Bioconjugate Chemistry

Review

pubs.acs.org/bc

Antibody–Drug Conjugates for Tumor Targeting—Novel Conjugation Chemistries and the Promise of non-IgG Binding Proteins

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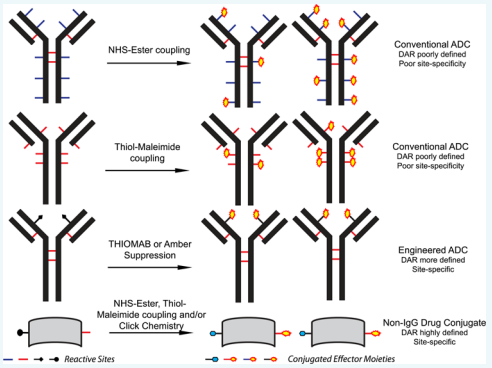
ABSTRACT: Antibody–drug conjugates (ADCs) have emerged as a promising class of anticancer agents, combining the specificity of antibodies for tumor targeting and the destructive potential of highly potent drugs as payload. An essential component of these immunoconjugates is a bifunctional linker capable 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 for 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 drug 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 responsible for the clinical failure of this first-generation ADC. In the meantime, the spectrum of tumor-selective antigens has increased and more potent anticancer agents with cellular IC_{50} values in the picomolar 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,

Special Issue: Antibody-Drug Conjugates

Received: May 6, 2015
Revised: June 14, 2015
Published: June 18, 2015

DOI: 10.1021/acs.bioconjchem.5b00260
Bioconjugate Chem. 2015, 26, 2176−2185

Table 1. ADCs FDA-Approved or Currently in Advanced Clinical Trials (www.clinicaltrials.gov June 2015)[a]

ADC	drug	linker	coupling reaction	antigen	lead indication
		Approved			
Brentuximab vedotin (Adcetris)	MMAE	Valine-citrulline	Cys + maleimide	CD30	HL, ALCL
Ado-trastuzumab emtansine (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 emtansine (Kadcyla)	DM1	Thioether	Lys + NHS (SMCC)	Her2	Gastric cancer, Breast cancer
		Phase II			
Glembatumumab vedotin	MMAE	Valine-citrulline	Cys + maleimide	gpNMB	Breast cancer, Melanoma
Lifastuzumab vedotin (DNIB0600A)	MMAE	Valine-citrulline	Cys +maleimide	NaPi2b	Ovarian cancer
Pinatuzumab vedotin	MMAE	Valine-citrulline	Cys + maleimide	CD22	NHL
Polatuzumab vedotin	MMAE	Valine-citrulline	Cys + maleimide	CD79b	B-cell lymphoma
PSMA-ADC	MMAE	Valine-citrulline	Cys + maleimide	PSMA	Prostate cancer
Coltuximab ravtansine	DM4	Disulfide	Lys+ NHS (SPDB)	CD19	B-cell lymphoma
Lorvotuzumab mertansine	DM1	Disulfide	Lys + NHS (SPP)	CD56	[b]SCLC

[a]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; SPDB, N-succinimidyl 4-(2-pyridyldithio)butyrate; SPP, N-succinimidyl 4-(2-pyridyldithio)pentanoate. [b]Halted.

such that the (usually) hydrophobic drug can cross the endolysosomal membrane into the cytosol. Currently, two categories of linkers, cleavable and noncleavable, 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 their large number in an IgG, will be stochastic and result in heterogeneous synthetic products characterized by a distribution of drug-to-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. Specifically, we focus on recent linker chemistries with potential for bioorthogonal drug conjugation and highlight potential advantages of alternative non-IgG scaffolds like Designed Ankyrin Repeat Proteins (DARPins), which can be engineered to generate homogeneous, site-specifically payloaded bioconjugates with precisely defined drug ratios and extended half-lives.

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).

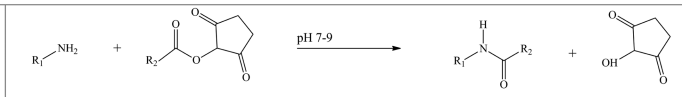
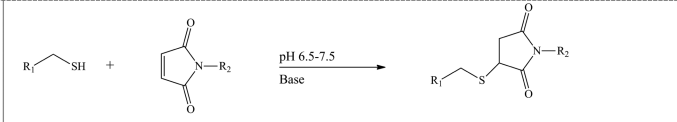
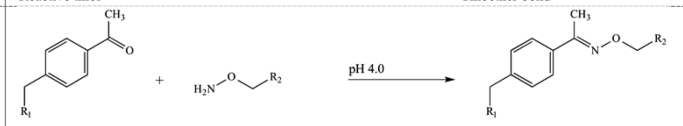
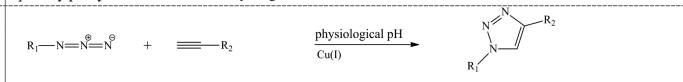
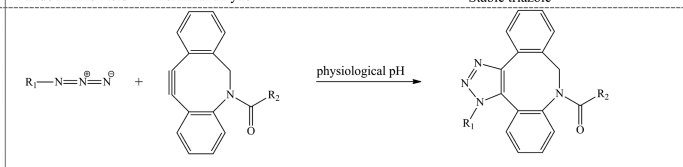
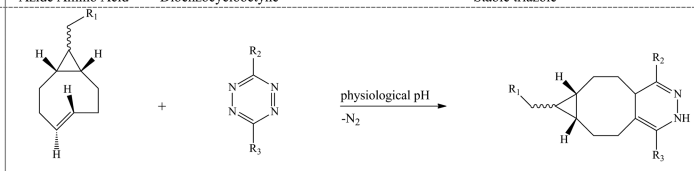
ADCs approved by the US Food and Drug Administration (FDA) or currently in advanced clinical trials are shown in Table 1.

So far, only three ADCs have been approved by the FDA. Two of them employ potent tubulin-binding agents, either monomethyl auristatin E (MMAE) or a derivative of maytansine (DM1), and one utilizes the DNA-strand breaking calicheamicin (Table 1). These payloads outperform the potency of classical chemotherapeutic drugs by a factor of about 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 antitumor activity in a postapproval 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 first-line

DOI: 10.1021/acs.bioconjchem.5b00260
Bioconjugate Chem. 2015, 26, 2176−2185

Table 2. Conjugation Chemistries for ADCs[a]

Reaction Chemistry	*Reaction scheme*	*ReactionRate*	*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

[a]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 refs 46,47.

treatment of HER2-positive metastatic breast cancer based on a positive phase III trial (Table 1).[18] Very recent primary results from a phase III randomized study (NCT01120184) could only demonstrate noninferiority of T-DM1 and T-DM1 plus pertuzumab in progression-free survival compared to the standard of care (taxane plus trastuzumab), but the trial showed a benefit of the T-DM1-containing arms over the control with regard to lower toxicity.[19] 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 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 noncleavable.[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 nontargeted tumor cells when hydrophobic drugs are used, which is particularly advantageous if antigens are heterogeneously expressed in tumors.[20] 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.[2,21] The serum stability of hydrazone linkers, however, is poor.[22,23] 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.[5,16,24] Cleavable disulfide linkers, in turn, exploit the high concentration of thiols, such as glutathione, in the cell, which reduce a disulfide bond in the linker to liberate the payload.[1,25] To decrease premature 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.[26] Although several disulfide-linked ADCs are currently under clinical investigation, 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.[27−29]

Noncleavable linkers, on the other hand, avoid premature drug release in the circulation by design.[1] Instead, the ADC

DOI: 10.1021/acs.bioconjchem.5b00260
Bioconjugate Chem. 2015, 26, 2176−2185

must be proteolytically degraded in lysosomes and the payload is released as an active metabolite along with the amino acid it was conjugated to, usually a cysteine or lysine.[20,30] Although the charge of a lysine-N^{ε}-coupled drug metabolite may hamper endolysosomal escape, it prevents externalization and bystander killing.[30,31] 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 PEG_4Mal linker.[32]

4. PAYLOADS

Natural products and synthetic chemistry provide a plethora of cytotoxins of which only a few have been found suitable for use in ADCs.[33,34] 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 would be well below the number which is delivered by ADCs, which depends on the drug-to-antibody ratio (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.[2,35] 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 and so forth, 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.[36]

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,[26,37,38] duocarmycins,[39] the tubulin-targeting maytansines,[40,41] and auristatins.[17,42] The mechanisms of action and the chemistry of the coupling and release reactions of these warheads have been described in detail elsewhere.[1,43,44] 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 (phase II and III) 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),[45] respectively (Table 1 and Table 2).

As IgGs carry a variable number of lysine side-chains, this yields heterogeneous products with variable DARs. 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.[48,49] If thiol-maleimide coupling is used instead, cysteine conjugation occurs after mild reduction of the four interchain disulfide bonds 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,[49,50] 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, at least in the context of MMAE conjugation to cysteine residues,[50] 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.[50,51] Unconjugated antibodies, if present, will block potential binding sites on the tumor surface and thus lower ADC efficacy, whereas drug conjugates with high DARs show increased off-target toxicity and are prone to aggregation, which narrows the therapeutic window.[52] 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.[51] 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.[53] It remains unclear whether the observed increased efficacy and safety of the resulting ADC[51] is a consequence of the more defined DAR or the different linker and thus due to a different final active metabolite. This must also be seen in the context of recent findings that, in terms of efficacy, less payload is more.[50]

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 the reagents used to activate the glycan. 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

DOI: 10.1021/acs.bioconjchem.5b00260
Bioconjugate Chem. 2015, 26, 2176−2185

bioorthogonal manner, is appealing also for antibody−drug conjugation. In this constellation, two components, which are fully complementary and, additionally, orthogonal to nature's reactive functional groups on protein (thiols, amines, alcohols, and carboxylates), will 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.[54] 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.[8,11,54]

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,[55] 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 a 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).[56,57] The higher reaction rate and the stability of the reactants has made this reaction appealing and widely used for bioconjugation.[58] 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).[59] To improve the modest reaction rates, various derivatives such as fluorinated,[60] benzoannulated,[61,62] and cyclopropanated[63] 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.[64] 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 in biological systems.[62,63,65] 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.[66] In another study, Borrmann et al.[67] 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 is 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.[68−75] So far, this technology was only used for imaging agents, as it shows unfavorably low expression yields.[69]

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[76] and adapted *E. coli* strains,[77] 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.

However, 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),[10,47,78] new biologicals could be generated. This technology, when applied to trastuzumab, 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 of blood serum components, may have partially inactivated the ADC.[78−80] However, the authors do not provide convincing data showing that their own control preparation of the THIOMAB antibody actually had a DAR of 2, as published by Junutula et al.[53] 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. Nevertheless, the technology of amber suppression is being significantly improved with the goal of achieving higher economically reasonable protein production levels of site-specific and homogeneous bioconjugates (www.ambrx.com).

5.4. 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.[81] As reported only recently, this results from a decreased closed-shell electron−electron repulsion between the reaction partners.[82] Nothing beats rate constants of up to 10^6 M^{-1} s^{-1} of *s*-tetrazines with trans-cyclooctene

DOI: 10.1021/acs.bioconjchem.5b00260
Bioconjugate Chem. 2015, 26, 2176−2185

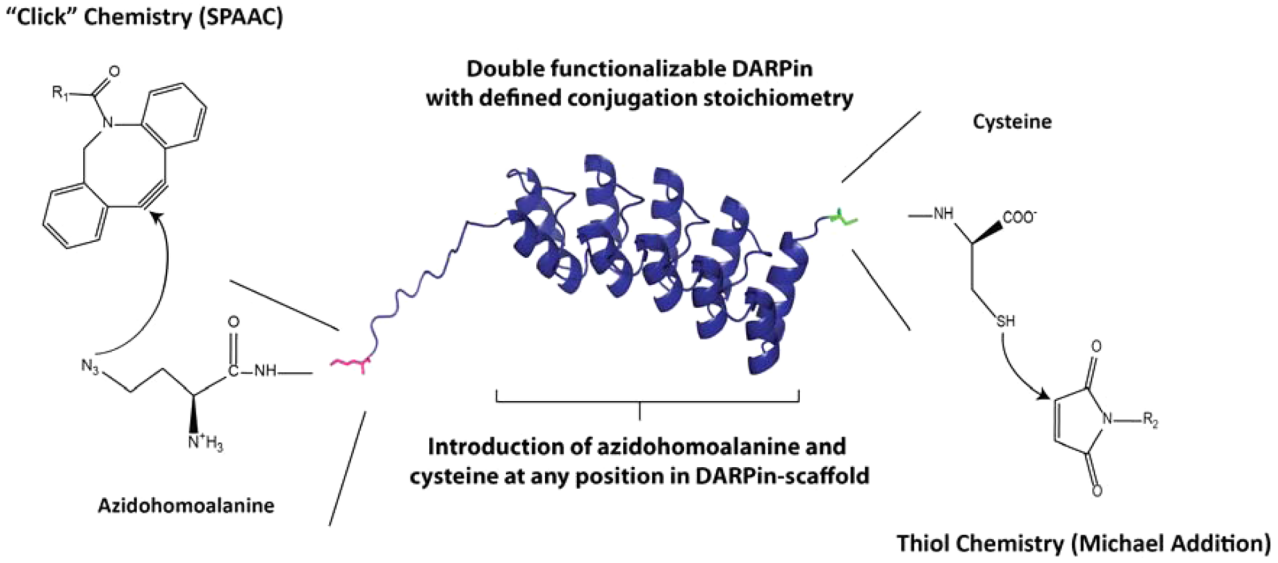


Figure 1. Double functionalized DARPin (blue) for bioconjugation of effector modules (R1 and R2) at defined positions in the DARPin scaffold. Figure adapted from ref 87.

(TCO)-derivatives (Table 2). Since TCO and tetrazines can be incorporated in proteins using amber suppression, numerous applications for imaging purposes were reported.[75,83,84] 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.[52] 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 drug exposure to the body.[85] The improved tumor penetration of smaller conjugates may at least partly compensate the shorter serum half-life and thus help explain this finding.[52]

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.[52] Axup et al.[86] 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. The robust nature of these scaffolds 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.[12,87] 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,[88] but similar approaches may be possible with other stable scaffolds too. The potential of bioorthogonally assembled peptide—drug conjugates for tumor targeting is described elsewhere.[89]

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.[90] Moreover, DARPins of very high affinity can be readily generated using well-established selection

DOI: 10.1021/acs.bioconjchem.5b00260
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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.[91−93]

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). 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.

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,[64,87] 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.[87] Additionally, DARPins are usually expressed with an N-terminal $MRGSH_6$ tag, from which the methionine is not cleaved,[94] and this is also true when an amino acid analogue is introduced.[95] Conversely, if the second amino acid is small (e.g., Ala, Gly, or Ser), the analogue will be cleaved off, allowing the introduction of a unique methionine surrogate such as Aha at desired positions elsewhere in the chain (Figure 1).[87] 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 monomethyl auristatin F (MMAF) and additional coupling of a half-life extension module like serum albumin.[88] 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 can the half-life be engineered, but bispecific binding proteins can also be easily designed from such scaffolds, allowing the optimization of drug conjugate performance for tumor targeting.[96]

7. CONCLUSIONS AND PERSPECTIVES

ADCs combine the specificity of antibodies for tumor-associated antigens to discriminate between normal and malignant cells, and the cytotoxic effect of anticancer agents as payload. 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, demonstrating that there is still a great need 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.

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Notes

The authors declare the following competing financial interest(s): A.P. is a cofounder and shareholder of Molecular Partners AG who are commercializing the DARPin technology.

ACKNOWLEDGMENTS

The author's work is supported by Swiss National Science Foundation grants 310030_31003A and 31003A_138201. We thank Dr. Annemarie Honegger for help with Figure 1.

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; MMAF, monomethyl auristatin F; NHS, *N*-hydroxysuccinimide; *p*AF, para-acetylphenylalanine; SPAAC, strain-promoted azide−alkyne cycloaddition; TCO, trans-cyclooctene

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DOI: 10.1021/acs.bioconjchem.5b00260
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DOI: 10.1021/acs.bioconjchem.5b00260
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