

Strategies and Advancement in Antibody-Drug Conjugate Optimization for Targeted Cancer Therapeutics

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Abstract

Antibody-drug conjugates utilize the antibody as a delivery vehicle for highly potent cytotoxic molecules with specificity for tumor-associated antigens for cancer therapy. Critical parameters that govern successful antibody-drug conjugate development for clinical use include the selection of the tumor target antigen, the antibody against the target, the cytotoxic molecule, the linker bridging the cytotoxic molecule and the antibody, and the conjugation chemistry used for the attachment of the cytotoxic molecule to the antibody. Advancements in these core antibody-drug conjugate technology are reflected by recent approval of Adectris® (anti-CD30-drug conjugate) and Kadcyla® (anti-HER2 drug conjugate). The potential approval of an anti-CD22 conjugate and promising new clinical data for anti-CD19 and anti-CD33 conjugates are additional advancements. Enrichment of antibody-drug conjugates with newly developed potent cytotoxic molecules and linkers are also in the pipeline for various tumor targets. However, the complexity of antibody-drug conjugate components, conjugation methods, and off-target toxicities still pose challenges for the strategic design of antibody-drug conjugates to achieve their fullest therapeutic potential. This review will discuss the emergence of clinical antibody-drug conjugates, current trends in optimization strategies, and recent study results for antibody-drug conjugates that have incorporated the latest optimization strategies. Future challenges and perspectives toward making antibody-drug conjugates more amenable for broader disease indications are also discussed.

Key Words: Antibodies, Antibody-drug conjugates, Immunotherapy, Targeted therapy

INTRODUCTION

The most commonly used treatment for cancer is combination therapy, such as surgery with chemotherapy, radiotherapy, or targeted immunotherapy. Since cancer is often present as a disseminated disease, it is imperative to target not only the primary tumor cells but also the distant metastases, without harming non-tumor cells. Therefore, targeted therapy for the tumor-specific antigens has become an invaluable tool in cancer therapy. In particular, antibody-based immunotherapies using monoclonal antibodies (mAbs) and antibody fragments have been the focus of the development of strategic anticancer drugs for many years. The mAbs and their derivatives, such as radionuclides, toxins, or cytotoxic molecule-labeled mAbs have become established as a new drug class for use in targeted cancer therapy. The significance of therapeutic antibodies is, in part, reflected by recent nomenclature regulations for antibody-based drugs as implemented by the

International Nonproprietary Names and the United States Adopted Names.

Clinical validation of therapeutic antibodies in combination with chemotherapy or another therapeutic antibody with a different mode of action is now becoming one of the standard therapeutic goals for exploratory drug delivery protocols in clinical oncology. This suggests the insufficient antitumor efficacy of the naked antibody when it is used alone. In an attempt to further improve clinical benefits for patients, the number of antibody-drug conjugates (ADCs), where the tumor antigen-specific antibody is conjugated to the potent cytotoxic molecule, has been rapidly growing, and may provide further promising treatments for cancer. Currently, approximately 45 ADCs are in clinical trials against ~35 targets, and ~70% of the therapeutic modalities in Phase I clinical trials are ADCs.

The principle of the ADC is quite simple; however, satisfactory efficacy of therapeutic ADCs has been more difficult to achieve than previously anticipated, as exemplified by the

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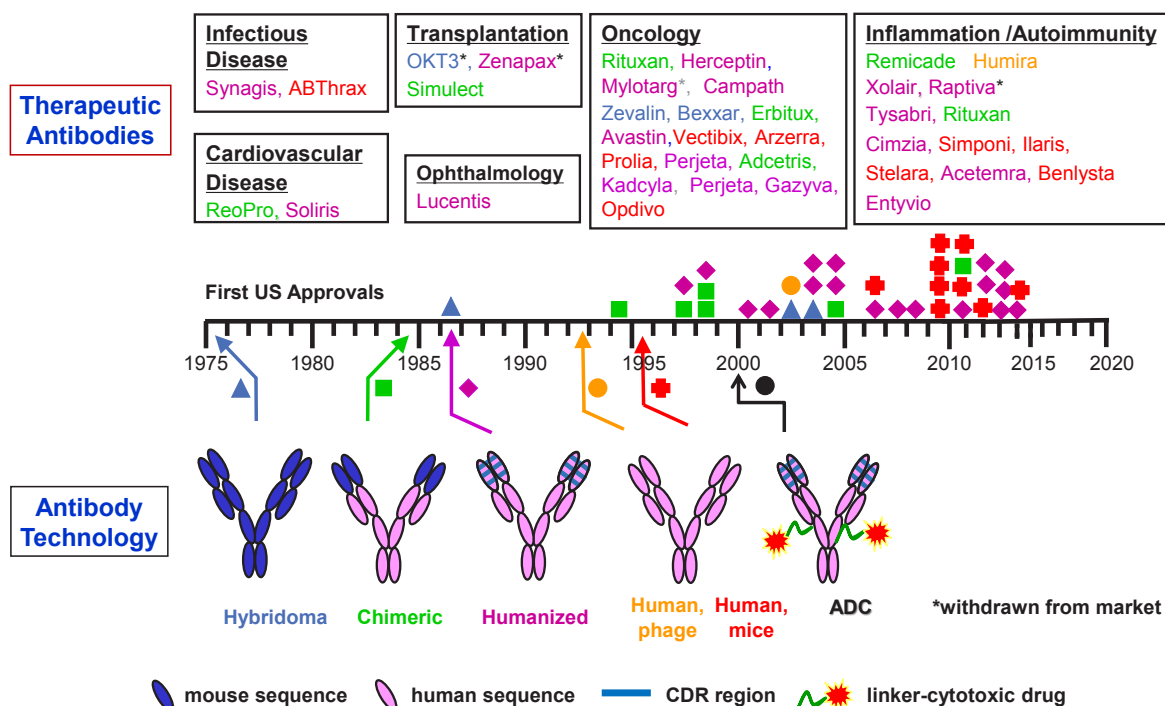


Fig. 1. Emergence of antibody technology and therapeutic antibodies. Modified from Kim, 2011¹.

number of ADCs that have been terminated during clinical trial phases. Therefore, optimization of ADCs, and identification of novel therapeutic combinations are needed to further improve the efficacy of immunotherapy. The progress in cancer therapy and emergence of ADCs as drug delivery vehicles for targeted immunotherapy are described in this review. In particular, the critical features of ADCs that contribute to the successful development and clinical implementation of their use, as well as the challenges and latest optimization strategies for therapeutic ADCs are reviewed. Results from current clinical and preclinical studies are also presented.

EVOLUTION OF TARGETED THERAPY FOR CANCER TREATMENT

Chemotherapy

Numerous cytotoxic molecules have been approved for use as chemotherapeutic agents. Most chemotherapeutic drugs target both proliferating cancer and normal cells, and are used near their maximum tolerated dose (MTD) to achieve therapeutic effects. As a result, the standard therapeutic modality for chemotherapy is often a combination therapy: chemotherapy regimens of CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) for non-Hodgkin's lymphoma (NHL) and CMF (cyclophosphamide, methotrexate, and 5-fluorouracil) for breast cancer are examples of combination modalities in chemotherapy (Corrie, 2008). However, a narrow therapeutic window due to severe off-target toxicity and lack of target specificity is a major drawback. To overcome the shortcomings of chemotherapeutic agents, drug development for the tumor-associated target based on its biological function

has led to the evolution of targeted cancer therapies.

DEVELOPMENT OF ANTIBODIES FOR TARGETED CANCER THERAPY

The basis for the development of antibodies for cancer therapy was initially to provide an alternative approach to reduce the undesirable systemic toxicity of chemotherapy; this approach has the advantage of antibody specificity for the tumor antigen to allow killing of the targeted tumor cells. Antibodies have become important therapeutic agents, as evidenced by the growing number of antibody-based drugs listed for US Food and Drug Administration (FDA) approval and for clinical development. The key to successful development of a therapeutic mAb is, in part, the rapid advancement and application of antibody engineering technology (Fig. 1). Currently, multiple approaches for immunotherapy are in development, including the use of unconjugated mAbs, mAb-toxin conjugates (immunotoxin conjugates), mAb-radionuclide conjugates (radio-immunoconjugates), and ADCs. Of these, the significance of therapeutic ADCs, with the emphasis on the emergence of ADC technology and optimization strategies, is highlighted in this review. Other antibody-based immunotherapies have been extensively reviewed elsewhere, and therefore are only briefly described herein, for comparisons with ADC technological development.

¹Kim, K. M. (2011) Trends in Antibody drug conjugate development. *KSBMB News* 31, 43-52.

Table 1. FDA-approved mAbs for cancer

Drug	Indication	Approved	Therapeutic Modality
Rituxan®	Non-Hodgkin's lymphoma (NHL)	1997	Unconjugated antibody (mAb)
Herceptin®	Breast cancer	1998	
Campath®	Chronic lymphocytic leukemia	2001	
Avastin®	Colorectal cancer	2004	
Erbix®	Colorectal cancer	2004	
Vectibix®	Colorectal cancer	2006	
Arzerra®	Chronic lymphocytic leukemia	2009	
Yervoy®	Melanoma	2011	
Perjeta®	Breast cancer	2012	
Gazyva®	Chronic lymphocytic leukemia	2013	
Opdivo®	Non-small cell lung cancer (NSCLC)/HL	2015/14	
Zevalin®	NHL (Yttrium-90 or Indium-111)	2002	Antibody-radionuclide conjugate (ARC)
Bexxar®	NHL (Iodine-131)	2003	
Adcetris®	Hodgkin's lymphoma (HL), ALCL	2011	Antibody-drug conjugate (ADC)
Kadcyla®	Breast cancer	2013	

Success and drawbacks of unconjugated mAbs for cancer therapy

A number of unconjugated mAbs have been approved for the treatment of various cancer types (Table 1), and have demonstrated promising clinical benefits. However, additional novel mAbs and improvements of current therapeutic mAbs are needed to further enhance the efficacy as exemplified herein. Rituxan® (Rituximab), a chimeric anti-CD20 antibody, was the first mAb approved by the FDA for NHL. CD20 is the surface marker present in >80% of NHL cases. A profound depletion of circulating B cells followed by complete recovery within a year of initial treatment was observed in most B cell NHL patients treated with Rituxan® (Maloney *et al.*, 1994). Although a ~50% overall clinical response rate in relapsed and refractory disease was observed, the complete response rate (CRR) was unacceptably low (<10%) (Eisenbeis *et al.*, 2003). A combination of Rituxan® with chemotherapy dosing regimens also lacked complete response, in spite of an improved response rate compared to standard therapy alone. In addition, acquired resistance to Rituxan® was observed for patients, which required an increased dose regimen for treatment of the recurrence (Trean *et al.*, 1991). In attempts to generate an improved anti-CD20 mAb, five FDA-approved mAb-based drugs for CD20 are currently available: rituximab, ibritumomab tiuxetan (Zevalin®), tositumomab-1131 (Bexxar®), ofatumumab (Arzerra®), and obinutuzumab (Gazyva®). These CD20-targeting therapeutic mAbs account for >30% of all current therapeutic mAbs for the treatment of hematologic and solid tumors, and reflect, in part, both the success and the need for further improvement of therapeutic mAbs.

Antibody-radionuclide conjugates (ARCs)

The next major advancement in immunotherapy following the use of unconjugated mAbs was arming the antibody with toxic molecules, such as diphtheria toxin and radionuclides (Moolten and Cooperband, 1970; Steiner and Neri, 2011). The concept of antibody-mediated delivery of radionuclides to the tumor site, using ARCs, hence radioimmune therapy, initially

failed in early clinical development due to an inadequate radiation dose delivered to the tumor site to obtain clinically meaningful responses. The considerations for ARC development, including the choice of antibody and radionuclide, have been discussed elsewhere (Koppe *et al.*, 2005; Steiner and Neri, 2011).

There are only two FDA-approved ARCs, Zevalin®, and Bexxar®, both of which were approved in early 2000, and both of which are conjugated to β -emitting radionuclides, ^{90}Y , and ^{131}I , respectively. Although both Zevalin® and Bexxar® utilize a murine-derived antibody, there are no other successful Zevalin® and Bexxar® antibodies (e.g., humanized or human mAb backbone), or other ARCs with FDA approval, despite the evidence for greater clinical efficacy compared to the unconjugated mAb (Morschhauser *et al.*, 2008). One of the possible explanations is the challenge associated with handling and scalability of ARCs, and the potential effects of radioactivity accumulation in normal cells.

ADCs

Since unconjugated mAbs possess modest antitumor efficacy as single agents, combination therapy with the mAb and a chemotherapeutic drug is now a routine clinical practice to achieve higher therapeutic efficacy. However, the off-target systemic toxicity of chemotherapy remains as a challenge. Therefore, an alternative approach to improve efficacy of mAb therapy, while integrating targeted selectivity of the chemotherapeutic drug, is to conjugate the mAb with a cytotoxic agent via a linker, known as an ADC (Fig. 2). Potent cytotoxic drug delivery of ADCs to tumors, with targeted specificity, would improve the therapeutic efficacy. However, because ADCs are drugs or potential drug candidates, in this review the cytotoxic molecule conjugated to the mAb will be referred to as a payload rather than a drug, unless the payload is the therapeutic drug, itself.

The first generation of ADCs was a mAb conjugated with an anticancer chemotherapeutic drug such as doxorubicin, because ADCs were designed to deliver cytotoxic agents to the

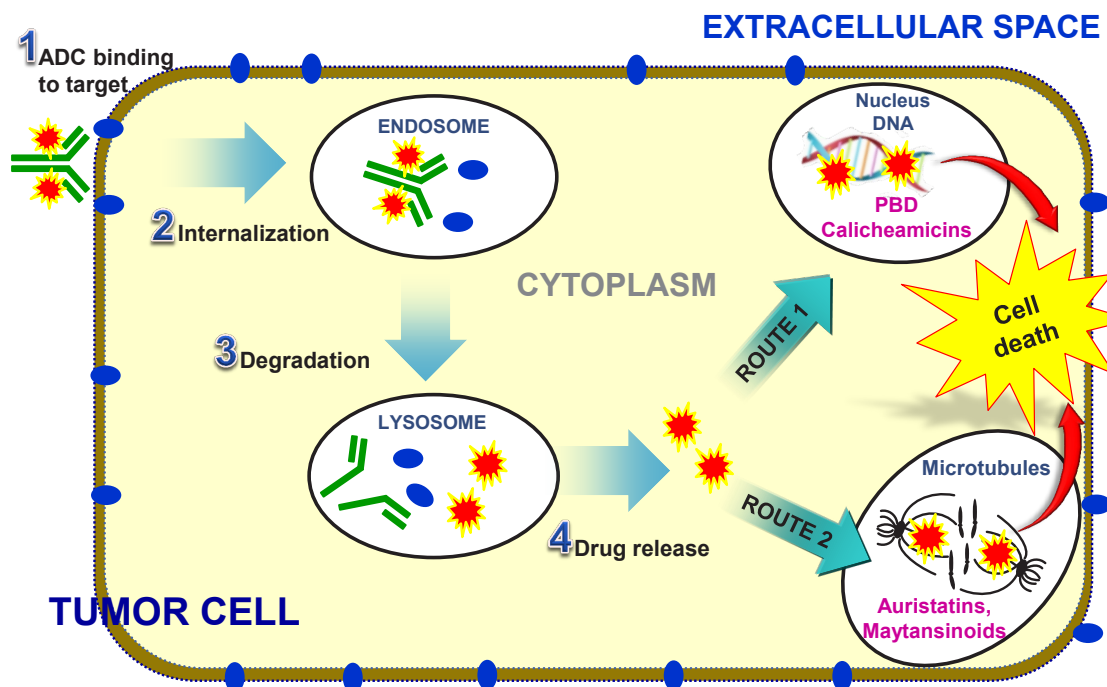


Fig. 2. Schematic representation of the mechanism of action of ADCs. Modified from Kitson *et al*, 2013².

specific tumor cells via tumor-specific antigens on the cancer cells (Yang and Reisfeld, 1988; Petersen *et al.*, 1991; Elias *et al.*, 1994). Unexpectedly, the clinical trials of these ADCs showed that they exhibited less potency than the corresponding free chemotherapeutic drugs. Thus, the lack of therapeutic efficacy in human clinical trials demonstrated that the chemotherapeutic drug was an unsuitable payload for ADCs (Trail *et al.*, 1993). Identification of potential issues in ADC development and optimization of ADC technologies, which are described in the following sections, have led to the development of FDA-approved ADCs including Mylotarg®, Adcetris®, and Kadcyla®. Currently, a number of ADCs are in clinical trials, and most of them are for cancer treatments, as listed in Table 2.

EMERGENCE OF THERAPEUTIC ADCs

Insufficient clinical benefits from the early ADCs were due to their lack of the core attributes for efficacious ADCs, which we now have a better understanding of the required potency of the cytotoxic agents, efficient internalization and stability of ADCs, and the microenvironment of the target antigen and the tumor. Some of the key drawbacks and historical significance of early preclinical and clinical studies of therapeutic ADC development are discussed below.

First generation of ADCs

Immunogenicity: ADCs with murine-derived antibody backbones were evaluated in clinical trials, but were soon discontinued due to an immune response involving development of human anti-murine antibodies (HAMA) in patients (Petersen *et al.*, 1991; Tolcher *et al.*, 1999). Much of this HAMA response

resulted from the antibody rather than the cytotoxic agents linked to the mAbs. This issue has been addressed with the advancement of antibody engineering technology for the generation of humanized and fully human antibodies (Fig. 1) (Kim, 2011¹).

Potency of cytotoxic payloads: BR96-Dox, an anti-Lewis^x mAb conjugated to doxorubicin via a hydrazone linker, failed in Phase II trials for metastatic breast cancer due to low potency of the doxorubicin as the payload and to the instability of the linker. Only 33% of the patients treated with BR96-Dox showed objective responses, despite high antitumor potency in preclinical studies (Trail *et al.*, 1993; Tolcher *et al.*, 1999; Saleh *et al.*, 2000). With the limitation of the ADCs' abilities to penetrate into tumors, along with limited target molecules on the cell surface ($<10^5$ copy numbers per cell), the need for highly potent payloads for ADCs was soon recognized. Current ADCs use cytotoxic agents to target tubulin (e.g., auristatin and maytansinoids), DNA (e.g., calicheamicin), or RNA (e.g., amanitin) with *in vitro* IC₅₀ values in the subnanomolar range. Paradoxically, these cytotoxic molecules are as much as 100~1000-fold more potent than the standard chemotherapeutic drugs and therefore have failed to enter the market.

Second generation ADCs

Gemtuzumab ozogamycin: Gemtuzumab ozogamycin (Mylotarg®) is considered a second generation ADC; but it was the first generation ADC drug to reach the market. Mylotarg® is an

²Kitson, S. L., Moody, T. S., Rozzell, D. and Caswell, J. (2013) Antibody-Drug Conjugates: Carbon-14 Labeling Requirements. Published on Drug Discovery & Development web site (www.dddmag.com), May 30, 2013.

Table 2. FDA-approved and selected ADCs in the clinic

ADC	Target	Antibody isotype	Payload	Phase	Indication(s)	Company
Brentuximab vedotin (Adcetis®, SGN-35)	CD30	ChlgG ₁	MMAE	Launched	HL/ALCL	Seattle Genetics/Takeda
Trastuzumab emtansine (Kadcyla®, T-DM1)	HER2	Hz IgG ₁	DM1	Launched	HER2+breast cancer	Roche(Genentech)/Immunogen
Inotuzumab ozogamicin (CMC-544)	CD22	Hz IgG ₄	Calicheamicin	III	ALL	Pfizer
Pinatuzumab vedotin (DCDT2980S, RG7593)	CD22	Hz IgG ₁	MMAE	II	NHL/DLBCL	Genentech
Polatuzumab vedotin (DCDS4501A, RG7596)	CD79b	Hz IgG ₁	MMAE	II	NHL/DLBCL	Genentech/Roche
SAR3419	CD19	Hz IgG ₁	DM4	II	NHL/DLBCL	Sanofi Pasteur
Milatuzumab doxorubicin (IMMU-110)	CD74	Hz IgG ₁	Doxorubicin	II	Multiple myeloma	Immunomedics
Lorvotuzumab mertansine (IMGN-901, huN901-DM1)	CD56	Hz IgG ₁	DM1	II	SCLC	Immunogen
BT-062	CD138	Hz IgG ₁	DM4	II	Multiple myeloma	Bio Test
Glembatumomab vedotin (CDX-011)	GPNCMB	Hu IgG ₂	MMAE	II	Breast cancer/Melanoma	Celldex Therapeutics
PSMA-ADC	PSMA	Hz IgG ₁	MMAE	II	Prostate cancer	Progenics
IMMU-130 (hMN-14-SN38, Labetuzumab-SN-38)	CD66e	Hz	SN-38	II	CRC	Immunomedics
IMMU-132 (hRS7-SN38ADC)	TROP-2	Hu IgG ₁	SN-38	II	Epithelial cancer	Immunomedics
SC16LD6.5	SC-16	n.d.	D6.5	I/II	SCLC	Stemcentrx
ABT-414	EGFR	Hu IgG ₁	MMAF	I/II	Squamous Cell Tumors	Abbvie
BAY 79-4620 (3ee9-ADC)	CAIX	Hu IgG ₁	MMAE	I	Solid tumor	Bayer/Seattle Genetics
DEDN6526A (RG7636)	ETBR	n.d.	MMAE	I	Melanoma	Genentech/Roche
HuMax-TF-ADC (TF-011-MMAE)	TF	n.d.	MMAE	I	Solid tumor	Genmab
Anti-NaPi2b (DNIB0600A, RG7599)	NaPi2b	Hz IgG ₁	MMAE	I	Ovarian cancer/NSCLC	Genentech/Roche
Anti-STEAP1 (DSTP3086S, RG7450)	STEAP1	n.d.	MMAE	I	Prostate cancer	Genentech/Roche
IMGN853	FRα	Hz	DM4	I	solid tumor	Immunogen
SGN-CD33A	CD33	Hz	PBD dimer	I	AML	Seattle Genetics
SGN-LIV1A	LIV-1	Hz	MMAE	I	Breast cancer	Seattle Genetics
ASG-22ME (ASG-22M6E, AGS-22CE)	Nectin-4	Hu IgG ₁	MMAE	I	Urothelial Cancer	Seattle Genetics/Agensys
ASG15E-13-1, ASG-15ME	SLITRK6	Hu	MMAE	I	Bladder cancer	Seattle Genetics
SAR566658	CA6	Hu IgG ₁	DM4	I	solid tumor	Sanofi Pasteur
SGN-CD19A	CD19	Hz	MMAF	I	ALL/NHL	Seattle Genetics
SGN-CD70A (superseding SGN-75)	CD70	Hz IgG ₁	PBD dimer	I	RCC/NHL	Seattle Genetics
AGS-16M8F	ENPP3	Hu IgG ₂ k	MMAF	I	RCC/Prostate cancer	Astellas/Agensys
MLN0264	GCC	n.d.	MMAE	I	Gastrointestinal malignancies	Millenium
SYD985	HER2	Hz	Duocarmycin	I	Breast cancer	Synthon BV
IMGN289, J2898A	EGFR	Hz	DM1	I	Solid tumor	Immunogen
BAY-94-9343	Mesothelin	Hu IgG ₁	DM4	I	Mesothelioma	Bayer
IMGN529, K7153A	CD37	Hz IgG ₁	DM1	I	NHL/CLL	Immunogen
AMG 595	HER3	n.d.	DM1	I	GBM	Amgen
AMG 172	CD70	n.d.	DM1	I	RCC	Amgen
PF-06263507	5T4	Hz	MMAE	I	Solid tumor	Oxford BioMedica/Pfizer
IGN523	CD98	Hz	n.d.	I	AML	Igenica Biotherapeutics

Ch: chimeric, Hu: human, Hz: humanized, n.d.: not disclosed, HL: Hodgkin's lymphoma, ALCL: anaplastic large cell lymphoma, ALL: acute lymphoblastic leukemia, NHL: non-hodgkin's lymphoma, DLBCL: diffuse large B cell lymphoma, SCLC: small-cell lung cancer, CRC: colorectal carcinoma, AML: acute myelogenous leukemia, NSCLC: non-small-cell lung cancer, RCC: renal cell carcinoma, CLL: chronic lymphocytic leukemia, GBM: glioblastoma multiforme.

anti-CD33 mAb conjugated to calicheamicin as the payload via an acid-labile hydrazone linker. Mylotarg[®] was given accelerated approval for treatment of acute myeloid leukemia (AML) during the first relapse of patients >60 years of age (Bross *et al.*, 2001; Larson *et al.*, 2005). However, it was voluntarily withdrawn from the market in 2010 due to relative therapeutic benefit concerns associated with hepatic veno-occlusive disease (VOD) and lack of sufficient activity (Giles *et al.*, 2001).

The clinical development of Mylotarg[®] involved humanization of murine P67.6 antibody and linker optimization. *In vivo* evaluation of calicheamicin conjugated to P67.6 demonstrated better antitumor efficacy for the conjugates using a carbohydrate than using an amide linkage group (Hamann *et al.*, 2002). In addition, insufficient conjugation efficiency of calicheamicin was observed; only ~50% of the mAb was conjugated with approximately 4-6 targeted calicheamicins per antibody, and the remaining 50% of the mAbs was unconjugated. The implication of these results is that no one linker fits all ADCs. Therefore optimization of the linker and the conjugation method is likely necessary for each targeted antigen with each ADC, on a case-by-case basis. Indeed, improved *in vivo* efficacy was observed using anti-CD70-MMAF conjugated with a 6-maleimidocaproyl hydrazone linker, compared to the corresponding conjugate using the 6-maleimidocaproyl-valine-citrulline (vc) linker (McDonagh *et al.*, 2008). Various strategies for optimization of ADCs have been applied toward the development of the third generation ADCs now on the market (Kadcyla[®] and Adcetris[®]), as well as following next-generation ADCs currently in clinical and pre-clinical development.

OPTIMIZATION STRATEGIES FOR ADCs

Target selection for ADCs

During therapeutic ADC development, most efforts involved the optimization of antibody, payload, and linker components of the ADC, which can be readily evaluated and optimized. However, the inherent features of the target are more difficult to address. Consequently, target selection remains one of the critical factors, especially in ADC development. Some of the principles and criteria that should be considered for the selection of good therapeutic ADC targets are discussed below.

High target expression level: Ideal ADCs targeting tumor-specific antigens are those that are exclusively and abundantly expressed on tumor cells and seldom expressed on normal cells. However, potential immunotherapeutic targets are often expressed on both tumor and normal cells. Thus, this is one of the most important criteria to be considered, as the level of target expression will ultimately dictate the therapeutic efficacy of ADCs. Since the antitumor activity of the ADC begins with binding to the target, followed by internalization into the tumor cells, higher expression levels of the target will result in more ADC localized on the tumor cells (Fig. 2). This ultimately results in higher intracellular concentration of the payload, which should enable more effective killing of the tumor cells. Considering only a fraction of administered antibody-based drugs are accumulated in the tumor, high target expression is therefore critically important (Scott *et al.*, 2007; Kim *et al.*, 2008). It was reported that significantly elevated CD30 expression in Hodgkin's lymphoma (HL) and an anaplastic large cell lymphoma (ALCL) was observed, but its expression was

limited to activated T and B cells (Gerber, 2010; Deutsch *et al.*, 2011). The anti-CD30 drug conjugate (Adcetris[®]) was successfully developed to target CD30-positive cancers. Another aspect of target expression levels to consider is the homogeneity of target expression within the tumor type and among target-positive patients. Consequently, a thorough evaluation of target expression profiles and selection of the target with the greatest difference in expression level between cancer and normal cells should be performed.

Internalization of target: Internalization of ADC upon binding to the target is often necessary for optimal efficacy of the ADC, because cytotoxic payloads typically act on intracellular targets. However, internalization of target antigen, alone, does not appear to be a prerequisite for ADCs to function. Non-internalizing or insufficiently internalizing antigens, such as alternatively spliced extra domains A and B of fibronectin and CD20, were successfully targeted by ADCs in preclinical *in vivo* xenograft models (Perrino *et al.*, 2014). Additionally, ADC internalization via target-mediated endocytosis can be impacted by the tumor microenvironment, as was observed for inhibition of ADC internalization of targeting CD19 by high CD21 expression (Ingle *et al.*, 2008).

Other attributes: Another characteristic of the antigens that may also reduce the binding of ADCs to the targets is the shedding or secreting of antigens, leading to a potentially higher risk of toxicity. Additionally, targets associated with non-solid tumors are expected to have better clinical responses to ADCs than do the solid tumors. Indeed, the first two FDA-approved ADCs, Mylotarg[®] and Adcetris[®], are approved for non-solid tumors. However, antigen shedding and tumor type are not absolute limiting factors. As one example, the expression levels of targets and internalization of the ADCs are exemplified by HER2 that is targeted by Kadcyla[®]. Only about 20% of breast cancer patients are HER2 positive, and soluble HER2 is systemically measurable and represents the target expressed on solid tumors (Wong, 1999; Gajria and Chandarlapaty, 2011). In addition, shedding of CD30 from HL-derived L540 cells was reported as an indication of disease activity, but was successfully targeted by Adcetris[®] (Horn-Lohrens *et al.*, 1995).

Antibody selection for ADCs

Strategies for optimization of therapeutic mAbs, such as increasing specificity, affinity, and pharmacokinetics (PK) can be applied to therapeutic ADC development. The tools for generation of more potent therapeutic antibodies have been extensively reviewed elsewhere, and are not described herein. This section is focused on the features of an antibody as a components of the ADC.

Structure of the Antibody: ADCs currently in development are comprised of the complete IgG antibody, which is likely optimal due to the favorable PK properties when compared to antibody fragments. Most ADCs on the market and in clinical development are the IgG1 isotype. Only a few of the ADCs in development are IgG2 or IgG4, as is AGS-16M8F (anti-ENPP3 IgG2-MMAF) and inotuzumab ozogamicin (anti-CD22 IgG4-calicheamicin), respectively. However, a systematic comparison of antitumor efficacy for a panel of anti-CD70 antibodies of various IgG isotypes conjugated to a monomethyl auristatin phenylalanine (MMAF) payload demonstrated comparable *in vivo* efficacy between IgG1 and IgG2 conjugates (McDonagh *et al.*, 2008). In contrast, a reduced therapeutic index for the

IgG4 conjugate compared to IgG1 and IgG2 conjugates could have been partially due to *in vivo* Fab arm exchange and/or the shorter half-life of IgG4 in mice than in humans (van der Neut Kolfschoten *et al.*, 2007). Antibody fragment drug conjugates with potent antitumor efficacy as the IgG-drug conjugate have been reported, although compensation by the dosing regimen was necessary (Kim *et al.*, 2008). Thus, the isotype of the antibody is one possible factor for the conjugation strategy of the payload and the stability of the ADC.

Effector function of the antibody: Antibodies having effector functions supported by IgG1 and bisecting N-glycosylation can further enhance the efficacy of the ADC. However, the efficacy of the ADC is less significant than the payload delivered by the ADC. Anti-CD70, the antibody component for SGN-70A ADC, has antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) functions. Nonetheless, equivalent efficacy among the anti-CD70-MMAF conjugates with IgG1, IgG1v lacking FcγR binding, and IgG2 isotypes was reported (McDonagh *et al.*, 2008). Additionally, the CD30 diabody-MMAF conjugate lacking the Fc domain to support effector function but having *in vivo* antitumor activity as the parent IgG-MMAF conjugate further supports the concept that the majority of ADC efficacy comes from the cytotoxic payload (Kim *et al.*, 2008).

Engineering of antibody: Binding specificity and affinity of ADCs for the targets are additional critical factors to efficacious immunotherapy. However, efforts to engineer antibodies for ADCs have been directed toward control of the conjugation site and for the stoichiometry of the payload for generation of homogenous ADC products. Detailed examples of antibody engineering for such approaches, including insertion of natural and artificial amino acids, are described below (under the *Methods for site- and stoichiometric-specific conjugated drugs* section of this review).

Efficient internalization of antibody: Internalization is critical for ADCs to exert cytotoxic functions on tumor cells. Therefore, failure of ADCs to internalize will result in poor efficiency of payload release, and thus, low efficacy. A recent report on the enhancement of internalization of cetuximab and a cetuximab-drug conjugate resulting in improved therapeutic efficacy further supports the importance of ADC internalization (Chen *et al.*, 2015). Current studies to identify ideal ADCs have been directed to the issue of the ADCs being rapidly internalized upon binding to their targets, while very high affinity of the antibody for the target is less desirable because this may inhibit the internalization of the ADC. However, there is no direct evidence to support a correlation between internalization rate and efficacy of ADCs. On the contrary, trastuzumab-DM1 drug conjugate demonstrated potent antitumor activity as ADC, even though internalization rates of trastuzumab is relatively slower than when other antibodies were used in the ADC (Hommelgaard *et al.*, 2004; Lewis Phillips *et al.*, 2008).

Cytotoxic payloads for ADCs

ADCs developed to date rely on the internalization of the ADC, and release of active cytotoxic molecules inside the tumor cell. Since expression levels of target antigens on the tumor cells are often limited, the inherent potency of the payload must be sufficient to kill the tumor cell, even at low concentrations. Consequently, an ideal payload for ADCs should have

in vitro subnanomolar IC₅₀ values toward tumor cell lines, and a suitable functional group with adequate solubility in aqueous solutions for conjugation reactions with the antibody and for solubility of the resulting ADC. Additionally, a Phase I clinical study with [¹¹¹In]-ch806 showed <0.1% of an injected antibody dose per gram of tumor was localized in humans (Scott *et al.*, 2007), which re-emphasizes the vital importance of payload potency for the ADC efficacy. Therefore, payloads suitable for ADCs are limited. Derivatives of auristatin, maytansinoid, calicheamicin, durcomycin, pyrrolbenzodiazepines, and amanitin are currently being used as payloads.

Auristatins: The highly potent antimitotic compound, dolastain 10, was discovered from the sea hare *Dolabella auricularia*; the antiproliferative effect of dolastain 10 is due to the inhibition of tubulin polymerization leading to cell death (Bai *et al.*, 1990). While dolastain 10 failed the clinical trials as a single agent for cancer therapy due to its off-target toxicity, its synthetic analog auristatins (MMAE and MMAF) are currently being used as payloads in ADCs (Table 2). Antibody conjugated with MMAE through a cleavable and self-immolative dipeptide-based linker releases free potent MMAE drug, which can diffuse through the cell membrane and induce bystander killing of neighboring target cells (Okeley *et al.*, 2010). In contrast, antibody conjugated with MMAF via a non-cleavable linker releases potent MMAF bound to the cysteine residue, and lacks bystander effects presumably due to impermeability of the drug from the carboxylic acid at the C-terminus of MMAF. The first and only auristatin-based FDA-approved ADC is anti-CD30-MMAE (brentuximab vedotin, Adcetris®) to treat HL and relapsed systemic ALCL.

Maytansinoids: Maytansine, isolated from the shrub *Maytenus ovatus* and its derivatives, also binds to tubulin and results in mitotic arrest (Remillard *et al.*, 1975; Oroudjev *et al.*, 2010). Maytansine was also tested in clinical trials due to its potent antimitotic effect, but did not demonstrate a significant therapeutic index among patients with different cancer types (Ravry *et al.*, 1985; Cassady *et al.*, 2004). However, antibody conjugated with maytansine derivatives, maytansinoids (DM1 and DM4), via cleavable disulfide linkers released free DM1 and DM4 that were membrane-permeable and induced a bystander effect. A number of ADCs with an average of 3–4 maytansinoids per antibody have entered clinical trials. Among these, trastuzumab-DM1 (adostrastuzumab emtansine, Kadcyla®) is the first and only maytansinoid-based ADC approved for metastatic breast cancer.

Calicheamicin: Chemical compounds that target DNA are also used as payloads for ADCs. Calicheamicin from *Micromonospora calichensis* is one of the more potent DNA cleaving agents (Lee *et al.*, 1992), and a derivative of calicheamicin-γ-1, N-acetyl-γ-calicheamicin (referred as calicheamicin, hereafter), is now being used to conjugate to a mAb. The cleavage site recognized by calicheamicin is the minor groove of DNA with preference for a TCCTAGGA sequence (Ellestad, 2011). Unlike the tubulin inhibitors, an acid-labile hydrazone linker is often used to conjugate calicheamicin to lysine residues of the antibody. For example, Mylotarg® is anti-CD33 IgG4 conjugated to calicheamicin through a hydrazone linker. Speculations concerning the systemic toxicity of Mylotarg® include instability of the hydrazone linker, poor conjugation efficiency of the drug to the antibody, Fab arm exchange of Mylotarg® with serum antibody, and the ADC binding to undesirable CD33-positive hepatic cells in the liver. It is also worth noting that inotuzumab

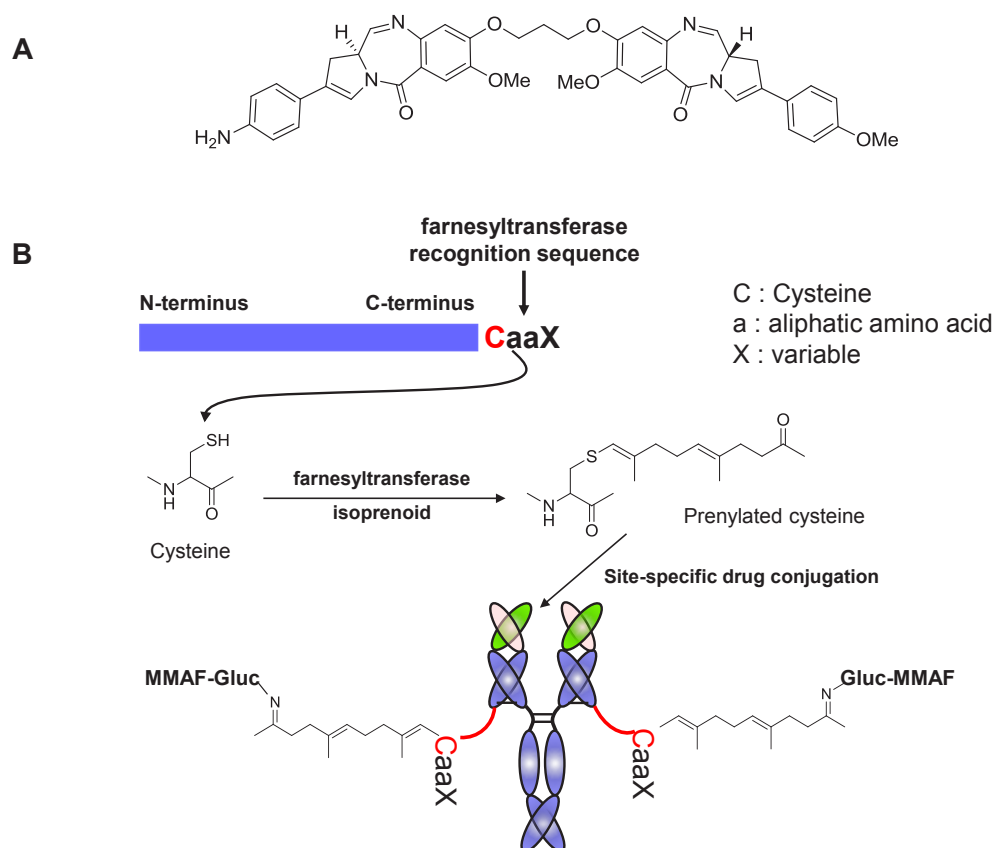


Fig. 3. Representative examples of emerging technologies for ADCs. (A) Structure of pyrrolobenzodiazepine dimers (PBD, SGD-1882), novel cytotoxic payload undergoing clinical evaluation for anti-CD33 and anti-CD70 conjugates. Modified from Kung Sutherland *et al.* (2013). (B) Schematic representation of chemo-enzymatic bioconjugation for site specific and stoichiometric specific attachment of cytotoxic payload using engineered CaaX tag.

ozogamicin, an anti-CD22 IgG4 calicheamicin conjugate via a hydrazone linker, is the most advanced calicheamicin-based ADC in clinical evaluation for aggressive and indolent NHL (Kantarjian *et al.*, 2012).

Duocarmycin: Duocarmycins, isolated from *Streptomyces* species represent one of the most potent antitumor antibiotics, with IC_{50} in the 40-100 pM range. Duocarmycins bind to the minor groove of DNA and alkylate adenine bases on DNA (Boger, 1993; Boger and Johnson, 1995). Currently, the synthetic derivatives and analogs of duocarmycin, such as CC1065, are being used for the development of ADCs. Of these, anti-HER2 antibody conjugated to a duocarmycin analog via novel SpaceLink technology is of particular interest. These ADCs are highly potent in P-glycoprotein-expressing multidrug-resistant (MDR) cell lines (DLD-1 and HCT-15) with subnanomolar IC_{50} values, and demonstrate *in vivo* antitumor efficacy at low doses (De Groot, 2011³).

MDX-1203 is an anti-CD70 conjugated to the duocarmycin derivative MED-2460 via a cleavable valine-citrulline linker. This ADC utilizes a multilayered mechanism for the activation of cytotoxic payloads to maximize the therapeutic index.

Following internalization of MDX-1203 into targeted cells, the prodrug MED-2460 is released from the antibody by cleavage of the linker via a lysosomal protease; the prodrug MED-2460 is then activated by carboxyl esterase to form an "active drug" form of MED-2460. This activated MED-2460 cytotoxic payload then alkylates AT-rich regions in the minor groove of DNA. The Phase I clinical evaluation in patients with clear cell renal cell carcinoma (ccRCC) or B cell NHL has been completed. However, the study results and the stability of this multilayered MED-2460 payload have not been reported.

Amatoxins: Amatoxins are bicyclic octapeptides found in poisonous mushrooms such as the green death cap mushroom *Amanita phalloides* (Hallen *et al.*, 2007). Amatoxins are potent and selective inhibitors of RNA polymerase II, a vital enzyme in the synthesis of mRNA, and thus inhibit protein synthesis (Lindell *et al.*, 1970). The chiHEA125-Ama, anti-EpCAM conjugated to α -amanitin, which is one of the predominant forms of the amatoxins, showed potent *in vitro* antiproliferative activity against multiple cancer cell lines, as well as *in vivo* antitumor efficacy in a pancreatic xenograft model (Moldenhauer *et al.*, 2012). Recently, improved *in vivo* antitumor efficacy of anti-PSMA- α -amanitin, conjugated via a protease-cleavable linker through a lysine residue on the antibody, was observed when compared to the cor-

³De Groot, V. (2011) Novel ADC linker-drug technology for next generation ADC products. In World ADC summit-Frankfurt.

responding conjugate through a cysteine linkage (Hechler *et al.*, 2014⁴). However, the details of the linkers were not disclosed and amanitin-based ADC is yet to be demonstrated in humans. Nonetheless, the potential advantages of the amanitin-based ADCs include higher solubility and uniformity, due to greater hydrophilicity than other cytotoxic payloads. This could further advance the development of therapeutic ADCs.

Other ADC payloads: In addition to the aforementioned cytotoxic payloads, other molecules being investigated include derivatives of pyrrolbenzodiazepines [(PBDs) SGD-1882], doxorubicin, and centanamycin (indolecarboxamide), which all bind to DNA and either alkylate or intercalate into the DNA (Fig. 3) (Beck *et al.*, 2011). Among the emerging and useful payloads, the PBD-containing ADCs (SGN-CD33A and SGN-CD70A) are currently in clinical evaluations. PBDs were originally isolated from *Streptomycin* species and covalently bound to discrete sequences in the minor groove of DNA, resulting in their antitumor activities. Doxorubicin also exerts its cellular cytotoxicity by inhibition of DNA synthesis via DNA intercalation and binding to topoisomerase, which is required for DNA replication. Although doxorubicin has shown a modest antitumor potency, milatuzumab-doxorubicin conjugate is currently in Phase I/II clinical trials for CD74-positive multiple myeloma, due to high uptake of anti-CD74 by targeted cells (Sapra *et al.*, 2005). Although optimization and identification of novel cytotoxic molecules for the future development of ADCs are actively being investigated, current cellular targets of payloads are often limited to tubulin, DNA, or RNA. Thus, the development of other targeted drugs having different modes of action that could improve the therapeutic index for cancers including MDR and tumor antigens with low and heterogeneous expression, are still needed.

Effect of payloads on ADC efficacy and stability

Additional cell killing via the bystander effect of membrane-permeable payloads (e.g., MMAE and PBD) compared to the less membrane-permeable payloads (e.g., MMAF) was reported (Li *et al.*, 2015⁵). Thus, variation of cell permeability via modification in combination payloads and linkers may offer a better selection of linker-payloads, and thus, improve the efficacy for the target of interest. The molar ratio of the payload attached to the antibody, also known as the drug-to-antibody ratio (DAR), has shown to adversely affect the PK of the antibody *in vivo*. ADCs with 8 DAR cleared more rapidly than the corresponding unconjugated antibody. In contrast, the PK of ADCs with 2~4 DAR were generally comparable to the unconjugated mAbs (Hamblett *et al.*, 2004), resulting in greater *in vivo* antitumor efficacy than ADCs with 8 DAR presumably due to increased exposure of the ADC, because slower clearance of ADC would result in a greater PK area under the curve (AUC).

Linker

One of the fundamental lessons learned from the first gen-

eration of ADCs is that a suitably stable linker is as vital as the antibody and payload for maximization of therapeutic efficacy of the ADC. The ideal linker is systemically stable so that biophysicochemical property of ADC are similar to that of the unconjugated antibody, but are still able to release the payload at the target site. Extensive research is being conducted to develop novel linkers for ADCs, and the most broadly evaluated and utilized linker platforms include both cleavable and non-cleavable linkers.

Cleavable linkers: The cleavable linkers include chemically-labile (e.g., hydrazones and disulfides) and protease-labile linkers. These cleavable linkers are designed to be stable in circulation, but release the toxic payloads due to differences between the extracellular and intracellular microenvironment following internalization of the ADC. For example, the acid-labile hydrazone linker of Mylotarg[®] liberates calicheamicin when encountering an acidic pH environment such as found in lysosomes and endosomes (pH 4~6) (van Der Velden *et al.*, 2001; Ulbrich and Subr, 2004). Similarly, disulfide linkers have the advantage of differential reduction potential in the cytosol, so that reduction of the disulfide bond can subsequently liberate payloads, as in the anti-CD56-maytansine conjugate (Saito *et al.*, 2003; Erickson *et al.*, 2006; Chanan-Khan *et al.*, 2010⁶). It is worth noting that the presence of a sterically-hindered carbon near the sulfur atom in the disulfide linker increases the stability of the disulfide bond, thus providing an equivalent or improved *in vitro* potency.

The peptide-based linkers are also designed to be retained in the ADC form in circulation, but to release their payload upon cleavage by specific intracellular proteases. For example, Adcetris[®] uses the valine-citrulline (vc) dipeptide linker, which is hydrolyzed by cysteine protease cathepsin B in lysosomes following endocytosis (Doronina *et al.*, 2003). Cathepsin B has been reported to be a tumor-specific protease due to its elevated expression and activity in certain tumors (Koblinski *et al.*, 2000; Mason and Joyce, 2011). The cleavage of ADCs containing the dipeptide-based linkers by a protease initially releases the cytotoxic payload-amino acid adduct, which then undergoes spontaneous self-immolation, and ultimately releases the free cytotoxic payload. The comparison of linker stability between hydrazone and dipeptide demonstrated greater stability, lower toxicity, and greater antitumor efficacy of ADCs linked with dipeptides than with hydrazone (Doronina *et al.*, 2003; Sanderson *et al.*, 2005).

Other optimization strategies for protease sensitive linkers include use of the β -glucuronide linker, which is recognized and hydrolyzed by β -glucuronidase for payload release (Jeffrey *et al.*, 2005). β -glucuronidase is a lysosomal enzyme overexpressed in some tumor types (Albin *et al.*, 1993). Thus, ADCs with a glucuronic acid-based linker provide a potential improvement for ADC stability in the circulation. Additionally, the hydrophilic nature of this linker can provide better solubility of the intact ADC compared to the dipeptide-based ADC. Indeed, ADCs with glucuronic acid-based linkers showed improved solubility of the intact ADC compared to the self-immolative p-aminobenzylcarbamate dipeptide ADC, while the

⁴Hechler, T., Kulke, M., Müller, C., Pahl, A. and Anderl, J. (2014) Amanitin-based antibody-drug conjugates targeting the prostate-specific membrane antigen [Abstract 664]. In AACR Annu. Meet. San Diego, CA. Philadelphia (PA)

⁵Li, F., Emmerton, K. K., Jonas, M., Zhang, X. and Law, C.-L. (2015) Characterization of ADC bystander killing in admixed tumor model [Abstract 5507]. In AACR Annu. Meet.

⁶Chanan-Khan, A., Wolf, J., Garcia, J., Gharibo, M., Jagannath, S., Manfredi, D., Sher, T., Martin, C., Weitman, S., O'Leary, J., Zildjian, S., Bulger, E. and Vescio, R. (2010) Efficacy Analysis from Phase I Study of Lorvotuzumab Mertansine (IMGN901) Used as Monotherapy in Patients with Heavily Pre-Treated CD56-Positive Multiple Myeloma [Abstract 1962]. ASH Annu. Meet.

efficacy was comparable to ADCs linked with vc linkers (Jeffrey *et al.*, 2006; Jeffrey *et al.*, 2007). Recently, Burke *et al* reported PEGylated β -glucuronide-MMAE linkers that improved PK stability of ADCs with eight DARs, and increased potency in xenografts compared to the non-PEGylated controls (Burke *et al.*, 2015⁷). However, clinical improvement of the therapeutic window for ADCs using glucuronic acid-based linkers has yet to be demonstrated.

Non-cleavable linkers: In contrast to the cleavable linkers, non-cleavable linkers that possess potent antitumor activity were unexpectedly discovered. Non-cleavable thioether and maleimidocaproyl (mc) linkers were initially synthesized for use as controls for the evaluation of cleavable linker conjugates. However, ADCs linked with these non-cleavable linkers, such as huC242-MCC-DM1 and cAC10-L4-MMAF, were as active as the conjugates with the cleavable linkers (Doronina *et al.*, 2006; Erickson *et al.*, 2006). Studies on the mechanism of action of the non-cleavable linker conjugates showed that antibody degradation of ADC components in lysosomes, following internalization, was necessary and resulted in release of “active” cytotoxic payload derivatives. Interestingly, the payload from non-cleavable ADCs remained covalently bonded to the linker via the residues to which the linkers were conjugated (Doronina *et al.*, 2006; Erickson *et al.*, 2006; Alley *et al.*, 2008). This payload derivative then subsequently killed the target cells. Thus, non-cleavable linkers can provide greater stability and tolerability, as well as potentially greater therapeutic windows compared to the conjugates with cleavable linkers. However, additional efficacy from bystander effects are not expected with non-cleavable drug conjugates, presumably due to the cell’s impermeability to the “hydrophilic” drug-linker complex. However, a potentially reduced off-target toxicity compared to the cleavable linker conjugates was observed (Polson *et al.*, 2009).

Some ADCs with non-cleavable linkers showed *in vivo* efficacy that was better than ADCs with cleavable linkers. For example, the anti-CD70-mcMMAF conjugate demonstrated an improved therapeutic index, as a result of higher MTD and antitumor efficacy in various renal cell carcinoma models compared with anti-CD70-vcMMAF conjugates (Ofiazoglu *et al.*, 2008). Similarly, the thioether-linked trastuzumab-DM1 (trastuzumab-MCC-DM1) conjugate showed improved efficacy, PK, and tolerability compared to the disulfide-linked trastuzumab-DM1 (Lewis Phillips *et al.*, 2008). The thioether-linked huC242-MCC-DM1 conjugate, however, showed more unfavorable *in vivo* efficacy in xenograft tumor models than the disulfide-linked huC242-SPDB-DM4 conjugate, even though the thioether-linked conjugate had improved stability with comparable *in vitro* efficacy as did the huC242-SPDB-DM4 conjugate (Tolcher *et al.*, 2003; Kellogg *et al.*, 2011). Therefore, the ultimately achieved therapeutic window of the ADC with non-cleavable linkers will likely be dependent on the biology of the target, the target cells, and the delivery of antibody for subsequent lysosomal degradation. Nonetheless, the expectation of potential enhancement of stability and tolerability for ADCs with non-cleavable linkers has directed research efforts toward novel linker development as an optimization strategy for ADCs.

⁷Burke, P. J., Hamilton, J. Z., Jeffrey, S. C., Hunter, J. H., Doronina, S. O., Okeley, N. M., Anderson, M. E., Senter, P. D. and Lyon, R. P. (2015) Optimization of a PEGylated glucuronide-auristatin linker for antibody-drug conjugates [Abstract 648]. In AACR Annu. Meet.

Innovative emerging linkers: Linker optimization has extended to the emergence of a “tunable” linker for payload conjugation at the linker site, rather than at the antibody. Novel SpaceLink technology developed by Syntarga (acquired by Synthron in 2011) utilizes highly flexible linkers, such that the linkers can reversibly attach the payload to the antibody in a modular fashion. This, in turn, enables selection and optimization of the payload and linker-payload combination to generate ADCs with maximal therapeutic potential for the target. SpaceLink technology uses unique linker chemistry to conjugate payloads via hydroxyl groups, and cleavage of the linker triggers spontaneous release of the payloads (De Groot *et al.*, 2007). The proof-of-concept for this emerging technology was demonstrated with anti-HER2-duocarmycin conjugates, which showed *in vivo* antitumor efficacy with minimal off-target toxicity (De Groot, 2011³). This technology may have broader utility and could be generalized for ADC production where the stoichiometry of drug loading is more important than the site of drug attachment.

Perhaps less intuitive in ADC development is the prediction of the optimal linker-payload combination to achieve the most efficacious and tolerable ADC for given targets. Therefore, a throughput systematic approach for the linker and payload selection that will minimize optimization would further advance ADC development. One potential method is the use of radiolabeled linkers and payloads for ADCs, which may help identify the metabolites and free payloads in the circulation using xenograft models (Kitson *et al.*, 2013²).

Methods for site- and stoichiometric-specific conjugated drugs

Homogeneous ADC production may become a prerequisite for FDA approval for future ADC development and use. It has been shown that optimal drug attachment for ADCs is 2–4 DARs for favorable efficacy with PK profiles comparable to that of the corresponding unconjugated mAbs (Hamblett *et al.*, 2004). However, ADCs generated through conventional conjugation methods on the solvent accessible residues result in heterogeneous ADCs containing a mixture of 0–10 DARs. Consequently, the development of conjugation methods for controlled site and stoichiometric drug attachment has been extensively investigated. Strategies for conjugation in generating such homogenous ADCs can include optimization of antibody, drug, and the linker.

Antibody engineering for conjugation: Typically, a cytotoxic molecule is attached to the antibody via alkylation of cysteine or acylation of lysine on the mAb through “controlled” but “random” conjugation reactions, which produces a mixture of ADCs. Modification at lysine is less preferable than cysteine, due to the greater number of lysine residues on mAbs that are solvent accessible for conjugation. Conjugation at cysteine following partial reduction of interchain disulfide bonds also produces heterogeneous ADCs. Thus, antibody engineering has been extended for controlled conjugation reactions to enable the production of homogeneous ADCs with defined sites and stoichiometric drug loading. Both insertion and deletion of cysteine residues in the mAb backbone have been approached to improve the homogeneity of ADCs, as used in anti-MUC16, anti-HER2, anti-CD70, anti-CD33, and anti-CD30 conjugates (McDonagh *et al.*, 2006; Junutula *et al.*, 2008a; Junutula *et al.*, 2008b; Kim *et al.*, 2008). THIOMAB, a mAb with an engineered cysteine for site-specific conjugation with

2 DARs, not only improved homogeneity and yields of ADCs, but also demonstrated improved efficacy and toxicity profiles in a cynomolgus monkey model compared with conventionally generated ADCs (Junutula *et al.*, 2008b; Junutula *et al.*, 2010; Shen *et al.*, 2012).

Other recombinant technologies employed for antibody engineering were the insertion of unusual amino acids such as selenocysteine (Se-Cys) and acetyl phenylalanine into the antibody backbone for site-specific conjugation. Se-Cys is a bio-orthogonal analog of cysteine with a selenol group in place of the thiol group. Utilization of engineered Se-Cys for site-specific conjugation of mAbs and Fab fragments has been recently demonstrated using rituximab as a prototype (Hofer *et al.*, 2009). Similarly, N-acetylphenylalanine utilizes an oxime linkage for conjugation between the alkoxy-amine group of the drug linker and the N-acetylphenylalanine of the antibody (Liu *et al.*, 2007; Axup *et al.*, 2012;). Although ADCs prepared with unnatural amino acids have *in vivo* efficacy, this conjugation method requires co-expression of properly paired unnatural amino acid tRNA synthetase and suppressor tRNA (Wang *et al.*, 2003; Young *et al.*, 2010).

Chemo-enzymatic bioconjugation: Other novel approaches investigated for the controlled conjugation of payloads to antibodies included the chemo-enzymatic bioconjugation approaches, using enzymes, such as glycosyltransferase, transglutaminase, and formyl glycine generating enzyme (FGE). The catalytic activity of mutant galactosyltransferase (1,4Gal-T1-Y289L) for the transfer of activated C2-keto-Gal glycan to the glycosylation site at Asn-297 of mAbs has been reported previously (Ramakrishnan and Qasba, 2002; Boeggeman *et al.*, 2007). Most importantly, antibodies with modified C2-keto-Gal enable subsequent selective conjugation to biomolecules with orthogonal reactive group, while retaining their target binding specificity and affinity (Boeggeman *et al.*, 2009). LegoChem Biosciences also developed a method which uses cysteine residues with the CaaX tag engineered into an antibody backbone for generation of functionally active conjugation sites via farnesyltransferase (Fig. 3) (Kim *et al.*, 2014).

Other chemo-enzymatic bioconjugation methods include the use of glutamine and aldehyde tag inserts. Use of transglutaminase for ADC generation utilizes the advantage that the enzyme does not recognize the naturally occurring glutamine residues, but recognizes glutamine in the glutamine tag (LLQG) located in a flexible region (Jeger *et al.*, 2010; Strop *et al.*, 2013). Conjugation of the glutamine tag-engineered antibodies with amine-containing dolastatin produced site-specifically labeled homogeneous ADCs by covalent bonding between the glutamine side chain of the tag and the primary amine of the drug. The *in vivo* efficacy was comparable to the conventional conjugates using cysteine residues. FGE recognizes cysteine in aldehyde tags (CxPxR peptide) and specifically oxidizes the cysteine to an aldehyde-bearing formyl glycine; hence, the aldehyde tag can be subsequently conjugated with aldehyde-specific chemical compounds (Carrico *et al.*, 2007; Rabuka *et al.*, 2012). This method also appears promising for site-specific conjugation of antibodies, although co-expression of enzymes with the aldehyde tagged antibody is required.

Various techniques for the incorporation of functional groups into proteins for controlled drug conjugation have been developed as discussed in this section. Some of these conjugation methods resulted in improvement of homogeneity, PK profiles,

efficacy, and greater tolerability, and resulted in additional improvements in ADC production. However, further investigations are needed to evaluate the generality and scalability of the conjugation technology, and to compare the efficacy and tolerability of ADCs prepared using different conjugation methods for identification and standardization of payload attachments.

ADCs IN THE CLINICAL AND PRECLINICAL PIPELINE

Advancement of ADC core technology development has led to their approval and strategically designed ADCs, including site- and DAR-specific ADCs, are currently in clinical and preclinical developmental stages (Table 2). Adcetris®, an anti-CD30-vcMMAE conjugate with ~4 DAR was approved to treat HL and relapsed systemic ALCL. Adcetris® is the first approved drug in over 30 years for HL treatment. CD30 is abundantly and selectively expressed on HL and Reed-Sternberg cells, while its expression is highly restricted to activated B and T lymphocytes and natural killer (NK) cells (Deutsch *et al.*, 2011). Unconjugated anti-CD30 antibody was also tested in clinical trials for HL and ALCL, but its modest antitumor clinical efficacy hampered further advancement beyond Phase II clinical trials (Ansell *et al.*, 2007; Forero-Torres *et al.*, 2009). In contrast, Adcetris® demonstrated overall response rates of 75% (34% CRR) for HL and 86% (53% CRR) for ALCL resulting in an accelerated approval of Adcetris® (Katz *et al.*, 2011; Pro *et al.*, 2012; Younes *et al.*, 2012). Adcetris® is currently in various clinical trials to broaden its therapeutic potential for HL treatments into earlier lines of therapy [Phase III AETHERA (ClinicalTrials.gov #NCT01100502) and ECHELON-1 (ClinicalTrials.gov #NCT01712490)], and for NHL indications [Phase III ECHELON-2 (ClinicalTrials.gov #NCT01777152)] (Seattle Genetics, 2014; Moskowitz *et al.*, 2015). Based on the positive results from the Phase III AETHERA, which demonstrated a significant increase in median progression-free survival from 24 months to 43 months (Moskowitz *et al.*, 2015), supplemental Biologics License Application for FDA approval of Adcetris® for HL patients at high risk of relapse post-autologous stem cell transplant, is anticipated in the last quarter of 2015.

The most advanced of the ADC drug candidates in the clinic, that has yet to be launched, is inotuzumab ozogamicin (CMC-544), an anti-CD22-calicheamicin conjugate in Phase 3 for relapsed or refractory CD22-positive acute lymphoblastic leukemia (ALL). CD22 is a cell surface sialoglycoprotein expressed on over 90% of leukemic lymphoblasts in a majority of B-lineage ALL patients. Remarkable clinical response rates from Phase I/II studies were observed for CD22-positive ALL patients treated with CMC-544 (Leonard *et al.*, 2004; Dijo-seph *et al.*, 2007). Notably, CMC-544 uses the same antibody backbone (humanized IgG₄), hydrazone linker, and payload as Mylotarg®. As would be expected, similar primary toxicities (thrombocytopenia and neutropenia) and development of hepatotoxicity for patients who underwent hematopoietic stem cell transplantation were observed for both CMC-544 and Mylotarg® (Advani *et al.*, 2010; Ricart, 2011; Jain *et al.*, 2014). Nonetheless, the stability of CMC-544 in systemic circulation appears to be better than Mylotarg®.

Most ADCs in clinical use, including Adcetris® and Kadcyla®, are heterogeneous ADCs that differ in drug conjugation

sites and DAR number. However, the most advanced stage for site- and DAR-specific ADC in clinical use is polatuzumab vedotin (DCDS4501A), indicated for diffuse large B cell lymphoma (DLBCL) and NHL (Dorman *et al.*, 2009; Morschhauser *et al.*, 2014⁹). DCDS4501A contains anti-CD79b THIOMAB which utilizes an engineered cysteine for conjugation to the MMAE drug. The preliminary results of Phase II ROMULUS trials (ClinicalTrials.gov #NCT01691898) showed higher clinical response rates for DCDS4501A when given in combination with rituximab, than for DCDS4501A, alone. However, approximately 35% of the patients experienced severe side effects with primary toxicities of neutropenia, peripheral neuropathy, and diarrhea (Morschhauser *et al.*, 2014⁹). Combination studies of DCDS4501A plus rituximab with other ADCs to reduce peripheral neuropathy are either ongoing or in the planning stages. Other THIOMAB-based conjugate in clinical studies include the anti-STEAP1-MMAE conjugate (RG7450) for prostate cancer (Phase I).

ADCs with recently emerging payloads are SGN-CD33A and SGN-70A, both of which use a PBD dimer cytotoxic agent, and are currently in Phase I trials for the treatment of AML (for SGN-33A), and NHL and renal cell carcinoma (for SGN-70A). It is important to note that these ADCs have the PBD dimer conjugated to the engineered cysteine (S239C) of the antibody via a protease-cleavable maleimidocaproyl-valine-alanine dipeptide linker for homogenous ADC products with a 2 DAR specification (Kung Sutherland *et al.*, 2013). Preclinical studies of SGN-CD33A demonstrated improved potency over its predecessor Mylotarg[®] against a panel of AML cell lines and xenograft models with the MDR phenotype (Kung Sutherland *et al.*, 2013). Additionally, interim efficacy and adverse event analyses from an ongoing Phase I trial of SGN-CD33A demonstrated promising therapeutic potential as an anti-leukemia drug primarily for the elderly, relapsed patients who were not candidates for other therapies; in the study, 77% of the patients treated with SGN-CD33A at ≥ 40 $\mu\text{g/kg}$ in ongoing Phase I trials showed at least a 50% reduction in bone marrow lymphoblasts with low off-target toxicity associated with underlying myelosuppression (Stein *et al.*, 2014¹⁰). Glematuzumab vedotin (CDX-011) is another interesting ADC in Phase II trials, used to target the glycoprotein NMB (gpNMB), which is overexpressed in 40-60% of breast cancers. CDX-011 is composed of an IgG2 antibody,

⁹Morschhauser, F., Flinn, I., Advani, R. H., Sehn, L. H., Kolibaba, K. S., Press, O. W., Salles, G. A., Diefenbach, C. S., Tilly, H., Assouline, S. E., Chen, A. T.-Y., Dreyling, M. H., Hagenbeek, A., Zinzani, P. L., Cheson, B. D., Yalamanchili, S., Akiko, Lu, D., Chai, A., Chu, Y. W. and Sharman J. P. (2014) Preliminary results of a phase II randomized study (ROMULUS) of polatuzumab vedotin (PoV) or pinatuzumab vedotin (PiV) plus rituximab (RTX) in patients (Pts) with relapsed/refractory (R/R) non-Hodgkin lymphoma (NHL) [Abstract 8519] In ASCO Annu. Meet.

⁹Morschhauser, F., Flinn, I., Advani, R., Diefenbach, C., Kolibaba, K., Press, O., Sehn, L., Chen, A. and Salles, G. (2014) Updated Results of a Phase II Randomized Study (ROMULUS) of Polatuzumab Vedotin or Pinatuzumab Vedotin Plus Rituximab in Patients with Relapsed/Refractory Non-Hodgkin Lymphoma. Lymphoma: Therapy with Biologic Agents, excluding Pre-Clinical Models: Poster III [Abstract 4457]. In ASH Annu. Meet.

¹⁰Stein, E. M., Stein, A., Walter, R. B., Fathi, A. T., Lancet, J. E., Kovacs, T. J., Advani, A. S., DeAngelo, D. J., O'Meara, M. M., Zhao, B., Kennedy, D. A. and Erba, H. P. (2014) Interim Analysis of a Phase I Trial of SGN-CD33A in Patients with CD33-Positive Acute Myeloid Leukemia (AML) [Abstract 623, oral presentation]. In ASH Annu. Meet.

Table 3. Discontinued ADCs

ADC	Target	Antibody/isotype	Drug/linker	Reasons for discontinuation	Year	Phasereached
Mylotarg	CD33	Hu IgG ₄	Calicheamicin/hydrazone	Failure to demonstrate clinical benefit	2010	III
IMGN242	CanAg	n.d.	DM4/SPDB	n.d.	2009	II
SGN-15	LeY carbohydrate	Ch	Doxorubicin/Hydrazone	Change in business strategy	2005	II
CMB-401	MUC1	Hu IgG ₄	Calicheamicin/hydrazone	Lack of clinical efficacy	1999	II
Vorsetuzumab mafodotin (SGN-75)	CD70	Hu IgG ₁	MMAF/mc	Lack of clinical efficacy; 2nd generation SGN-CD70A	2013	I
ASG-5ME	SLC44A4	Hu IgG ₂	MMAE	Strategic reasons	2013	I
IMGN388	Integrin α v β 3	Hu	DM4/SPDB	Change in business strategy	2011	I
BAY79-4620	CAIX	Hu IgG ₁	MMAE/vc	n.d.	2011	I
BLIB015	Cripto	Hu IgG ₁	DM4/SPDB	n.d.	2011	I
AV/E9633	CD33	Hu IgG ₁	DM4/SPDB	Lack of clinical efficacy	2008	I
Bivatuzumab mertansine	CD44v6	Hu	DM1/SPP	Safety issues: fatal case of toxic epidermal necrolysis	2006	I
MLN2704	PSMA	Hu	DM1/SPP	n.d.	2006	I
CMD-193	Le ^y carbohydrate	Hu	Calicheamicin/hydrazone	n.d.	2006	I
MED1547	EphA2	n.d.	MMAF/mc	Safety issues: bleeding and coagulation events	2012	n.d.

Ch: chimeric, Hu: human, Hz: humanized, n.d.: not disclosed.

a less common IgG backbone used for ADC in clinical trials (Table 2). The Phase II primary end point data showed a progression-free survival of 33% and was efficacious for patients who had advanced triple-negative breast cancer (i.e., tumors lacking estrogen, progesterone, and HER2, but were gpNMB positive). Additional Phase II METRIC trials (ClinicalTrials.gov #NCT0199733) will provide confirmation of whether CDX-011 can improve potential clinical benefits for the drug-resistant metastatic breast cancers associated with current treatments.

Additional ongoing clinical studies of ADCs include CD19-targeting ADCs, including SAR3419 and SGN-19A, both of which appear to have a similar MTD and therapeutic potential as the newer drug candidates. SAR3419 is an anti-CD19-DM4 conjugate under development by Sanofi using ImmunoGen ADC technology. Although SAR3419 for ALL has been discontinued due to lack of therapeutic efficacy compared to its competitors (Sanofi, 2014), promising clinical results against DLBCL were observed. The Phase II STARLYTE (ClinicalTrials.gov #NCT01472887) trial of SAR3419 showed >40% response rate as a single agent in patients with relapsed or relapsed/refractory CD19-positive DLBCL, and among the responding patients whom had not responded to first line treatment (Trneny *et al.*, 2014¹¹). SGN-19A, being developed by Seattle Genetics, uses MMAF as its payload and releases Cys-mcMMAF that induces apoptosis of CD19-positive targeted cells. Preclinical results of SGN-19A in combination with standard of care R-ICE or R-CHOP in NHL (Heather *et al.*, 2015¹²), and in combination with CAVD in ALL models showed superior antitumor efficacy over SGN-19A alone (Stone *et al.*, 2015¹³). The results from a Phase I open-label and dose-escalation study in NHL showed a 40% response, and 30% of the patients achieved a complete response (Law *et al.*, 2011¹⁴). The randomized Phase II trial for relapsed DLBCL planned for 2015 may provide stronger efficacy data to support this new therapeutic drug candidate. Additional comparative clinical results between SGN-CD19A and SAR3419 may further provide insight into the improvement of therapeutic ADC development.

CHALLENGES AND PERSPECTIVES FOR FUTURE ADC DEVELOPMENT

Extensive research focused on each component of the ADC that contributes to successful therapeutic ADC development, and a more informed selection of ADC target strategies

have led to the approval of ADCs and increases in ADCs in the pipeline (Table 2). The addition of inotuzumab ozogamicin for ALL indications and the promising clinical data for extension of Adectris® in additional therapeutic indications are anticipated sometime later this year. The evolution of cancer therapy from targeted unconjugated mAbs to ADC for better clinical outcomes will drive the development of the next generation of immunotherapies for cancer.

However, discontinuation of some ADCs in clinical development (Table 3) may also occur due to insufficient clinical efficacy or safety concerns related to the payload toxicology. Inotuzumab ozogamicin, for example, was recently discontinued for NHL indication in Phase III trials due to lack of clinical efficacy that did not correlate well with the preclinical *in vivo* disease model studies. These are limitations that are difficult to resolve, and thus, remain as challenges that need to be addressed to further enhance the therapeutic window of ADCs. Future challenges and perspectives for therapeutic ADCs are discussed below.

Homogeneous ADC products are likely required to obtain FDA approval in the near future. And such homogeneous products are also desired by ADC drug manufacturers since better PKs and safety profiles are anticipated with reduction in undesirable higher DAR mixtures in the ADC product. Consequently, the technological development in site-directed conjugation chemistry, along with antibody engineering, will continue to emerge for the development of homogenous ADCs; these are the gold standard attributes for conjugated biological drugs. Future research must improve the solubility of the payload or the linker to mask payload hydrophobicity and thus improve the current site-directed conjugation technology to further enhance physiobiochemical and PK stability of ADCs (Zhao *et al.*, 2011). The solubility of the payload, however, needs to be carefully chosen to control the bystander cytotoxicity effects.

Recently emerging cytotoxic payloads, including the PBD dimer and α -amanitin, targeting at the DNA and RNA levels, respectively, have demonstrated clinical or preclinical antitumor efficacy. Likewise, the development of novel cytotoxic payloads with different cellular targets and metabolic processes could be additional areas of focus to improve clinical responses and broaden therapeutic options for cancer treatment. In particular, greater opportunities and challenges exist for the development of payloads and linkers that are non-substrates for drug-efflux pumps to bypass MDR resistance for cancer therapies. Clinically proven payloads for ADCs (e.g., calicheamicin, MMAE, and DM1) are the substrates for MDR. Recently developed ADCs with a PBD dimer payload (e.g., SGN-CD33A) or with PEGylated linkers (e.g., anti-EpCAM-PEG4Mal-DM1) generated metabolites that were efficacious in MDR-expressing tumor cells, further demonstrating the potential enhancement of the therapeutic window for ADCs.

Perhaps the most intriguing preclinical developments of anticipated ADCs are the bispecific antibody-drug conjugates (BDCs) and antibody fragment drug conjugates (FDCs). Blinatumomab (Blinicyto®) is the first bispecific anti-CD19 and anti-CD3 mAbs approved by the FDA in 2014 for ALL, and ~20 bispecific mAbs are currently in clinical development. Cytotoxic drugs conjugated to the antibodies that target two tumor-specific antigens could provide better efficacy and safety, which in turn, would increase the therapeutic index above the corresponding conventional monospecific ADCs or the unconjugated bispecific antibodies. Thus, extension of ADC technol-

¹¹Trneny, M., Verhoef, G., Dyer, M. J., Yehuda, D. B., Patti, C. and Canales, M. (2014) Starlyte phase II study of coltuximab ravtansine (CoR, SAR3419) single agent: Clinical activity and safety in patients (pts) with relapsed/refractory (R/R) diffuse large B-cell lymphoma (DLBCL; NCT01472887). [Abstract 8506]. In ASCO Annu. Meet.

¹²Heather, A. V. E., Kerry, K., Martha, A., Weiping, Z., Devra, O., Maureen, R., Tina, A. and Law, C. L. (2015) Preclinical results of SGN-CD19A in combination with R-ICE or R-CHOP in non-Hodgkin lymphoma models [Abstract 2541]. In AACR Annu. Meet.

¹³Stone, I. J., Albertson, T. and Law, C. L. (2015) Preclinical combination activity of SGN-CD19A and CVD in patient-derived B-lineage acute lymphoblastic leukemia models [Abstract 1339]. In AACR Annu. Meet.

¹⁴Law, C. L., Sutherland, M., Miyamoto, J., Hayes, D., Duniho, S., Boursalian, T., Stone, I., Jonas, M., Smith, L. and Benjamin, D. (2011) Preclinical characterization of an auristatin-based anti-CD19 drug conjugate, SGN-19A [Abstract 625]. In AACR Annu. Meet.

ogy into bispecific antibody use for BDC development could provide further ADC optimization. The challenges associated with the identification of two targets that are preferentially expressed on the same tumor or in the microenvironment, that favors bispecificity and production of homogenous BDCs, must first be overcome to gain popularity.

FDC is an alternative ADC platform that may be developed again in the future. Antibody fragments such as diabody and Fabs were investigated in the past to improve tumor penetration. *In vivo* antitumor efficacy with faster drug accumulation in tumors for FDCs were demonstrated in preclinical studies (Kim *et al.*, 2008). Selection of appropriate antibody fragment backbones with balanced PKs via antibody modification and/or dosing regimens may provide additional clinical efficacy. If successful, both BDCs and FDCs would have significant impact on future ADC development.

The lessons learned from both unpromising and successful ADCs, along with the continued emergence of diverse ADC core technologies, will make future ADC development more successful for cancer treatments. The strategic design of effective ADCs for the treatment of other conditions, such as autoimmune diseases, could result from the current clinical trials, as some of the chemotherapeutic drugs, such as methotrexate and cyclophosphamide, are already used in diseases other than cancer. The improvement of the therapeutic window, elucidation of the ADC mechanism of action, and decrease of off-target toxicities remain as challenges for future ADC development.

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CONFLICT OF INTEREST

K.M.K is a former employee of Seattle Genetics and holds stocks in Seattle Genetics. The authors have no other potential conflicts of interest to disclose.

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