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Enzymatic strategies for (near) clinical development of antibody-drug conjugates

Sander S. van Berkel[a], Floris L. van Delft[a,b,*]

[a]Synaffix BV, Kloosterstraat 9, 5349 AB, Oss, The Netherlands
[b]Wageningen University and Research, Laboratory of Organic Chemistry, Stippeneng 4, 6708 WE, Wageningen, The Netherlands

Abstract

Target-specific killing of tumor cells with antibody-drug conjugates (ADCs) is an elegant concept in the continued fight against cancer. However, despite more than 20 years of clinical development, only four ADC have reached market approval, while at least 50 clinical programs were terminated early. The high attrition rate of ADCs may, at least in part, be attributed to heterogeneity and instability of conventional technologies. At present, various (chemo)enzymatic approaches for site-specific and stable conjugation of toxic payloads are making their way to the clinic, thereby potentially providing ADCs with increased therapeutic window.

Section editor:
Vijay Chudasama – Department of Chemistry, University College London, London, UK

Introduction

Antibody-drug conjugates (ADCs) constitute a valuable class of anticancer drugs, based on the concept of target-specific delivery of a cytotoxic payload to a tumor [1]. However, the development trajectory of ADCs, like for any other chemotherapeutic, is marked by hurdles and setbacks [2,3]. Besides the requirement of a specifically upregulated tumor-associated surface receptor (the 'target') [4], each of the structural elements of an ADC (*i.e.* monoclonal antibody, conjugation technology, linker design, choice and stoichiometry of payload) must be optimized for potential clinical success [5,6]. With all these moving parts, it is not surprising that it took more than 40 years from the preclinical proof-of-concept [7] until an ADC (SGN-15) was put into clinical trials [8]. Unfortunately, clinical development was rapidly discontinued due to target-related gastrointestinal toxicities as well as poor efficacy, attributed to the lack of potency of the payload and the instability of the linker [9]. Moreover, it speaks volumes that basically all of its design aspects of this first ADC -a chimeric IgG, randomly conjugated to a variable number of doxorubicin molecules via an acid-sensitive hydrazone linker-have been aborted. In fact, with ~50 clinical failures, ADCs as a class show attrition rates that are at best slightly improved versus traditional chemotherapeutics: more than 125 ADCs have entered the clinic over the years, but only four have successfully reached market approval to date (while clinical trials on 75 others are still ongoing) [10]. Obviously, over the years many valuable lessons have been learned regarding antibody, linker and payload. For example Fig. 1, shows the evolution of technologies from (random) conjugation to native lysine or cysteine side-chains in early days, to site-specific conjugation approaches (engineered cysteine or non-natural amino acids) making their entry in the past five years.

*Corresponding author: F.L. van Delft (f.vandelft@synaffix.com)

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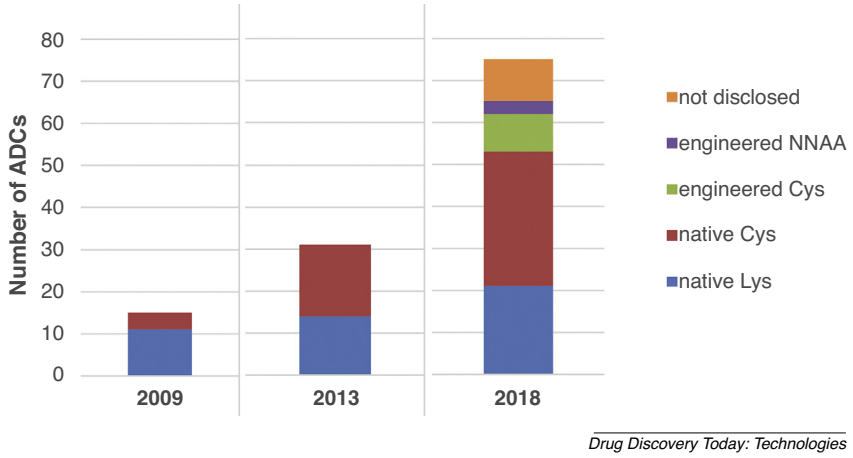

Fig. 1. Historical overview of ADCs in the clinic, categorized by conjugation chemistry (NNAA = non-natural amino acid) [1,4,10].

Marketed and clinical stage conjugation technologies

Conjugation to native or engineered amino acids

The vast majority of clinical ADCs are based on conjugation to native lysine or cysteine side-chains, respectively with an active ester (Table 1, entry 1) or with a maleimide reagent (entry 2). However, due to the plethora of lysine and cysteine side-chains in a typical antibody, the resulting ADC is obtained as a heterogeneous mixture of regioisomers with varying drug-to-antibody ratio (DAR) [11], although some hydroxysuccinimide esters may conjugate preferentially to the mAb light-chain constant domain (*e.g.* Concortis' K-lock™ technology) [12]. A useful solution towards homogeneous ADCs was introduced by Genentech in 2008, based on engineering of an additional cysteine at a specific site in the antibody (THIOmab technology) [13] an approach that has inspired the clinical development of multiple ADCs (9 today). In more recent years, a range of alternative reagents for site-specific alkylation of either lysine [14] or cysteine [15] have been reported, as well as bifunctional reagents for rebridging of interchain cysteine disulfides leading to DAR4 ADCs (*e.g.* bisbromomaleimide [16], C-Lock [17], THIObridge [18] and dibromopyridazinediones [19]), some of which are nearing clinical stage.

Nevertheless, many ADCs obtained by conjugation to lysine or cysteine, including site-specific ADCs, have been discontinued. Although an overarching explanation for clinical failure of these ADCs cannot be provided, an important shortcoming of ADCs obtained by thiol-maleimide alkylation is premature payload deconjugation due to retro-Michael reaction [20], which impacts both efficacy and tolerability of the conjugate. As a solution, various cysteine conjugation technologies with enhanced stability have been developed over the years [21–23], most notably Seattle Genetics' DPR-technology [24] (entry 3) as applied in SGN-CD48A, which entered clinical trials in November 2017. It has been found that the rate of payload release from cysteine-engineered ADCs is highly dependent on the specific location in the antibody sequence [13]. However, the observed structure-activity relationship of ADCs cannot be accounted for by instability alone, but the exact location of toxic payload(s) on the antibody also has a direct influence on ADC properties like pharmacokinetic profile [25–27] and aggregation [28], in particular due to the high lipophilicity of most payloads. For example, a consistent set of data illustrates that the C-terminus of the heavy chain is a problematic point of attachment for ADC payloads [27], while the antibody hinge region may be considered as privileged [29].

Genetic encoding of non-natural amino acids

Clinical application of technologies based on genetic encoding of a non-natural amino acid (NNAA), has been explored by Axup et al. [30] and Zimmerman et al. [31] for *p*-acetylphenylalanine and *p*-azidomethylphenyl alanine, respectively (entries 4 and 5), leading to site-specific and highly stable ADCs. Also in this context, comprehensive site-scanning studies have demonstrated [31] that the *in vivo* efficacy of ADCs is highly dependent on the specific location of the NNAA, and hence the payload, on the antibody.

(Chemo)enzymatic conjugation technologies

The insight that the therapeutic windows of ADCs can generally be improved by ensuring site-specificity and linker stability has generated interest to develop next-generation technologies based on enzymatic antibody modification. Whereas chemical antibody modification typically proceeds with little regiocontrol, enzymes are renowned for their high selectivity and efficiency. It is remarkable that enzymes have to date found little application in the post-recombinant modification of biological drugs, with only two notable examples (outside the field of ADCs), *i.e.* abciximab

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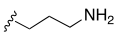
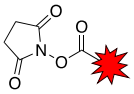
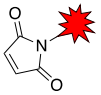
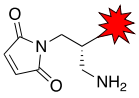
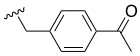
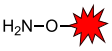
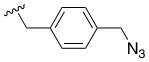
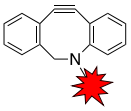
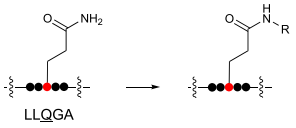
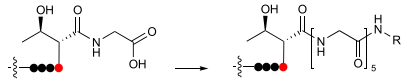
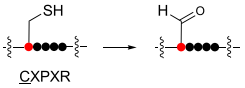
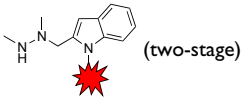
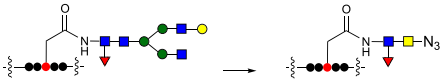
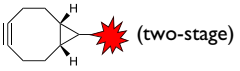
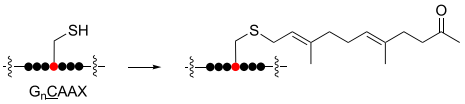
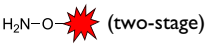
Table 1. Overview of clinically validated conjugation technologies (September 2018).

Entry	Amino acid	Side-chain(s)	Reactive group	# Marketed	# Clinical	# Failed
1	Lysine			3[a]	20	17
2	Cysteine			1 (random)	32 (random) 9 (site-specific)	23 (random) 8 (site-specific)
3				0	1 (site-specific)	0
4	*p*-AcPhe			0	2	0
5	*p*-AzPhe			0	1	0

[a] Kadcyla® is prepared by lysine conjugation with a bifunctional linker containing both an active ester and a maleimido group, followed by nucleophilic addition of thiol-containing toxic payload (DM1). Mylotarg® and Besponsa® are prepared in one step with a linker-payload containing an acid-labile hydrazone functionality.

Table 2. Enzymatic approaches for antibody conjugation, by direct attachment of payload or via a two-stage strategy.

Entry	Enzymatic transformation	Conjugation probe (approach)	Ref
1	*Bacterial transglutaminase (TGase)*  LLQGA	 (direct)	[26,35,36]
2	*Sortase*  C-terminal LPETG	 (direct)	[39,40]
3	*Formylglycine-generating enzyme (FGE)*  CXPXR	 (two-stage)	[41–43]
4	*Glycosyl transferase*  N297 (G1F-glycoform)	 (two-stage)	[50,51]
5	*Isoprenoid transferase*  G_nCAAX	 (two-stage)	[53]
		 = linker-drug	

(Reopro®) and lipegfilgrastim (Lonquex®). Abciximab, a drug used for inhibition of platelet aggregation after coronary artery procedures, is a F(ab) fragment obtained by papain digestion of the recombinant antibody above the hinge region [32]. Lipegfilgrastim was introduced for the sustained treatment of chemotherapy-induced neutropenia, based on post-recombinant two-stage modification of human granulocyte colony-stimulating factor (G-CSF [33]): (i) transfer of *N*-acetylgalactosamine (GalNAc) to a unique threonine *O*-glycosylation site followed by (ii) transfer of a (synthetic) 20 kDa-PEGylated sialic acid derivative under the action of sialyltransferase. While these drugs illustrate the general usefulness of enzymes for generation of improved biologicals, enzymes are now also emerging in the development of ADCs for the site-specific attachment of toxic payloads to monoclonal antibodies. Although multiple approaches have been reported in scientific literature, below is summary is provided of five strategies that (appear to) have reached a level of maturation suitable for clinical evaluation (Table 2). Conceptually, two strategies can be recognized: direct enzymatic

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attachment of payload (entries 1–2) or a two-stage approach employing (i) enzymatic tagging with a functional handle, then (ii) chemical attachment of payload (entries 3–5).

Bacterial transglutaminase (TGase)

Trailblazer in the field of enzymatic preparation of ADCs is Rinat Laboratories (now Pfizer), by recognizing that microbial transglutaminase (TGase), is able to form a stable amide bond between a glutamine-containing pentapeptide sequence and an aminoalkyl-containing substrate (Table 2, entry 1). Thus, glutamine tag LLQGA was introduced at multiple positions in an antibody (internal, N-terminus or C-terminus), and screened for efficiency of conjugation with a lysine-modified payload [26]. It was found that the site of conjugation had significant impact on *in vivo* ADC stability and pharmacokinetics, which was attributed to the proteolytic degradation of the Val-Cit linkage by proteases in rodent blood, but not to chemical instability. In follow-up work, it was described that TGase-prepared conjugates were substantially more efficacious *in vivo* than random ADCs, while retaining good PK properties and tolerability. [34] Various other TGase-based approaches with potential future clinical application have also been reported in more recent years. For example, Jeger et al. had demonstrated that heavy chain glutamine Q^{295}, after PNGase F removal of the glycan, becomes a substrate for TGase [35], a technology currently applied by Innate Pharma [36]. Also, a specifically engineered bacterial TGase is able to recognize a unique glutamine in the antibody heavy chain [37] Finally, Spidel et al. recently showed that, by preventing carboxypeptidase clipping of the endogenous C-terminal lysine (K^{447}), TGase treatment induces the formation of a covalent bond with a glutamine-containing dipeptide (marketed as Respect^{TM}-H technology by Morphotek) [38].

Sortase

Sortase is another bacterial enzyme that catalyzes a transpeptidation between a protein containing a C-terminal pentapeptide ''sort-tag'' and an N-terminal oligoglycine and can be applied for the clean and efficient modification of proteins (Table 2, entry 2), as pioneered by Popp et al. [39] Applying this technology, NBE Therapeutics have shown that by engineering of a C-terminal LPETG tag on the antibody heavy chain and/or light chain and treatment with excess Gly_5-modified payloads, ADCs can be prepared with tailored DAR2 or DAR4 (SMAC^{TM} technology) [40].

Formylglycine-generating enzyme (FGE)

Formylglycine-generating enzyme (FGE) also recognizes a specific pentapeptide consensus sequence (CxPxR), thereby oxidizing cysteine to an aldehyde-bearing formylglycine (Table 2, entry 3). [41] Formylglycine in turn provides a unique chemical handle for conjugation of payload by ligation with *O*-alkyl hydroxylamines, however the resulting oximes are susceptible to hydrolysis [42]. An elegant solution involves reaction of formylglycine with a dimethylated 2-(hydrazinomethyl)indole (entry 3), leading to the fast formation of a stable carbon–carbon bond by hydrazino-Pictet-Spengler ligation (HIPS) at near neutral pH [42]. This technology, termed SMARTtag^{TM}, is currently being marketed by Catalent for site-specific ADC generation. Conveniently, recombinant co-expression of CxPxR-engineered antibody with FGE leads to direct generation of formyl-containing antibody by *in situ* enzymatic oxidation [43].

Glycosyl transferase

Monoclonal antibodies carry a globally conserved glycosylation site in the C_H2 domain, at or around asparagine-297. This glycan is a mixture consisting of primarily complex glycoform (mainly G0, G0F, G1F), but also includes minor fractions, such as hybrid type and/or mannosylated glycans. As a strategy, conjugation through the antibody glycan was already explored in the early days of ADCs, based on (i) oxidative cleavage of 1,2-diols followed by (ii) hydrazone or oxime formation [44]. However, this approach has never reached the clinic as it leads to complex and unstable mixtures, while some antibodies are sensitive to periodate oxidation [45,46]. Various groups have demonstrated that glycosyl transferases can be applied for generation of stable antibody conjugates. For example, Zeglis et al. showed that a mutant galactosyl transferase GalT(Y289L) developed by Qasba et al. [47] enables the selective introduction of azido-modified GalNAc, which may be subsequently conjugated using copper-free click chemistry [48]. A similar approach reported by Li et al. employed sialyltransferase to introduce 9-azido-modified sialic acid [49]. To improve homogeneity, Synaffix introduced the concept of endoglycosidase trimming prior to introduction of yet another azidosugar (6-azidoGalNAc) with a native GalNAc-transferase (Table 2, entry 4) [50] This technology, called GlycoConnect^{TM}, has the advantages that all glycoforms are used (including hybrid and mannosylated), a shorter linker remains between antibody and payload (improvement of the pharmacokinetic profile) and offers the option to tailor drug-antibody ratio (DAR2 or DAR4) [51].

Isoprenoid transferase

Finally, isoprenoid transferases (*e.g.* farnesyl transferase, FTase) [52] can be applied for conjugation of a prenyl derivative to a C-terminal CAAX sequence engineered into light or heavy chain, preferably *via* a short glycine spacer (Table 2, entry 5). Thus, treatment of engineered antibody with FTase in the presence of farnesyl pyrophosphate, leads to alkylation of cysteine. By prior chemical modification of farnesyl pyrophosphate with a ketone functionality, subsequent oxime ligation of payload can be ensured [53].

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Clinical or near-clinical ADCs prepared by (chemo) enzymatic conjugation

Fig. 2 shows the structure of various ADCs prepared by the enzymatic technologies highlighted above, which have already been clinically tested (PF-06664178) or are expected to reach that status in the next 1–2 years.

PF-06664178

In August 2014, a phase I study (NCT02122146) for the treatment of neoplasms was initiated by Pfizer on PF-06664178, based on convincing preclinical data showing the power of site-specific conjugation [34]. PF-06664178 is a TROP-2 targeting ADC prepared by TGase-mediated conju-

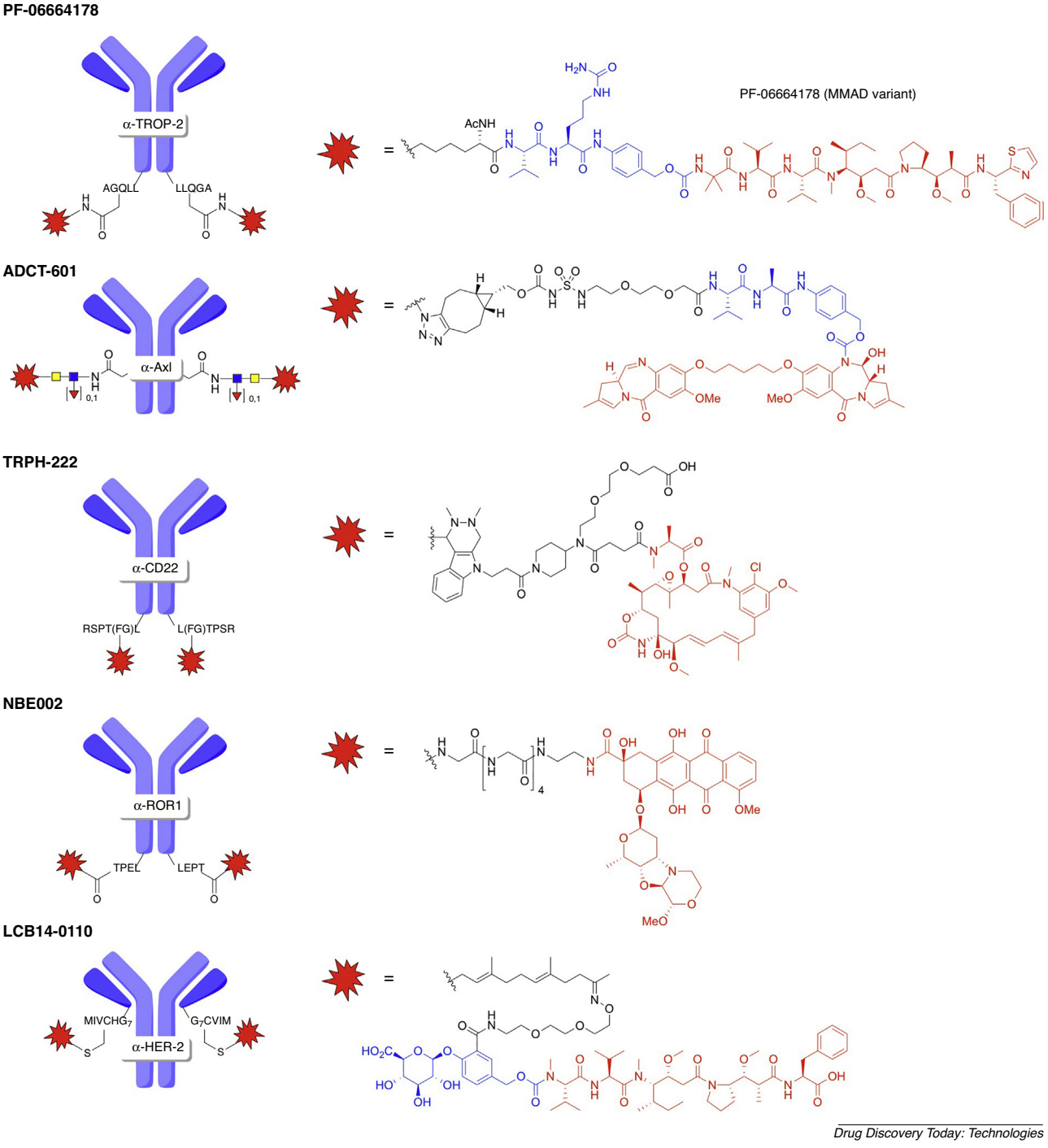

Fig. 2. Overview of (near) clinical ADCs produced by means of enzymatic processes.

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gation of a highly potent auristatin analogue (pelidotin) to an engineered C-terminal glutamine tag. However, the clinical trial was discontinued rather soon, after dose escalation phase, in June 2016. Details have not been disclosed.

ADCT-601

ADC Therapeutics is developing ADCT-601, an ADC conjugated to a highly potent PBD payload using Synaffix' glycan remodeling (GlycoConnect™) [50] and polar spacer (HydraSpace™) [51] technologies. Interestingly, HydraSpace™ was found to significantly increase the MTD of ADCT-601 in rats versus traditional PEG spacer alone (6 mg/kg versus 3 mg/kg). ADCT-601 is expected to reach the clinic in Q1 2019 latest and will be evaluated in patients with solid tumors expressing Axl receptor [54].

Trph-222

In October 2016, Triphase licensed Catalent's CD22-targeting ADC (now Trph-222) based on a humanized antibody conjugated at its C-terminus to a maytansinoid payload by FGE-mediated oxidation, hydrazino-Pictet-Spengler (HIPS™) chemistry and a polar linker (4AP) [55]. CD22 is a sialoglycoprotein that is an important modulator of B-cell signaling and survival, which is expressed on 90% of B-cell malignancies, such as non-Hodgkin's lymphoma (NHL) and acute lymphoid leukemia (ALL). Trph-222 is expected to enter clinical trials in 2019.

NBE-002

Based on sortase-mediated C-terminal antibody conjugation (SMAC™ technology) [40] of the highly potent anthracycline analogue PNU159,682, NBE Therapeutics is developing a clinical pipeline of which NBE-002 is the frontrunner (projected for clinical evaluation in 2020) [57] PNU159,682 is a promising payload as it provides ADCs with a high therapeutic window, and moreover led to a durable response by induction of tumor type-specific immunity mediated by tumor infiltration of activated CD8+ T cells. NBE-002 is a DAR2 ROR-1 targeting ADC with potential clinical application against lung cancer, triple negative breast cancer and leukemia.

LCB14-0110

LegoChemBio is developing ADCs employing isoprenoid transferase technology (ConjuAll™ technology). Frontrunner in the LegoChemBio pipeline [58] is a HER2-targeting ADC (LCB14-0110) based on trastuzumab, genetically modified at both light chains harboring a C-terminal CVIM recognition sequence via a heptaglycine linker. Conjugation of the toxic payload MMAF, via a cleavable linker with glucuronic acid, is ensured via enzymatic prenylation and oxime ligation to provide a DAR2 ADC [53,59]. LCB14-0110 shows high *in vivo* stability and improved response versus Kadcyla® in various xenograft models (JIMT-1 and N87).

Conclusions

The vast majority of all ADCs that are or have been in the clinic are based on conjugation to lysine or cysteine sidechain. However, the resulting ADCs are typically a heterogenous mixture of components and may be unstable (or a combination thereof), which compromises the therapeutic window and therefore are at least in part responsible for the disappointing clinical results. A promising approach to mitigate some of the shortcomings of current ADC technologies involves the modification of immunoglobulins with enzymes. By encoding of a specific peptide sequence or by judicious targeting of a native amino acid or glycan, a linker-drug can be installed onto an IgG that first of all does not suffer from deconjugation and secondly can be positioned at a privileged site for improved pharmacokinetic profile. Various enzymatic approaches have led to the development of ADCs that are expected to reach clinical status in the coming years. Besides, a multitude of additional enzymatic approaches [60,61] for example based on the use of phosphopantetheinyl transferase, trypsiligase/subtiligase, tub tyrosine ligase, intein, lipoic acid ligase, tyrosinase and others, offer promise for the development of next generation of ADCs with improved therapeutic index.

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