, A., Devarajan, D., Gustafson, S. J., Fernandez, I., Bickelhaupt, F. M., and Ess, D. H. (2014) Activation-Strain Analysis Reveals Unexpected Origin of Fast Reactivity in Heteroaromatic Azadiene Inverse-Electron-Demand Diels-Alder Cycloadditions. *J. Org. Chem. 80*, 548-558.

(81) Selvaraj, R., and Fox, J. M. (2013) trans-Cyclooctene--a stable, voracious dienophile for bioorthogonal labeling. *Curr. Opin. Chem. Biol. 17*, 753-760.

(82)   Seitchik, J. L., Peeler, J. C., Taylor, M. T., Blackman, M. L., Rhoads, T. W., Cooley, R. B., Refakis, C., Fox, J. M., and Mehl, R. A. (2012) Genetically encoded tetrazine amino acid directs rapid site-specific in vivo bioorthogonal ligation with trans-cyclooctenes. *J. Am. Chem. Soc. 134*, 2898-2901.

(83)   Kim, K. M., McDonagh, C. F., Westendorf, L., Brown, L. L., Sussman, D., Feist, T., Lyon, R., Alley, S. C., Okeley, N. M., Zhang, X., Thompson, M. C., Stone, I., Gerber, H. P., and Carter, P. J. (2008) Anti-CD30 diabody-drug conjugates with potent antitumor activity. *Mol. Cancer Ther. 7*, 2486-2497.

(84)   Axup, J. Y., Bajjuri, K. M., Ritland, M., Hutchins, B. M., Kim, C. H., Kazane, S. A., Halder, R., Forsyth, J. S., Santidrian, A. F., Stafin, K., Lu, Y., Tran, H., Seller, A. J., Biroc, S. L., Szydlik, A., Pinkstaff, J. K., Tian, F., Sinha, S. C., Felding-Habermann, B., Smider, V. V., and Schultz, P. G. (2012) Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proc. Natl. Acad. Sci. U. S. A. 109*, 16101-16106.

(85)   Simon, M., Zangemeister-Wittke, U., and Plückthun, A. (2012) Facile double-functionalization of designed ankyrin repeat proteins using click and thiol chemistries. *Bioconjug. Chem. 23*, 279-286.

(86)   Simon, M., Frey, R., Zangemeister-Wittke, U., and Plückthun, A. (2013) Orthogonal assembly of a designed ankyrin repeat protein-cytotoxin conjugate with a clickable serum albumin module for half-life extension. *Bioconjug. Chem. 24*, 1955-1966.

(87)   Bohme, D., and Beck-Sickinger, A. G. (2015) Drug delivery and release systems for targeted tumor therapy. *J. Pept. Sci. 21*, 186-200.

(88)   Binz, H. K., Stumpp, M. T., Forrer, P., Amstutz, P., and Pluckthun, A. (2003) Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J. Mol. Biol. 332*, 489-503.

(89)   Zahnd, C., Kawe, M., Stumpp, M. T., de Pasquale, C., Tamaskovic, R., Nagy-Davidescu, G., Dreier, B., Schibli, R., Binz, H. K., Waibel, R., and Pluckthun, A. (2010) Efficient tumor targeting with high-affinity designed ankyrin repeat proteins: effects of affinity and molecular size. *Cancer Res. 70*, 1595-1605.

(90)   Steiner, D., Forrer, P., and Pluckthun, A. (2008) Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display. *J. Mol. Biol. 382*, 1211-1227.

(91)   Martin-Killias, P., Stefan, N., Rothschild, S., Plückthun, A., and Zangemeister-Wittke, U. (2011) A novel fusion toxin derived from an EpCAM-specific designed ankyrin repeat protein has potent antitumor activity. *Clin. Cancer Res. 17*, 100-110.

(92)   Hirel, P. H., Schmitter, M. J., Dessen, P., Fayat, G., and Blanquet, S. (1989) Extent of N-terminal methionine excision from Escherichia coli proteins is governed by the side-chain length of the penultimate amino acid. *Proc. Natl. Acad. Sci. U. S. A. 86*, 8247-8251.

(93)   Wang, A., Winblade Nairn, N., Johnson, R. S., Tirrell, D. A., and Grabstein, K. (2008) Processing of N-terminal unnatural amino acids in recombinant human interferon-beta in Escherichia coli. *ChemBioChem 9*, 324-330.

(94)   Boersma, Y. L., Chao, G., Steiner, D., Wittrup, K. D., and Pluckthun, A. (2011) Bispecific designed ankyrin repeat proteins (DARPins) targeting epidermal growth factor receptor inhibit A431 cell proliferation and receptor recycling. *J. Biol. Chem. 286*, 41273-41285.

(95)   Lang, K., and Chin, J. W. (2014) Bioorthogonal reactions for labeling proteins. *ACS Chem. Biol. 9*, 16-20.