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Jeffrey Wang
Wei-Chiang Shen
Jennica L. Zaro *Editors*

Antibody-Drug Conjugates

The 21st Century Magic Bullets for Cancer

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Editors
Jeffrey Wang
Western University of Health Sciences
Department of Pharmaceutical Sciences
Pomona
California
USA

Wei-Chiang Shen
University of Southern California
Department of Pharmacology and
Pharmaceutical Sciences
Los Angeles
California
USA

Jennica L. Zaro
University of Southern California
Department of Pharmacology and
Pharmaceutical Sciences
Los Angeles
California
USA

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Contents

About the Editors

Dr. Jeffrey Wang received his PhD in pharmaceutical sciences from the University of Southern California in Los Angeles. Dr. Wang's research focuses on the application of principles of pharmacokinetics and drug metabolism in drug discovery and delivery, particularly in anti-cancer drug discovery and peptide and protein drug delivery. He has published more than 100 peer-reviewed articles, book chapters and meeting abstracts and is the co-inventor of several U.S. and international patents. Dr. Wang is currently Associate Professor of Pharmaceutical Sciences and Director of Graduate Education at the College of Pharmacy, Western University of Health Sciences, Pomona, California.

Dr. Wei-Chiang Shen is John A. Biles Professor in Pharmaceutical Sciences at the University of Southern California's School of Pharmacy in Los Angeles. Dr. Shen received his PhD in bio-organic chemistry from Boston University, Massachusetts. He has many years of experience on the conjugation of drug to macromolecules, including poly (amino acids), transferrin and antibodies. His current focus of research is on recombinant fusion proteins and cell-penetrating peptides. Dr. Shen has published about 150 papers, mostly in the area of drug delivery. He is an inventor or co-inventor of 16 issued U.S. patents and a Fellow of the American Association of Pharmaceutical Scientists and the American Association for the Advancement of Science.

Dr. Jennica L. Zaro is a Research Assistant Professor at the School of Pharmacy, University of Southern California, Los Angeles. Her main research projects are focused on targeted delivery of peptide and protein drugs and on recombinant bifunctional fusion proteins as therapeutics. Dr. Zaro is also the Core Director of the Translational Research Laboratory. Prior to joining the university, Dr. Zaro received her PhD in pharmaceutical sciences from the University of Southern California, where she focused on the design and characterization of peptide carriers for protein drugs. In addition, she has held several roles in the pharmaceutical industry, where she developed formulations for aerosol products and designed and validated analytical methods for protein drugs.

Contributors

Laird Bloom Global Biotherapeutic Technologies, Pfizer, Cambridge, MA, USA

Gregory Carven Global Biotherapeutic Technologies, Pfizer, Cambridge, MA, USA

Karen J. Cha Regulatory Affairs, Agensys, Inc., Santa Monica, CA, USA

Nancy Chan Department of Medicine, Weill Cornell Medical College, New York, NY, USA

Ravi Chari Chemistry and Biochemistry, ImmunoGen, Waltham, MA, USA

Bruce W. Hart Regulatory Affairs, Seattle Genetics, Bothell, WA, USA

E. Erica Hong Chemistry and Biochemistry, ImmunoGen, Waltham, MA, USA

Junyan A. Ji Late Stage Pharmaceutical Development, Genentech, South San Francisco, CA, USA

Jérôme J. Lacroix Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA, USA

Kedan Lin Preclinical and Translational Pharmacokinetics, Genentech Research and Early Development, South San Francisco, CA, USA

Jun Liu Late Stage Pharmaceutical Development, Genentech, South San Francisco, CA, USA

Yun Luo Department of Pharmaceutical Sciences, Western University of Health Sciences, Pomona, CA, USA

Sunil Prabhu Department of Pharmaceutical Sciences, Western University of Health Sciences, Pomona, CA, USA

Michael Ritchie Bioconjugates Discovery and Development, Pfizer, Pearl River, NY, USA

Puja Sapra Bioconjugates Discovery and Development, Pfizer, Pearl River, NY, USA

Wei-Chiang Shen Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, CA, USA

Siddharth Sukumaran Preclinical and Translational Pharmacokinetics, Genentech Research and Early Development, South San Francisco, CA, USA

Chalet Tan Department of Pharmaceutical Sciences, Mercer University, Atlanta, GA, USA

Linda T. Vahdat Department of Medicine, Weill Cornell Medical College, New York, NY, USA

Jeffery Wang Department of Pharmaceutical Sciences, Western University of Health Sciences, Pomona, CA, USA

Y. John Wang Late Stage Pharmaceutical Development, Genentech, South San Francisco, CA, USA

Zhijun Wang Center for Advancement of Drug Research and Evaluation, Western University of Health Sciences, Pomona, CA, USA

Jonathan Hirohiko Watanabe Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, CA, USA

Chen Xie Center for Advancement of Drug Research and Evaluation, Western University of Health Sciences, Pomona, CA, USA

Jennica L. Zaro Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, California, USA

Part I
Introduction

Chapter 1
Antibody-Drug Conjugates: A Historical Review

Wei-Chiang Shen

The origin of the antibody–drug conjugate (ADC) concept of has been attributed mostly to the "magic bullet" idea conceived by Paul Ehrlich more than 100 years ago (Strebhardt and Ullrich 2008). Indeed, Ehrlich was able to demonstrate the selective absorption of dyes with different chemical structures by various tissues, and thus the possibility of achieving a targeted delivery of drug to the disease site. More importantly, Ehrlich was one of the scientists responsible for the discovery of antibodies and was the first one to describe the unique "receptors" on the target cells that could be recognized by antibodies (Strebhardt and Ullrich 2008). Ehrlich's early work on small molecular dyes to target disease tissues, in combination with his later work on the specific recognition of target cells by antibodies, laid the foundation for the field of ADC for targeted drug delivery to treat human diseases.

However, the development of ADC, especially in cancer treatment, has made very little progress in the first half century since Ehrlich introduced the "magic bullet" concept in the early 1900s. The lack of any significant progress in ADC before 1970 is understandable. It was difficult to isolate and purify antibody from animal or human serum. It was almost impossible to produce a large quantity of a specific antibody that could be used to prepare ADC and to test its efficacy in therapy. Nevertheless, several pioneers in the ADC field used partially purified immunoglobulin preparations, mostly from rabbits or goats, to make drug conjugates. They demonstrated the feasibility of using antibody as a targeted carrier for the treatment of different types of cancer. Since this book focuses on recent developments of ADC with recombinant monoclonal antibodies, this chapter only covers the early history of ADC development from the late 1960s to early 1980s, i.e., during pre- and early post-monoclonal antibody era.

Mathé in 1957 reported that cell-specific antiproliferation activity against L1210 leukemia cells could be achieved when methotrexate was conjugated via a diazo

W.-C. Shen (✉)
Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA, 90033, USA
e-mail: weishen@usc.edu

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coupling reaction to antileukemia 1210 antigen immunoglobulins, but not to normal gamma globulin (Mathé et al. 1958). Although Mathé subsequently made many important contributions in the field of cancer immunotherapy (Mathé 1969), he did not follow-up his work on methotrexate–antibody conjugate after his historical report. After Mathé's publication, several other groups made more extensive investigations on ADC in the late 1960s and early 1970s. The most serious challenge at that time was how to translate the studies with animal immunoglobulins into clinical applications. The research was performed mostly in academic laboratories, with very little support from the pharmaceutical industry. Yet, with limitations of the quantity and purity of the immunoglobulins, early publications reported many observations that provided the foundation for further studies in the ADC field. For example, with an alkylating chemotherapeutic agent, it was demonstrated in early 1970s by Ghose and collaborators at Dalhousie University in Canada (Ghose and Nigam 1972; Ghose et al. 1975) and Rowland and colleagues at Searle Research Laboratory in the UK (Rowland et al. 1975) that a covalent conjugation between the immunoglobulin and the drug is essential to achieve the tumor targeting effect. Sela and colleagues at the Weismann Institute of Science in Israel reported in 1975 that daunomycin and Adriamycin could be linked covalently to anti-bovine serum albumin (BSA) immunoglobulins with various covalent reactions, but the retention of both drug and antibody activities was observed only with the periodate oxidation method (Hurwitz et al. 1975). This was the first report that indicated, with identical antibody and drug, the activity of ADC was dependent on the conjugation method. This finding opened a new area in ADC, i.e., the linker chemistry, which played an important role in the later design of ADC (Blair and Ghose 1983). More impressively, with the use of isolated animal immunoglobulins, ADC was already tested in human patients in several studies in the mid-1970s and showed promising results (Ghose et al. 1977; Oon et al. 1974). Ghose and Blair published one of the first comprehensive reviews on ADC in 1978 which covered ADC studies with immunoglobulins isolated from animal antiserum before 1977 (Ghose and Blair 1978). It is worthy to mention here that Moolten and Sigband reported in 1970 on the preparation of a conjugate between antileukemia immunoglobulins and diphtheria toxin with a specific toxicity against the leukemia cells (Moolten and Cooperband 1970). Their finding initiated a new class of cancer therapeutics, i.e., immunotoxin (Vitetta and Uhr 1985), which focuses on the use of protein toxins rather than conventional drugs.

The field of ADC surged in the 1980s for several reasons. First, and most importantly, was the successful production of monoclonal antibody by Milstein and Koch (Kohler and Milstein 1975). Monoclonal antibody technology has solved the issue in antibody production and purification. The first monoclonal antibody drug, Muromonab-CD3 (OKT3®), was approved by the Food and Drug Administration (FDA) in 1986 as an immunosuppressive agent for kidney transplantation (Ortho Multicenter Transplant Study Group 1985). Subsequently, the application of recombinant technology to produce humanlike antibody, first as chimeric antibody (Morrison et al. 1984) and later as humanized antibody (Jones et al. 1986), has greatly reduced the concern of immunogenicity of immunoglobulins as the classical murine monoclonal antibodies encountered. Second, many new biomarkers have been identified, such as HER2 (van de Vijver et al. 1988) and vascular endothelial growth

factor (VEGF; Kim et al. 1993), which allowed immunologists to focus not only on the structure but also on the function of antigens as targets in the design of antitumor monoclonal antibodies. Another reason that specifically helped the progress of the ADC field in 1980s was the better understanding of protein uptake in mammalian cells. Scientists were able to understand the intracellular processing of ADC in antigen-bearing cells through the knowledge of endocytosis. It became clear that one of the limiting steps in drug action of drug–macromolecular conjugates was the intracellular release of pharmacologically active drug (Shen and Ryser 1979). Thus, different types of linkages have been designed to facilitate the release of drug from the carrier macromolecules inside the cells based on the knowledge of cell biology such as endosomal/lysosomal proteolytic activity (Duncan et al. 1980; Monsigny et al. 1980; Trouet et al. 1982) and acidification (Shen and Ryser 1981). Many successful applications of linkage design in monoclonal antibody ADC preparation have been reported in 1980s (Gallego et al. 1984; Blattler et al. 1985; Dillman et al. 1988). To overcome the limitation of the amount of the active drug released inside the antigen-bearing cells, another approach was to increase the number of drugs that could be carried by an antibody molecule. Several approaches have been developed in 1980s to increase the drug loading per each antibody, such as using dextran (Manabe et al. 1984), albumin (Garnett and Baldwin 1986), or liposomes (Allen et al. 1995) as intermediate carriers between antibody and drug, as well as using IgM isotype that could accommodate more drug per each immunoglobulin molecule (Shen et al. 1986).

Based on many successful feasibility studies, the field of ADC became a mature research area in immunotherapy in 1990s. Pharmaceutical and biotech companies began to invest into this new area of the 100-year-old Ehrlich's "magic bullet" concept. New technology in the production of human monoclonal antibody such as the single-chain Fv polypeptides, scFv (Bird et al. 1988) and the phage-display method (McCafferty et al. 1990) has further promoted the idea of using monoclonal antibodies, including ADC, in therapeutics. There were seven monoclonal antibodies that were approved by FDA in 1990s for the treatment of various human diseases, and the number tripled in the next decade. The first ADC, gemtuzumab ozogamicin (Mylotarg®), was approved by FDA in 2000 for the treatment of acute myeloid leukemia (Ducry and Stump 2010). Even though gemtuzumab ozogamicin was withdrawn from the market in 2010; it was a milestone in the clinical application of ADC as a therapeutic drug. With recent approved brentuximab vedotin (Adcetris®) in 2011 and trastuzumab emtansine (Kadcyla®) in 2013, as well as several in clinical trials as described in following chapters of this book, ADC has finally been accepted as an established category of therapeutics.

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Part II
The ADC Construct

Chapter 2
Payloads of Antibody-Drug Conjugates

Chalet Tan

2.1 Introduction

Over the past several decades, three classes of exceedingly potent toxins have been discovered and evaluated for their antiproliferative activities, including maytansinoids, auristatins, and calicheamicins (Thorson et al. 2000; Luesch et al. 2002; Cassady et al. 2004). However, these toxins displayed serious toxicities *in vivo* at the dosing levels required for the anticancer efficacy, which precluded their further development as anticancer drugs.

The therapeutic application of these potent toxins in cancer therapy is now revived via the conjugation to monoclonal antibodies (mAbs) as the payloads (Chari 2008; Anderl et al. 2013). The molecular targets of these toxins are tubulin (for maytansinoids and auristatins) and DNA (for calicheamicins). Because of the ubiquitous presence of such targets in all normal and tumor cells, it is of crucial importance to maximize the delivery of these toxins to the tumor cells while minimizing the exposure to normal cells. The stable linkage of the toxins to the mAbs imparts the pharmacokinetic characteristics of mAbs to the payloads, which greatly increases the elimination half-lives of the toxins, restricts their nonspecific distribution to the healthy organs, and enhances the drug accumulation in the tumor tissues. Following receptor-mediated endocytosis of antibody–drug conjugates (ADCs), the payloads are released intracellularly via enzymatic cleavages and exert cytotoxicity against the tumor cells at the picomolar to nanomolar concentration range, which is about 100- to 1000-fold more potent than the conventional cytotoxins currently used in the clinic. In essence, ADCs offer a unique approach to achieve the targeted delivery of the payloads to the tumor cells with markedly improved therapeutic windows.

C. Tan (✉)
Department of Pharmaceutical Sciences, Mercer University, Atlanta, GA, USA
e-mail: tan_c@mercer.edu

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2.2 Maytansinoids

2.2.1 Chemistry and Anticancer Activity

Maytansine was initially isolated from an alcoholic extract of the bark of the African shrub *Maytenus serrata* and *Maytenus buchananii* (Kupchan et al. 1972; Kupchan et al. 1974). It was the first compound discovered in a class of ansa macrolide antibiotics named maytansinoids, which showed potent anticancer activity in human nasopharynx carcinoma KB cells (EC_{50}=8 pM), murine lymphocytic leukemia P-388 cells (EC_{50}=0.6 pM), and murine leukemia L1210 cells (EC_{50}=2 pM; Wolpert-Defilippes et al. 1975; Kupchan et al. 1978). A series of maytansine analogs bearing a disulfide or thiol substituent were recently synthesized to allow for covalent linkage with mAbs (Widdison et al. 2006). Among these, DM1 and DM4 are currently being pursued in the clinic as the ADC payloads.

The chemical structures of maytansine, DM1, and DM4 are shown in Fig. 2.1.

Studies on the structureactivity relationship of maytansinoids reveal that the C3 *N*-acyl-*N*-methyl-L-alanyl ester side chain, the C4–C5 epoxide moiety, the C9 carbonyl functional group, and the conjugated C11 and C13 double bonds are all essential to the antitumor activity of maytansinoids (Kupchan et al. 1978; Widdison et al. 2006). By contrast, the phenyl ring and the *N*'-acyl group are modifiable. Importantly, the nature of the acyl group can be varied without a significant loss in the activity. DM1 and DM4 form a stable covalent bond with mAbs in aqueous solution with high efficiency and yield (Widdison et al. 2006). Sulfhydryl groups or their respective thiolated anions react readily with maleimido moieties in a Michael-type addition reaction to form thioethers and with disulfide groups in a disulfide exchange reaction to form new disulfide bonds.

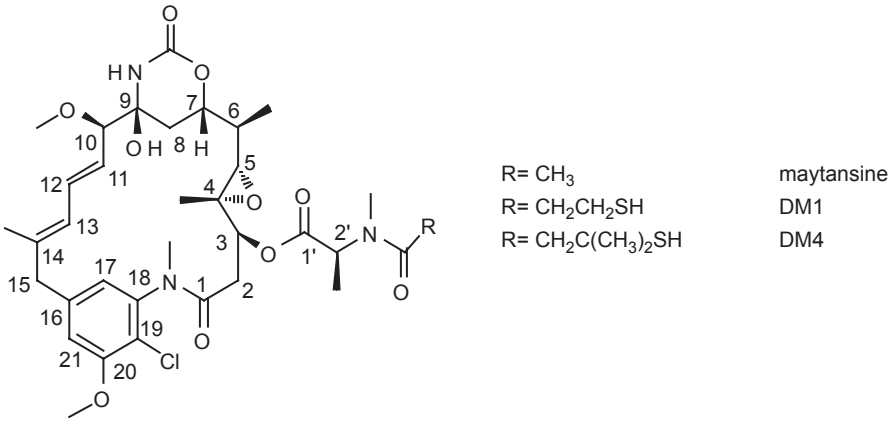


Fig. 2.1 Structures of maytansine, DM1, and DM4

2.2.2 Mechanism of Action

Maytansine blocks the polymerization of tubulin, arrests the cell cycle at G2/M phase, and inhibits mitosis (Remillard et al. 1975; Wolpert-DeFilippes et al. 1975). Maytansine appears to share a common binding site on tubulin with vinca alkaloids (Mandelbaum-Shavit et al. 1976), but is about tenfold more potent in inhibiting the binding of guanine nucleotides to tubulin at the exchangeable site (Huang et al. 1985). For DM1- or DM4-loaded ADCs to exert cytotoxicity, lysosomal processing is required irrespective of the linker type (Erickson et al. 2006). In addition to the intracellular release of DM1 and DM4 from the ADCs, *S*-methyl-DM4 is also formed in the tumor cells, which is shown to be more potent than maytansine in suppressing the dynamic instability of the microtubules (Lopus et al. 2010).

2.2.3 Early Preclinical and Clinical Experiences

Maytansine exerted potent growth inhibition against P-388 and L-1210 leukemia, Lewis lung carcinoma, and B-16 melanoma in mice at an intraperitoneal dose as low as 25 μg/kg/day for 9–10 consecutive days (reviewed in Issell and Crooke 1978).In mice, maytansine was rapidly eliminated with a terminal half-life less than 20 min (Chari et al. 1992). Encouraged by its preclinical activity, maytansine was evaluated in several phase I clinical trials in patients with advanced solid tumors in the late 1970s (reviewed in Issell and Crooke 1978). Maytansine was given intravenously, and the maximum tolerated dose (MTD) was found to be 2–2.5 mg/m^2 every 3–4 weeks as a single dose or divided over three daily doses. The dose-limiting toxicities of maytansine included nausea, vomiting, and neurotoxicity. Subsequently, the efficacy of maytansine was investigated in a number of phase II clinical trials in patients with ovarian, cervical, breast, head, and neck, small-cell lung, or other advanced cancers, at a dose of 0.75–1.8 mg/m^2 divided over three daily doses every 1–2 weeks. These studies demonstrated that maytansine had little antitumor efficacy at its tolerated doses in cancer patients. The conjugation of DM1 with mAbs was shown to increase the MTD of maytansine by at least twofold, allowing the safe delivery of therapeutically effective levels of the payload to the tumor (Tolcher et al. 2003; Krop et al. 2012).

2.2.4 ADC Development

Maytansinoids, DM1, and DM4 are being employed as the payloads in a dozen of ADCs that have been advanced to the clinic. The ADCs and their corresponding mAb targets, therapeutic indications, and payloads are summarized in Table 2.1.

Table 2.1 Summary of the clinically tested ADCs that employ maytansinoids as the payloads

ADC	Status	Therapeutic area	Target	mAb	Payload
Trastuzumab emtansine (T-DM1)	Approved	Breast cancer	HER2	Trastuzumab	DM1
Lorvotuzumab mertansine (IMGN901)	Phase I	SCLC, MM	CD56	huN901	DM1
IMGN529	Phase I	NHL, CLL	CD37	K7153A	DM1
SAR3419	Phase II	LBCL, ALL	CD19	Anti-CD19	DM1
BT-062	Phase II	MM	CD138	Anti-myeloma	DM1
AMG 172	Phase I	RCC	CD27L	Anti-CD27L	DM1
AMG 595	Phase I	Glioma	EFGRvIII	Anti-EGFRvIII	DM1
MLN2704	Discontinued	Prostate cancer	PSMA	MLN591	DM1
Cantuzumab mertansine (huC242-DM1)	Discontinued	Solid tumors	CanAg	huC242	DM1
IMGN388	Discontinued	Solid tumors	Integrin α_v	Anti-integrin α_v	DM4
IMGN853	Phase I	Solid tumors	Folate receptor 1	Anti-FOLR1	DM4
SAR566658	Phase I	Solid tumors	CA6	huDS6	DM4
AVE9633	Discontinued	AML	CD33	huMy9-6	DM4
BAY 94-9343	Phase I	Solid tumors	Mesothelin	Anti-mesothelin	DM4
BIIB015	Phase I	Solid tumors	Crypto	Anti-cripto	DM4

SCLC small-cell lung cancer, *RCC* renal cell carcinoma, *MM* multiple myeloma, *NHL* non-Hodgkin's lymphoma, *HL* Hodgkin's lymphoma, *LBCL* large B-cell lymphoma, *ALCL* anaplastic large-cell lymphoma, *ALL* acute lymphocytic leukemia, *CLL* chronic lymphocytic leukemia, *AML* acute myelogenous leukemia, *ADCs* antibody-drug conjugates

2.3 Auristatins

2.3.1 Chemistry and Anticancer Activity

Dolastatin 10 was initially isolated from the sea hare *Dolabella auricularia* at a vanishingly low yield (~1 mg from each 100 kg) (Pettit et al. 1987). It is a unique linear pentapeptide comprising of several unusual amino acids (Fig. 2.2a). Dolastatin 10 was found to possess a wide spectrum of anticancer activity against murine L1210 leukemia cells (IC_{50}=0.01–1 nM) (Bai et al. 1990), lymphomas (IC_{50}=0.1–1 pM) (Beckwith et al. 1993), ovarian (IC_{50}=0.05–1.8 nM) (Aherne et al. 1996),colon (IC_{50}=0.02–0.2 nM) (Aherne et al. 1996), and lung cancer cells (IC_{50}=0.03–0.18 nM) (Kalemkerian et al. 1999). Nevertheless, it was shown to be toxic towards hematopoietic progenitor cells (IC_{50}=0.1–1 pM) (Jacobsen et al. 1991), which

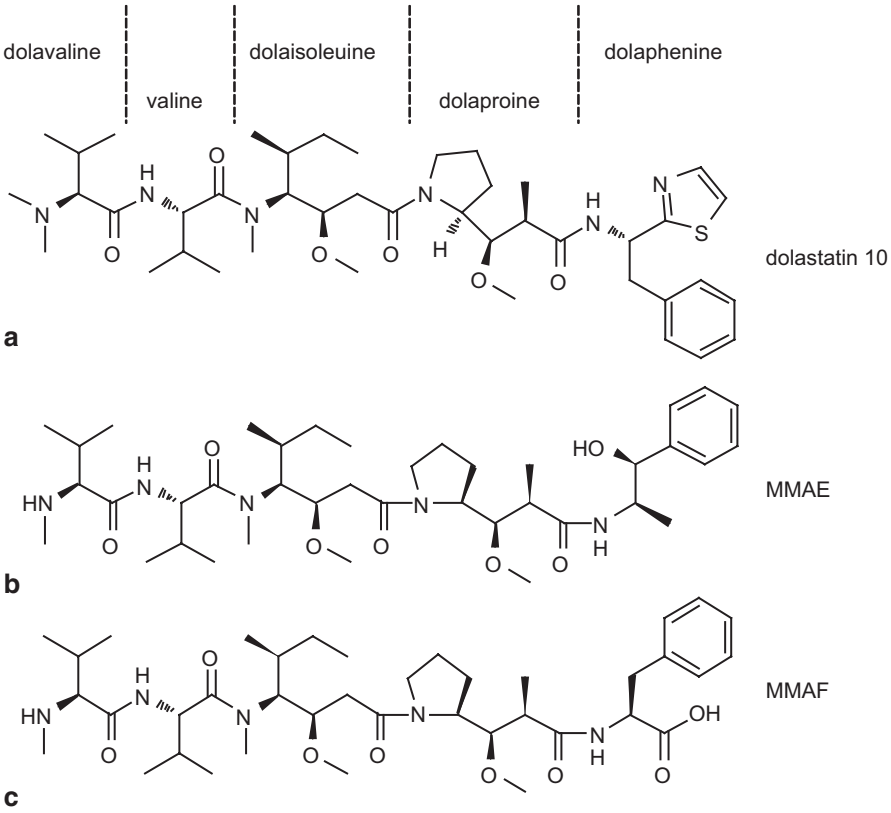


Fig. 2.2 Structures of dolastatin 10, MMAE, and MMAF. MMAE monomethyl auristatin-E, MMAF monomethyl auristatin-F

explains potent myelosuppression associated with dolastatin 10 treatment *in vivo*. The C-terminal portion of dolastatin 10 is shown to be important for its cytotoxicity, as the tripeptides missing dolaproine and dolaphenine moieties lack inhibitory activity against the cancer cells (Bai et al. 1993).

The analogs of dolastatin 10, monomethyl auristatin-E (MMAE, Fig. 2.2b) (Doronina et al. 2003), and monomethyl auristatin-F (MMAF, Fig. 2.2c) (Doronina et al. 2006) were designed as the toxic ADC payloads. Both MMAE and MMAF are peptide analogs, which have limited impact on the physicochemical properties of the mAbs. MMAF differs from MMAE owing to a phenylalanine moiety at its C-terminus, contributing to its membrane impermeability. MMAE and MMAF are both highly stable molecules, showing no signs of degradation in plasma, human liver lysosomal extracts, or proteases such as cathepsin B. As free toxins, the cytotoxicity of MMAE and MMAF is about 200- and 1000-fold less potent than that of dolastatin 10 in lymphoma cells, respectively. The conjugation of these toxins with cAC10, an mAb specific for CD30, restored their cytotoxicity against $CD30^+$ lymphoma cells similar to the level of dolastatin 10 (Francisco et al. 2003; Doronina et al. 2006).

2.3.2 Mechanism of Action

Investigation into the mechanism of cell cycle arrest by dolastatins 10 reveals that it impedes the polymerization of microtubules, suppresses tubulin-dependent guanosine-5'-triphosphate (GTP) hydrolysis, and inhibits the binding of vinca alkaloids to tubulin (Bai et al. 1990). As a result, dolastatin 10 blocks the microtubule assembly and mitosis, arresting the cancer cells in the G2/M phase.

2.3.3 Early Preclinical and Clinical Experiences

In vivo, dolastatin 10 demonstrated exceptional anticancer efficacy against murine P388 leukemia at doses as low as 1 μg/kg (Pettit et al. 1987). Preclinical pharmacokinetic study demonstrated that dolastatin 10 was highly protein-bound in plasma (>80%) and rapidly metabolized in the liver with an elimination half-life of 3.7 h in mice (Newman et al. 1994). The MTD in mice, rats, and dogs of dolastatin 10 was 1.35 μg/m^2 (0.45 μg/kg), 0.45 μg/m^2 (0.075 μg/kg), and not greater than 0.4 μg/m^2 (0.02 μg/kg), respectively (Mirsalis et al. 1999).

In patients with advanced solid tumors, the MTD of dolastatin 10 was reached at 0.3 μg/m^2 with granulocytopenia being the dose-limiting toxicity (Pitot et al. 1999; Madden et al. 2000). Peripheral neuropathy was also observed in some patients, a common toxicity for antimicrotubule agents. A three-compartment model adequately described the plasma concentration of dolastatin 10 versus time profile showing a rapid distribution phase ($t_{1/2,\alpha}$=4–6 min). Metabolism turned out to be a minor elimination route in humans. Subsequently, dolastatin 10 was evaluated in phase II trials in patients with soft-tissue sarcomas (Von Mehren et al. 2004), prostate (Vaishampayan et al. 2000), ovarian (Hoffman et al. 2003), and pancreatic/hepatobiliary carcinomas (Kindler et al. 2005). Although well tolerated at an i.v. dose of 0.4 μg/m^2 given every 3 weeks, dolastatin 10 failed to yield objective responses in cancer patients. These results clearly indicate the narrow therapeutic window of dolastatin 10. The conjugation of MMAE with anti-CD30 mAb allowed for the delivery of the payload in patients with CD30-positive hematological cancers at a dose over fivefold higher than the MTD of dolastatin 10, which was shown to be clinically efficacious (Katz et al. 2011).

2.3.4 ADC Development

MMAE and MMAF are being employed as the payloads in a number of ADCs (Table 2.2), which are currently being used or evaluated in the clinic for the treatment of leukemia, lymphoma, and solid tumors.

Table 2.2 Summary of the clinically tested ADCs that employ auristatins as the payloads

ADC	Status	Therapeutic area	Target	mAb	Payload
Brentuximab vedotin (SGN-35)	Approved	HL, ALCL	CD30	cAC10 (SGN-30)	MMAE
Glembatumumab vedotin (CDX-011)	Phase II	Breast, melanoma	GPNMB	CR001	MMAE
SGN-LIV1A	Phase I	Breast	LIV-1 (SLC39A6, ZIP6)	Anti-LIV-1	MMAE
DCDS4501A	Phase II	NHL, CLL	CD79B	Anti-CD79B	MMAE
DSDT2980S (RG7593)	Phase II	NHL, CLL	CD22	Anti-CD22	MMAE
AGS-22M6E (ASG-22CE)	Phase I	Solid tumors	Nectin-4	Anti-Nectin-4	MMAE
AGS15E	Phase I	Bladder	SLITRK6	Anti-SLITRK6	MMAE
BAY79-4620	Discontinued	Solid tumors	Carbonic anhydrase (CAIX)	Anti-CAIX	MMAE
PSMA ADC	Phase I	Prostate	PSMA	Anti-PMSA	MMAE
Vorsetuzumab mafodotin (SGN-75)	Discontinued	NHL, RCC	CD70	H1F6	MMAF
SGN-CD19A	Phase I	ALL, NHL	CD19	Anti-CD19	MMAF
AGS-16C3F	Phase I	RCC	ENPP3	Anti-ENPP3	MMAF
MEDI-547	Discontinued	Solid tumors	EphA2	Anti-EphA2	MMAF

ADCs antibody-drug conjugates, *HL* Hodgkin's lymphoma, *ALCL* anaplastic large-cell lymphoma, *NHL* non-Hodgkin's lymphoma, *CLL* chronic lymphocytic leukemia, *RCC* renal cell carcinoma, *ALL* acute lymphocytic leukemia, *MMAE* monomethyl auristatin-E, *MMAF* monomethyl auristatin-F

2.4 Calicheamicins

2.4.1 Chemistry and Anticancer Activity

Calicheamicin γ_1^I was initially isolated from a broth extract of a soil microorganism *Micromonospora echinospora calichensis*, which was found to possess the most potent antitumor activity among all members of the calicheamicins and was active against murine tumors at 0.5–1.5 μg/kg dose range (Lee et al. 1987a; Lee et al. b). It is an enediyne-containing anticancer antibiotic with unique structural features including a glycosylated hydroxyamino sugar and a labile methyltrisulfide group (Fig. 2.3a). A semisynthetic derivative of calicheamicin, *N*-acetyl-γ-calicheamicin

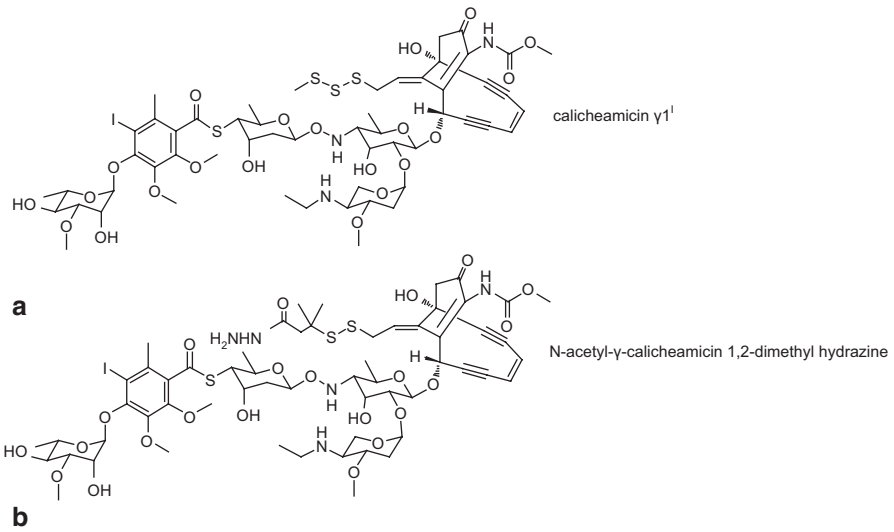
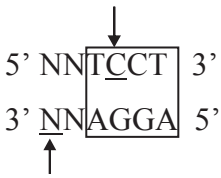


Fig. 2.3 Structures of calicheamicin γ_1^I and its dimethyl hydrazide

1,2-dimethyl hydrazine (Fig. 2.3b), has been developed as a payload toxin for ADCs (Hinman et al. 1993). While γ_1^I hydrazide itself was tenfold less cytotoxic than the parent γ_1^I, γ_1^I hydrazide-conjugated CT-M-01 antibody displayed the cytotoxicity equivalent to calicheamicin γ_1^I at pM (8 ng/ml) in human breast carcinoma MX-1 cells.

2.4.2 Mechanism of Action

The potent cytotoxicity of calicheamicin γ_1^I is ascribed to its remarkable ability to cleave double-strand DNA at specific sites, resulting in cell death (Zein et al. 1988). Without exception, the preferred site of attack is at the following DNA sequence with the cleavage positions indicated by arrows:



Calicheamicin γ_1^I binds to the double-helical DNA in the minor grove. The staggered nature of the cleavage sites on the double-stranded DNA and the remarkable efficiency/specificity of the scission reaction result from the unique fit between the

compound and the DNA (Zein et al. 1989; Ikemoto et al. 1995). For a double-strand scission to occur, the enediyne portion of the aglycone binds to the complementary cleavage site on the DNA. The presence of intracellular thiol cofactors initiates the aromatization of the diyne-ene moiety, followed by the generation of a non-diffusible 1,4-dehydrobenzene-diradical that mediates oxidation strand scission by hydrogen abstraction on the deoxyribose ring (Zein et al. 1989). The association of the thiobenzoate-carbohydrate moiety with the minor grove not only enhances the DNA-binding affinity with the aglycone but also help to position the biradical for the optimal reactivity with the DNA (Drak et al. 1991).

2.4.3 Early Preclinical Experiences

In the P388 leukemia mouse model, the MTD and the therapeutic dose of calicheamicin γ_1^I following the intraperitoneal administration were 1.25 and 5 μg/kg, respectively (Hinman et al. 1993). It became evident that although it had highly potent anticancer activity, calicheamicin γ_1^I caused lethal toxicities at a dose much lower than its tolerated dose. Calicheamicin γ_1^I had never been evaluated in the clinic due to its unfavorable therapeutic window. The conjugation of the dimethyl hydrazine derivative of calicheamicin γ_1^I with mAbs increased the MTD of calicheamicin γ_1^I more than tenfold, allowing for the delivery of a therapeutically effective dose of the toxin to the tumor (Hinman et al. 1993). The dose-limiting toxicities of calicheamicin γ_1^I-loaded ADCs included thrombocytopenia and neutropenia.

2.4.4 ADC Development

The dimethyl hydrazine derivative of calicheamicin γ_1^I has been employed in two clinically tested ADCs (Table 2.3). In both cases, the calicheamicin derivative is covalently linked to mAbs via the acid-labile 4-(4′-acetylphnoxy)butanoic acid linker.

Table 2.3 Summary of the clinically tested ADCs that employ calicheamicin as the payload

ADC	Status	Therapeutic area	Target	mAb	Payload
Gemtuzumab ozogamicin	Withdrawn	AML	CD33	hP67.6	Calicheamicin γ_1^I
Inotuzumab ozogamicin	Phase III	NHL, ALL	CD22	G544	Calicheamicin γ_1^I

NHL non-Hodgkin's lymphoma, *ALL* acute lymphocytic leukemia, *AML* acute myelogenous leukemia

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Chapter 3
Selecting an Optimal Antibody for Antibody-Drug Conjugate Therapy

Michael Ritchie, Laird Bloom, Gregory Carven and Puja Sapra

3.1 Target Selection for an ADC

3.1.1 Characteristics of an Optimal ADC Target

Selection of appropriate targets is likely the most important consideration for the success of an antibody–drug conjugate (ADC) development program. Target selection for ADCs can be classified into two principal approaches: target-first and antibody-first approach or agnostic approach (Fig. 3.1). In the target-first approach, a target is chosen based on a set of factors, including expression pattern, abundance and internalization properties, and an antibody-generation campaign is focused on isolation of antibodies against this target. In the agnostic approach, antibodies capable of binding to and internalizing in tumor cells are isolated, followed by retrospective identification of their targets. While the target-first approach may have the advantage of a higher degree confidence in the suitability and novelty of the target based on prior knowledge of target properties, it requires significant work before internalizing antibodies become available. By contrast, the agnostic approach leads quickly to reagents suitable for testing hypotheses directly, although this strategy tends to favor highly expressed surface antigens, which are likely to have been described previously.

P. Sapra (✉) · M. Ritchie
Bioconjugates Discovery and Development, Pfizer, Pearl River, NY, USA
e-mail: Puja.Sapra@pfizer.com

L. Bloom · G. Carven
Global Biotherapeutic Technologies, Pfizer, Cambridge, MA, USA

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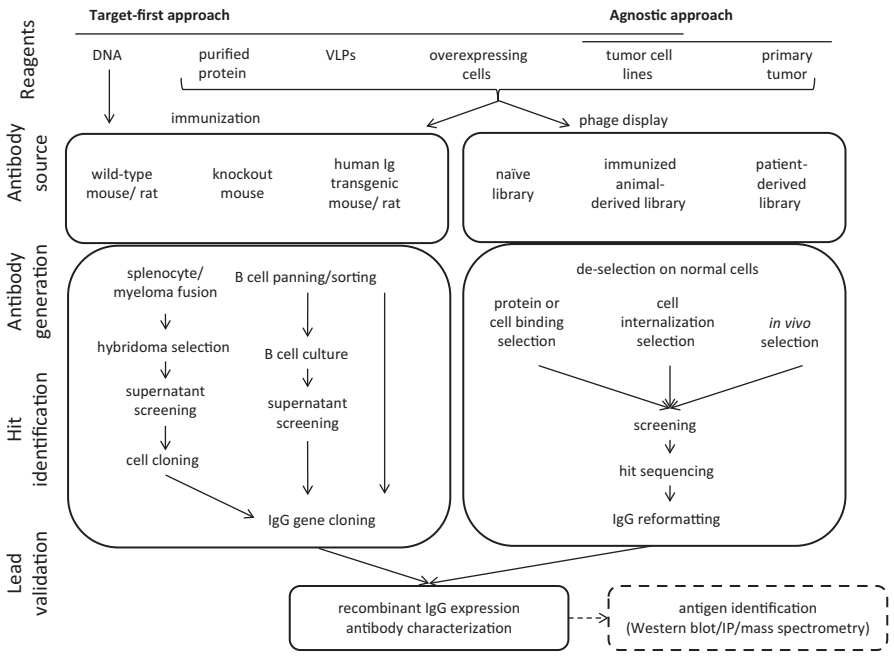

Fig. 3.1 Methods for antibody isolation and development via the agnostic and target-first approaches. Antibody isolation and validation can be performed with specific targets in mind ("target-first approach") or on tumor cells of interest without bias toward specific targets ("agnostic approach"). When the target is known, reagents for antibody generation can be as diverse as cDNA constructs for DNA immunization; purified target protein (full-length or domain); engineered cells or cell surrogates: virus-like particles (*VLPs*) or other target-enriched membranes; or tumor cells known to express the target. A target-agnostic approach will usually utilize primary tumor cells or tumor lines for antibody generation. The source for antibodies can be immunized animals, cancer patients, or phage display libraries with antibodies from naïve or tumor-exposed sources using a range of techniques, from conventional hybridoma generation or phage panning to B cell sorting and V gene isolation from immune sources or selection of phage binding to or internalizing in cells expressing the target. Once lead antibodies are cloned and selected, the antibodies can be recombinantly expressed and validated. If the target of an internalizing antibody is not known, target identification can often be done through mass spectrometry once a recombinant antibody is expressed

3.1.2 Selection and Validation of Tumor-Associated Targets Using Omics Approaches

Most strategies used for the identification of targets amenable to the ADC approach resemble strategies used to identify tumor-associated cell-surface antigens for other oncology biotherapeutic approaches, though with a few notable differences. The ADC concept of arming antibodies or antibody fragments with toxins or cytotoxic payloads relies on the selectivity of the antibody towards the target antigen to increase efficacy, while reducing toxicity compared to nontargeted delivery. Thus,

the ideal ADC target will be expressed highly in tumor cells and minimally across normal tissues. Multiple methods have been employed to identify differentially expressed proteins. Usually, this involves comparison of tumor samples (tissue biopsies, tumor cell lines, patient-derived xenografts, etc.) with closely-related nontumor cells. Comparison can be done at the DNA, messenger RNA (mRNA) or protein levels using genomic (e.g., continuous stacking hybridization (CSH) and fluorescence in situ hybridization (FISH)), transcriptomic (microarray) and/or proteomic techniques (two-dimensional gel electrophoresis/mass spectrometry (2DE/MS) and isotope-coded affinity tag (ICAT)) reviewed elsewhere (Guo 2003; Shiio and Aebersold 2006; Yaziji and Gown 2004). The output of such omics studies is typically a list of genes for which expression is increased in tumor samples relative to normal tissues.

The actual number of tumor-associated targets that are identified using such techniques is dependent on the scale and the methodology used in the selection process. It is quite common for a single study to identify tens to hundreds of potential targets that meet the criteria for tumor association. However, since selectivity is only one of multiple critical factors involved in target selection for ADCs, such a list is often just a starting point for validation of multiple targets in parallel.

Narrowing the list of potential targets can be achieved by additional diligence around each target to assess druggability, internalization, and other factors. For ADCs, the target molecule must reside on tumor cells in a location accessible to a systemically-administered therapeutic antibody. Confirmation that a target is localized to the plasma membrane and contains extracellular regions that could be targeted with an antibody is a critical early validation step. For annotated proteins, assessment of topology may be performed using a protein knowledge base, such as UniProt (UniProt 2013), to ensure that the protein contains at least one predicted transmembrane region, or is anchored to the cell surface through a glycosylphosphatidylinositol (GPI) link. If the target is novel, the expert protein analysis system (EXPASY) bioinformatic portal (www.expasy.org; Gasteiger et al. 2003) contains numerous structure prediction tools that can be used to define features such as transmembrane helices, secondary structural elements such as intra-/extracellular domains, as well as sites for predicted protein modification.

For confirmation of cell-surface localization, flow cytometry is commonly used if flow-compatible detection antibodies are available. Quantitative flow analysis can be useful to provide data on antigen density as the level of tumor (vs. normal) expression is an important component of target selection. For targets for which flow-compatible tools are unavailable, recombinant expression using genetic fusion to epitope tags (such as FLAG or myc) can be used. Alternatively, the target protein can be fused to green fluorescent protein and subsequently probed by microscopy.

Determining whether (and to what extent) a given antigen can be shed from the cell membrane is another important consideration. Many cell-surface receptors are shed, resulting in a portion (usually the extracellular domain(s)) being released into the circulation. Shed target protein at high levels may function as a sink, diverting the therapeutic antibody and its associated payload away from the tumor, and thereby lowering the potency of the ADC and potentially causing increased

liver clearance and associated systemic toxicity (Lin and Tibbitts 2012). It is known that a number of tumor-specific surface antigens, such as carcinoma antigen 125 (CA125), prostate-specific antigen (PSA), human epidermal growth factor receptor 2 (HER2), and others, are actively shed from cancer cells (Kulasingam and Diamandis 2008). The presence of circulating antigen can be expected to have a significant influence on the delivery of ADC payloads that use these antigens as the delivery target. While it is generally thought that targets that are shed from the cell surface should be avoided for the development of ADCs, the clinical efficacy of Kadcyla (anti-HER2–ADC; Burris et al. 2011) indicates that the presence of shed extracellular domain need not rule out an otherwise-promising ADC target. Indeed, a recent study suggests that the presence of soluble antigen may be beneficial for targeting solid tumors (Pak et al. 2012).

3.2 Antibody Characteristics

3.2.1 Optimal Antibody Characteristics

In addition to the careful selection of the tumor-specific antigen, characteristics of the antibody, such as affinity and molecular size, will have a significant impact on the efficacy of an ADC. Accordingly, there are a vast number of strategies available to characterize antibody characteristics. While high-affinity antibodies often stand out in screening campaigns, it is worth noting that high affinity does not always correlate with high efficacy, as a high-affinity antibody binding to a rapidly internalizing target may be rapidly eliminated from circulation. High affinity may also reduce the ability of the antibody to penetrate far within the tumor due to tight binding to and/or rapid internalization into the first cells it encounters, a phenomenon termed the binding-site barrier (Adams et al. 2001; Rudnick et al. 2011). A recent study has examined how efficiently a panel of anti-HER2 antibodies with differing affinities can penetrate through a solid tumor. All of these antibodies bind to the same epitope of HER2, but do so with log-fold differences in affinity (Rudnick et al. 2011). The antibody with the lowest affinity (~200 nM K_D) penetrated through the tumor with the highest efficiency; however, this antibody possessed the lowest tumor retention due to its low affinity and resultant inability to remain bound to the receptor (Rudnick et al. 2011). Conversely, antibodies with the higher affinity ($K_D < 1$ nM) for HER2 were largely confined to perivascular space of the tumor (Rudnick et al. 2011). These high-affinity antibodies exhibited lower penetration because they are rapidly internalized and catabolized by the first cells encountered after extravasation. Conversely, the lower-affinity antibodies were not bound long enough to internalize, and thus were able to continue moving through the tumor space (Rudnick et al. 2011). Accordingly, there exists a threshold whereby antibodies that possess a k_{off} faster than the K_e of receptor internalization can avoid internalization and catabolization by the cell, whereas

antibodies with k_{off} slower than the K_e of receptor internalization are internalized and catabolized more readily (Rudnick et al. 2011). A balance of the two rates, allowing effective delivery of toxin throughout the tumor, appears to be achieved by antibodies of moderate affinity (between 7 and 23 nM, in the case of the HER2 antibodies examined in Rudnick et al. (2011)).

Modeling and experimental analyses have shown that there is a fine balance between molecular size and affinity of a therapeutic protein and the degree of tumor retention and penetration. Accordingly, a complex correlation exists in which modalities of intermediate size (~25 kDa) have the lowest tumor uptake, while therapeutic modalities of extremely small (<20-kDa) and large (>100-kDa) size exhibit higher tumor uptake and retention (Schmidt and Wittrup 2009). This is due to the fact that small biotherapeutics can penetrate through a solid tumor rapidly without constraint, although a high affinity for the target antigen is required for the therapeutic to be retained (Schmidt and Wittrup 2009). However, while these smaller modalities can penetrate further through the tumor, they often suffer from faster serum clearance and shorter half-lives (Schmidt and Wittrup 2009). Alternatively, larger proteins usually exhibit reduced tumor penetration; however, they can achieve high accumulation due to their increased serum half-life (Schmidt and Wittrup 2009). Accordingly, intermediate-sized therapeutics (~25 kDa) appear to be the least optimal because they suffer from both a degree of restriction in tumor penetration and possess a reduced serum half-life.

Species cross-reactivity is also a practical consideration for many antibody discovery programs and is particularly important for ADC development due to the complex nature of toxicology studies. Alignment of the target amino acid sequence with that of its orthologs in other species can be useful to determine the identity or homology between human and another species such as nonhuman primate or rodent target. Sequence identity serves as a rough predictor of the likelihood for obtaining cross-reactive antibodies (i.e., there is a higher probability for an antibody to be cross-reactive to an ortholog when the antigens share high sequence identity). Similarly, it is difficult to raise cross-reactive antibodies when target identities are low (60 % identical or lower). Thus, when faced with multiple potential targets coming from -omics studies, priority may be given to follow up on targets with high-sequence homology. Linear sequence identity, however, does not always translate to the three-dimensional structure of a protein. For example, a ligand-binding site may be structurally well conserved across species, despite lower overall homology in other areas of the protein. For this reason, it is a common practice during an antibody generation campaign to screen for antibody candidates that bind to the human target and its nonhuman primate and/or rodent orthologs.

Finally, as cellular uptake of an antibody is a critical component of its efficacy, assessment of a target's ability to internalize and subsequently shuttle its antibody and payload to the appropriate intracellular compartment (usually the lysosome) is of the utmost importance. Strategies to measure internalization and trafficking of ADCs are presented in subsequent sections.

3.2.2 Engineering pH Dependence of Antibody Binding

Recently, the successful engineering of antibodies to have dramatically lower affinity at pH 6.0 than at neutral pH has attracted interest as a method for manipulation of the kinetics of antibody delivery to the lysosome (Chaparro-Riggers et al. 2012). The pH dependence is achieved through the introduction of histidine residues at or near the antibody/antigen binding interface. These histidine residues are protonated $<$pH 6.0 and deprotonated $>$pH 6.0 and, if placed correctly, can have a profound effect on binding affinity in different pH environments. Antibodies against PCSK9 and IL-6R have been engineered to dissociate from their targets in the early endosome (pH$\leq$6.0), while retaining binding to the FcRn receptor, which returns them to the cell surface and prevents degradation in the lysosome (Chaparro-Riggers et al. 2012). A similar approach has been used to *increase* antibody trafficking to the lysosome in cells lacking endosomal FcRn expression, which would be desirable for the delivery of ADCs requiring degradation for the release of the payload. However, the tumor microenvironment is often weakly acidic, which would reduce the cell-surface binding of pH-sensitive ADCs. Further investigation is required to understand the optimal degree of pH dependence for ADCs targeted to solid tumors, such that binding at the cell surface is not compromised.

3.2.3 Engineering Fc-Mediated Interactions

Fc-mediated effector function accounts for most of the in vivo efficacy imparted by unconjugated mAbs in cancer therapy. However, the role of Fc effector function in inhibition of tumor growth by an ADC in the clinic remains unclear. Several strategies have been employed to enhance immune-mediated effector functions of unconjugated mAbs, including afucosylation as well as mutations in the antibody-Fc region, both of which can enhance FcγR binding and amplify antibody-dependent cell-mediated cytotoxicity (ADCC). While this could potentially increase the potency of an ADC, effector mechanisms may lead to undesirable side effects such as cross-linking-related agonistic effects, cytokine storm, or platelet aggregation (Chatenoud et al. 1990; Wing et al. 1996; Langer et al. 2005). Thus, effector functions may not be desirable when the targeted delivery of the payload is sufficient for antitumor activity.

Antibody isotypes IgG2 and IgG4 have inherently reduced binding to FcgRII. Accordingly, these isotypes have been utilized in ADCs where effector function is not required for tumor killing (e.g., calicheamicin-based ADCs). However, given the high potency of these payloads, the concentration at which these ADCs are dosed in patients is low enough (1.8–9 mg/m^2) where eliciting effector function is likely to be minimal. However, ADCs possessing less potent payloads, such as microtubule inhibitors, may be dosed at higher levels (1–5 mg/kg) in the clinic, in which case effector function may come into play.

While advances in antibody engineering have allowed for the enhancement and elimination of effector function-mediated cytotoxicity of a tumor, little has been done to dissect what role(s), if any, effector function has in ADC-mediated tumor killing. As a result, three isotypes (IgG1, IgG2, and IgG4) are being utilized for ADC development programs. One preclinical study of an anti-CD70 antibody conjugated to maleimidocaproyl monomethyl auristatin F (mcMMAF) compared the activity of this ADC with isotype variants (IgG1, IgG2, and IgG4) and mutations that eliminate FcγR binding (IgG1v1 and IgG4v3). Interestingly, the IgG1v1-ADC lacking FcγIIIa binding was the most potent ADC in preclinical models of renal cancer and glioblastoma. Compared to the parental IgG1 ADC, this mutant displayed enhanced tumor exposure of the drug and an improved therapeutic index in mice (McDonagh et al. 2008). Accordingly, the importance of eliciting effector function in ADC-mediated tumor killing remains an important, unanswered question.

3.3 Agnostic Approach to Antibody Production

Whole-cell immunization has been used for decades to identify antigens located on the surface of cells (Williams et al. 1977). Immunophenotyping of hematopoietic cells based on surface markers identified by antibodies raised against cells at different stages of growth became the basis for the "clusters of differentiation" (or CD antigen) classification system (Bernard and Boumsell 1984). Targets with selective expression on tumor cells can also be identified using hybridomas generated via immunization with tumor cells. Many well-known tumor antigens such as prostate-specific membrane antigen (PSMA; Israeli et al. 1993) or Lewis^Y (Hellstrom et al. 1990) were identified in this manner. Typically, this includes a differential binding screen on tumor cells and a related nontumorigenic cell line. Screening can be performed using cell-based enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS) based methods using intact cells.

The efficiency of identifying tumor antigens via whole-cell immunization approaches can be improved using techniques such as subtractive immunization (Bickel et al. 2000; Sleister and Rao 2002). Subtractive immunization is a technique that can be used to produce monoclonal antibodies specific for antigens (such as tumor antigens) that are present in low abundance in a protein mixture or are poorly immunogenic due to the presence of immunodominant epitopes present within the sample. Here, neonatal mice are tolerized by injection of nontumorigenic tissue before thymic selection occurs, followed by more traditional immunization with tumor tissues after maturation of the mouse immune system.

Phage display has been used to identify panels of antibodies to surface antigens on a large variety of tumor cell lines, primary tumors, tumor tissue sections, cells captured by laser capture microdissection (Ruan et al. 2006), and even primary tumors in cancer patients, the latter injected with phage several hours prior to tumor surgery (Shukla et al. 2013). In order to identify tumor-specific antibodies, libraries are typically exposed to nontarget-bearing cells (e.g., noncancerous cell lines,

normal cells from the same tissue, different cancer types from the same tissues, etc.; Poul et al. 2000; Liu et al. 2004) in order to remove antibodies to common antigens, although this step is often applied following an initial round of selection on tumor cells to avoid depletion of rare antibodies (Zhou et al. 2012). Methods for isolating tumor cell-binding phage include simple washing and elution with standard low- and high-pH buffers (Zhou and Marks 2012; Poul et al. 2000; Becerril et al. 1999), centrifugation through an organic solvent to separate cells from unbound phage (Akahori et al. 2009), and internalization (see below). Some extensive campaigns have examined over 50 tumor lines and over 10,000 resulting scFv (Bouchard et al. 2009; Kurosawa et al. 2009).

Selection for phage that internalize into tumor cells can reduce the recovery of antibodies that bind nontumor-specific or noninternalizing targets and has been used successfully on many tumor types (Liu et al. 2004); reviewed in (Zhou et al. 2012); also (Zhou and Marks 2012; Poul et al. 2009; Fransson and Borrebaeck 2009). Selection for internalizing phage involves allowing phage to bind to the cell surface at 4 °C to prevent internalization, transfer to 37 °C for a brief period (typically 15–60 min) to allow internalization, followed by washing and stripping surface-bound phage with a low-pH buffer, and recovery of internalized phage by cell lysis and infection of *Escherichia coli* with cell lysate. Factors that can favor recovery of internalized phage include high abundance of the target on the cell surface, rapid constitutive target internalization, high affinity, accessible epitope, and multivalent phage (Burris et al. 2011; Zhou and Marks 2012; Zhou et al. 2012). While multivalent display (usually five copies per phage) allows greater recovery of lower affinity clones than does monovalent display (Burris et al. 2011), and, therefore, is likely to lead to pools of higher diversity, numerous examples exist of monovalent phagemid systems giving rise to internalizing clones from naïve human scfv library selections (McDonagh et al. 2008; Osborn et al. 2013; Sapra et al. 2013).

Identification of antibody targets from whole-cell phage selection or whole-cell immunization can be challenging. Antigens are frequently identified by immunoprecipitation with the isolated cell-surface-binding antibodies, followed by proteolytic digestion and mass spectrometry to identify bound peptides (Kurosawa et al. 2008, 2009); see also (Poul et al. 2000; Goenaga et al. 2007). Overexpression of the candidate gene on a test cell or reduction of the candidate gene expression via small interfering RNA (siRNA) can be used to confirm the identity of the candidate antigen. In one example, selection for phage internalizing in MDA-MB-231 breast cancer cells was followed by selection on yeast cells expressing either of two candidate proteins, leading to the isolation of antibodies recognizing the two candidates (Zhou et al. 2010). The authors suggest that this method could be applied on a larger scale to isolate cell-binding antibodies to a large panel of candidate genes.

Large-scale tumor antibody screening has required a series of steps to narrow down candidates. Kurosawa and coworkers (Kurosawa et al. 2008, 2009, 2012) first used patterns of binding to multiple tumor cell lines or tissue sections to narrow down a panel of several hundred antibodies into clusters, followed by a combination of ELISA and western blotting against known antigens and immunoprecipitation/MS to identify new antigens. Liu et al. (2004) selected and characterized a large

panel of internalizing antibodies on prostate cancer cells, similarly using binding profiles to group cells into different epitope clusters.

Studies that have attempted to address the spectrum of tumor surface proteins from several approaches in parallel have suggested that certain common antigens are detected by different technologies (e.g., phage display, hybridoma, and proteomic approaches) but that the majority of antigens identified are different, pointing to the biases and technical limitations of relying on any single method (Rust et al. 2013).

3.4 Target-First Approach to Antibody Generation

3.4.1 Considerations and Preparation of Target for Antibody Screening

A panel of high-quality reagents is key to a successful campaign. Many of the tools used for target validation may also be useful during antibody generation and screening. Such tools typically include target DNA, expression plasmids, native and recombinant cell lines, purified proteins, and control or reference antibodies. When generating screening reagents, it is important to consider generating target orthologs in parallel which can be used to test for species cross-reactivity of antibody hits.

The nature and inherent characteristics of target molecules selected for ADC intervention vary considerably. Nearly all are expressed on the cell surface, whether directly on tumor cells or tumor vasculature. As integral membrane proteins, they are permanently attached to the cell membrane, and typically contain one or more membrane-spanning alpha helices. Such proteins can only be separated from the biological membranes using detergents, nonpolar solvents, or denaturing agents. For this reason, the vast majority of protein antigens used for antibody generation and screening are broken into smaller soluble subdomains, or extracellular domains of cell-surface receptors. These fragments can be designed to be recombinantly expressed for use as immunogens or selection reagents. The precise determination of the domain boundaries is often critical, and small changes in an expression construct may result in drastically different expression yields.

Recombinant production of target antigens can be done using either prokaryotic or eukaryotic hosts. The choice of host likely depends on the nature of target molecule. Most importantly, the yield should be a homogenous, properly folded antigen such that the critical epitopes are accessible (Ebersbach and Geisse 2012). For rapid generation of material, transient expression in HEK293 or Chinese hamster ovary (CHO) cells is preferred. For many proteins, addition of tags, such as influenza hemagglutinin (HA), FLAG, or His_6, will allow easy purification from complex cell culture media. Fc-based fusion proteins, composed of an immunoglobulin Fc domain genetically fused to the target antigen, may also be used. The Fc domain folds independently and can improve the solubility and stability of the partner molecule both in vitro and in vivo, while also providing a convenient purification handle for protein A/G affinity chromatography.

For oncology targets, transformed cell lines expressing the target are often readily available. These cell lines are potent immunogens for the generation of antibodies, resulting in a strong immune response in animals (Kung et al. 1979). Whole cells are complex antigens, made up of many cell-surface proteins, each a potential antigen. Thus, extensive screening is often required to generate antibodies specific for a singular antigen of interest. To reduce complexity during cell-based screening, recombinant overexpressing cell lines can be used. The full-length target antigen can be expressed in commonly used cell systems such as HEK293 or CHO cells. Nontransfected, parental control cells expressing low or no target molecule can be used for counter screening.

To maximize the specific antibody response in the mouse upon immunization with whole cells, human target antigens can also be overexpressed on the surface of mouse cell lines derived from a strain syngenic to the immunization host (Ashley et al. 1997; Uchida et al. 2004). Unlike human cells, murine cells expressing recombinant antigen will typically exhibit a reduced background response as their entire cell-surface components (apart from the transfected and expressed target molecule) are of mouse origin and, therefore, tolerized in the animal. An ideal antibody generation campaign may employ native-expressing tumor cell lines and recombinant proteins as well as engineered cell lines on different backgrounds.

3.4.2 Approaches to Antibody Isolation

Many of the currently marketed therapeutic antibodies have been generated using traditional hybridoma technology developed by Kohler and Milstein (1975). Hybridoma cell lines are generated from the stable fusion of immortalized myeloma cells with immunoglobulin-producing B cells, which is inherently an inefficient process. Despite its limitations, hybridoma technology has been highly successful and has historically been the most widely used method for antibody generation. More recently, methods to identify antibodies in a more efficient manner have been employed. These include culture and expansion of antibody-producing B cells from immunized rodents using CD40/CD40L (Wen et al. 1987; Weber et al. 2003) as well as flow cytometry-based sorting of antibody-producing cells (APCs) using fluorescently labeled antigen followed by reverse transcription polymerase chain reaction (RT-PCR) from cells (Berry et al. 2009; Kurosawa et al. 2012).

Each of the above methods relies on the immunization of rodents or other animals as a source of antibody-producing immune cells. Mice are often used for immunization due to their well-defined genetics, short generation times, and low maintenance costs. The use of knockout mouse strains that have a targeted deletion of a particular gene of interest makes it easier to raise an immune response to some protein targets that are highly conserved among species. When knockout mice are not available, rats or other species can be used to facilitate the generation of human/mouse cross-reactive antibodies.

Human immunoglobulin transgenic rodents provide a method of obtaining fully human monoclonal antibodies using conventional hybridoma technology (Fishwild et al. 1996; Mendez et al. 1997; Osborn et al. 2013; Ma et al. 2013). Transgenic rodents offer a powerful way to generate antibodies to validated targets relatively rapidly without the need for humanization. The benefits of transgenic rodents must be balanced, however, against generally weaker immune responses, requiring more effort to generate equivalent panels of antibodies compared to wild-type rodents (Lonberg 2005). The relatively high technology access cost and limited availability of transgenic animals further contribute to the continued preference for nontransgenic animals among most researchers.

Immunization methods vary depending on the nature of the target and the reagents available during an antibody campaign. When the target is novel, there may be few available tools at the outset of a project. Requiring only plasmid DNA, genetic immunization can be initiated before other tools such as recombinant protein or engineered cell lines can be generated. DNA can be delivered by several means, including injection, a gene gun using DNA-coated gold beads, or intramuscular electroporation (Robinson and Pertmer 2001). The encoded antigen is taken up, translated and presented by immune cells such as dendritic cells, and elicits both a strong humoral and cellular immune response. Coadministration of DNA-encoding immune mediators such as granulocyte-macrophage colony stimulating factor (GMCSF) and Flt3L has been shown to increase the number of infiltrating dendritic cells at the immunization site, resulting in overall increased antigen-specific responses.

Recent improvements to the technique have shown that DNA immunization is capable of generating immune responses against antigens such as G protein-coupled receptors (GPCRs) and ion channels, which are typically considered intractable to antibody generation (Hazen et al. 2014). Generally, immune responses from DNA immunization take longer to develop than those elicited by other methods, but the delayed response time is often offset by the time required to generate immunogens for more traditional approaches.

Rapidly generating a panel of antibodies against multiple targets can be very useful for ADC target validation efforts. Typical rodent immunization methods require two or more months of injections before immune responses are of sufficient titer to be used for antibody generation. Rapid immunization methods such as repetitive immunization, multiple sites (RIMMS) can be used to reduce the period of time between initiation and screening for a panel of hits, without sacrificing either the quantity or the quality of hits in a given campaign (Kilpatrick et al. 1997). RIMMS capitalizes on rapid hypermutation and affinity maturation events that occur in secondary lymphatic tissue early in response to antigenic challenges. Small concentrations of antigen are injected at several subcutaneous sites three times per week over the course of 2–3 weeks. Following antigen challenge, draining lymph nodes from each of the injection sites are collected and used for fusion or B cell-based antibody screening.

Phage display provides the opportunity to guide antibody populations toward those with particular characteristics: for example, toward antibodies binding to a

tumor cell type and not to a set of normal cells, toward antibodies binding to a known epitope on a known target, toward antibodies that are internalized rapidly, or a combination of these traits. Phage-derived antibodies generally have lower affinities than those derived from immunized animals (K_D ranging from 1 to 100 nM vs 0.1 to 10 nM), but due to considerations of the binding-site barrier described above and the likelihood that moderate-affinity antibodies will avoid internalization into low-expressing normal cells, while still internalizing in high-expressing tumor cells, antibodies in the low double-digit nanomolar range are likely to be suitable for ADCs (Rudnick et al. 2011).

The most straightforward phage display approaches have used purified or recombinant antigens for phage selection in either solution or solid phase, followed by screening for binding to the antigen expressed on the surface of engineered cells or tumor cells (Zhou et al. 2012). The advantage of this approach is that the target specificity of the antibodies is known, and binding to the more complex cell surfaces is only confirmatory. The chief disadvantages are that the approach requires the generation of high-quality protein reagents, and even following successful selections, antibodies selected for binding to purified antigen frequently fail to recognize the target in the context of the tumor cell. Selection of phage for binding to cells, including tumor cell lines, tumor tissue samples, or mammalian expression lines engineered to overexpress a specific target, reduces the risk that clones will fail to bind cell-surface target, but as cell-surface complexity increases, the task of separating clones that bind to targets of interest from clones that do not becomes increasingly challenging.

Selection on cells overexpressing the surface protein of interest is also a path to isolating antibodies of known specificity. The advantage of this method is that it favors the isolation of antibodies that bind specifically to the cell surface form of a known target (Hoogenboom et al. 1999). Generation of stable mammalian cell lines can be time consuming, but often a transfected cell line and its untransfected parent can be used for both selection and screening of antibodies and for providing clear evidence for target specificity. Recombinant lines may overestimate the ability of low-affinity antibodies to bind cells expressing lower levels of target, and may have altered internalization mechanics, glycosylation, or association with other proteins, so validation of antibody properties on tumor cells is essential.

Often a combination of methods is appropriate. If the target is known, phage selections using alternating rounds on purified proteins and cells can improve the likelihood that the resulting clones will bind specifically to the desired target as presented on tumor cells. Similarly, combining selection on purified protein and internalizing selections on tumor cells can focus the selected pool on clones of the desired phenotype. It is difficult to predict what characteristics observed for scFv of Fab displayed on phage will translate to characteristics in immunoglobulin G (IgG) format, and for this reason, it is often advantageous to convert as diverse a set of primary isolates to IgG as possible to confirm their binding and internalization profiles.

3.5 Screening for Internalization and Intracellular Trafficking

3.5.1 Screening by Microscopy and Flow Cytometry

Current linker chemistry has been designed such that cytotoxic payloads are cleaved from the antibody, via an assortment of mechanisms, within the intracellular space of the cell, while remaining highly stable in the extracellular space. Thus, it is necessary to demonstrate efficient internalization of the selected antibodies. Depending on reagent availability, antibody binding and specificity can be determined in relatively high throughput by ELISA or other protein-binding assays, and in somewhat lower throughput by cell-based ELISA or flow cytometry on target-expressing engineered cell lines or tumor lines. Internalization is frequently tested in a subsequent step by either fluorescence-based internalization assays (Fig. 3.2a) or by testing the ability of the antibody to deliver toxins intracellularly (Fig. 3.2b).

When using fluorescence-based internalization assays, the screened antibodies can either be complexed with a fluorophore-conjugated modality (such as a Fab fragment or liposome) or directly conjugated with a fluorophore. The use of fluorophore-labeled secondary antibodies is the quickest way to tag an antibody or pool of antibodies. However, since the size and valency of a ligand can effect internalization, the presence of a secondary antibody may alter the internalization properties of the primary antibody (Nielsen et al. 2006). Alternatively, antibodies can be biotinylated with NHS-SS-biotin and subsequently complexed with streptavidin-linked fluorophore moieties (Nielsen et al. 2006). While this process has a higher throughput than direct labeling, it also carries a risk of altering binding affinity of the antibody to its antigen (Vira et al. 2010). The direct conjugation of a fluorophore to an antibody, while suitable only for a small number of antibodies in parallel, allows the user to monitor internalization and intracellular trafficking in real time and, importantly, also reduces the background noise that often arises from the use of secondary antibodies. Antibodies can be directly labeled with fluorophores via a number of commercially available conjugation kits that utilize random lysine or cysteine coupling of the fluorophore. Direct conjugation carries with it the risk of placing fluorophores at the antigen-binding interface, which may interfere with binding (Vira et al. 2010). Accordingly, after direct conjugation, it is important to verify that the binding affinity of the antibody has not been compromised (Vira et al. 2010).

To measure internalization, the fluorescent antibodies are added to cells seeded in a 96-well plate for a period of time at 4 ℃ to allow for binding without internalization (Nielsen et al. 2006; Harper et al. 2013). The cells are then moved to 37 ℃ to allow for target-mediated internalization (Nielsen et al. 2006; Harper et al. 2013). After a selected period of time, noninternalized fluorescent antibodies are gently removed from the cell surface (e.g., by washing with a low pH buffer or, if NHS-SS-biotin was utilized to conjugate the fluorophore, by addition of a reducing agent), or the extracellular fluorescence is quenched by an antifluorophore antibody (Nielsen et al. 2006; Harper et al. 2013). Internalized fluorescence can then be quantified via

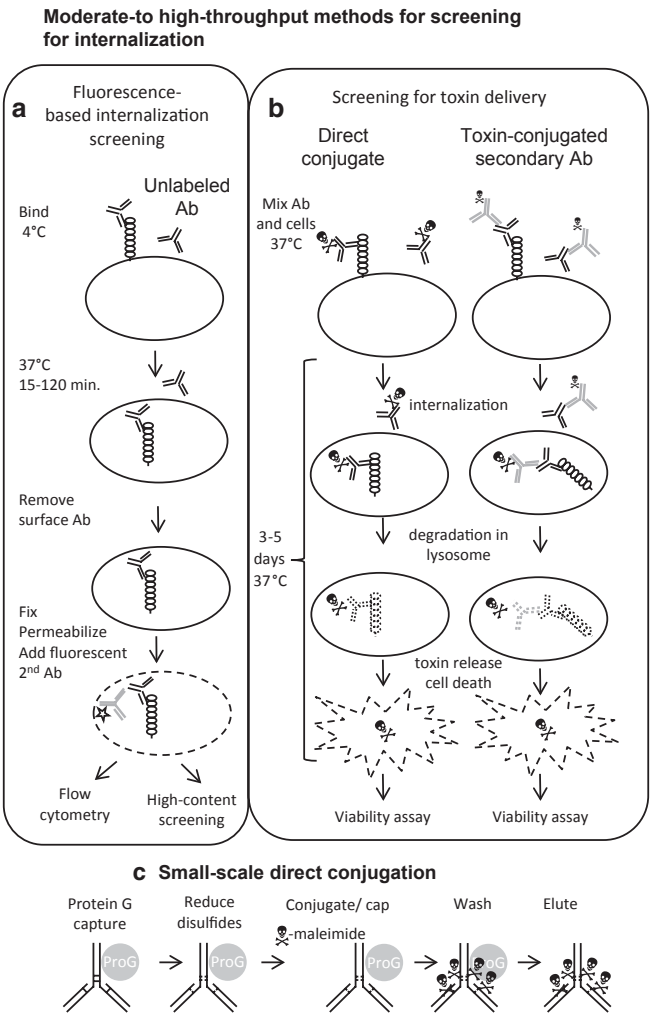

Fig. 3.2 Screening for internalizing antibodies. **a** Screening for internalization using fluorescence-based approaches, In fluorescence-based assays, target cells can be bound to antibodies at 4 ℃, warmed to 37 ℃ to allow for internalization, washed to remove extracellular antibodies, and fixed and stained with fluorescent secondary reagents to visualize internalized antibodies. Alternatively (not shown), antibodies directly conjugated to fluorophores can be used for internalization, and secondary antibodies omitted. Internalization can be measured qualitatively or quantitatively by microscopy or flow cytometry. **b** Screening for internalization by testing for intracellular toxin delivery. For target-mediated toxin delivery assays (*right panel*), antibodies can be directly conjugated to a toxin or can be complexed with a secondary antibody that is directly conjugated to a toxin. The conjugated antibody is then added to target cells for a period of 3–5 days and cell viability is assessed. **c** Small-scale direct conjugation of antibodies. In a method described by Lyon et al. (2012), IgG is captured onto protein G beads from small volumes of tissue culture medium and coupled to toxins via maleimide chemistry, while remaining immobilized on the beads. A mixture of toxin/linker and a sulfhydryl-capping reagent keeps the drug–antibody ratio (DAR) within a controlled range.

a high-content fluorescence reader or flow cytometer, and the degree of internalization can be assessed by comparison with cells kept at 4 ℃ (Nielsen et al. 2006). High-resolution images can also be captured via confocal microscopy to verify the results (Fransson et al. 2004).

The engineering of second-generation ADCs that possess noncleavable, stable payload linkages to the antibody requires delivery to the lysosomal compartment of the cell for liberation of the payload from the antibody and subsequent delivery to the cell in an active form. Accordingly, screening for this intracellular delivery has become of paramount importance and can also be achieved with moderately high throughput. This methodology requires the fluorescent labeling of the intracellular compartment of interest as well as the labeling of the internalizing antibodies with a different fluorophore by the methods described above. Organelle labeling can be achieved through various cell-permeable organelle fluorescent probes, through indirect immunostaining of the organelles, or through ectopic expression of a fluorophore-tagged organelle resident protein (Morelli et al. 2006; Starkuviene and Pepperkok 2007). Similarly to the approach described above, cells are exposed to fluorescently labeled antibodies at 4 ℃ and are then moved to 37 ℃ for a period of time before fixation. However, this method does not require quenching of noninternalized antibodies (Morelli et al. 2006; Starkuviene and Pepperkok 2007). The plates are read in a high-content fluorescence reader, and the magnitude of colocalization of the labeled antibody and the labeled organelle of interest are quantified (Starkuviene and Pepperkok 2007).

Conjugation to liposomes has also been used to measure antibody internalization (Nielsen et al. 2006; An et al. 2008). The antibodies can be coupled to fluorophore-labeled liposomes via either His_6/Ni-NTA or thiol coupling (An et al. 2008; Harper et al. 2013). In this method, conjugated liposomes are incubated with cells for a period of time, before noninternalized mAb/liposomes are gently removed and the cells are lysed (Harper et al. 2013). The remaining internalized fluorescence is measured via a microfluorimeter (Harper et al. 2013). Alternatively, liposomes can be loaded with a cytotoxin or immunotoxin in order to measure the ability of the antibody to confer target-mediated killing (An et al. 2008; Harper et al. 2013). However, the high valency of conjugated liposomes may result in internalization and trafficking properties that differ significantly from those of conventional IgG.

3.5.2 Screening for Intracellular Toxin Delivery

The ability of antibodies to internalize and deliver toxins to the cell type of interest can also be measured directly, and increasing numbers of methods are becoming available to allow rapid screening of hundreds to thousands of antibodies. Some investigators starting with antibodies derived from phage display have screened for internalization by generating genetic fusions of scFv to protein toxins such as *Pseudomonas* exotoxin and testing for cytotoxicity of the resulting immunotoxins (Cizeau et al. 2011; Yoshikawa et al. 2013). Expression in *E. coli* makes this method suitable

for screening of large numbers of scFv, although (Yoshikawa et al. 2013) showing that the cytotoxic potency of the selected fusion proteins was ~100-fold lower than that of the same molecules as neocarzinostatin-conjugated IgG, suggesting that only the most potent internalizing antibodies may be identified by this method.

The development of toxin-conjugated secondary reagents has facilitated screening of antibodies derived from both hybridoma and phage display. Conjugates of protein toxins to anti-IgG secondary reagents have been reported, either chemical conjugates to ricin A (Till et al. 1988) or pokeweed antiviral protein (Weltman et al. 1987) or genetic fusions of *Pseudomonas* exotoxin A to an antikappa single-domain antibody (Kellner et al. 2011) or to the Z domain of *Staphylococcus* protein A (Mazor et al. 2007). Commercially available secondary antibodies conjugated to the ribosome-inactivating protein saporin have been widely used to confirm antibody internalization (Kohls and Lappi 2000; Nguyen et al. 2006; Fransson and Borrebaeck 2009; Sawada et al. 2011). Antibodies can be incubated with saporin-conjugated antimouse, antirat, or antihuman reagents and then incubated with tumor cells for 3–4 days. Uptake of the complexes and release of saporin from the endosome into the cytoplasm leads to cell death (Fig. 3.2b), which can be measured in high-throughput format by viability reagents such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or CellTiterGlo (Promega). The assay can be increased in sensitivity through the use of bivalent saporin-conjugated secondary IgG, which allows higher order cross-linking of surface receptors and may enhance internalization, while Fab–saporin secondary conjugates maintain the level of cross-linking arising from the targeting antibody itself. For His-tagged scfv or Fabs, a saporin-conjugated anti-His_6 antibody provides a tool suitable for screening internalization of His_6-tagged antibody fragments such as scFv. In addition to their use in antibody screening, saporin-conjugated secondary antibodies provide an opportunity to validate the internalization of newly identified cell-surface proteins. For example, Fransson et al. (Fransson and Borrebaeck 2006) used a saporin conjugate to confirm the surface exposure and internalization of the nuclear protein Ku70/80.

The sensitivity of assays with secondary toxin conjugates can be further increased by using potent small-molecule toxins. Klussman et al. (2004) showed that the cytotoxicity of an anti-$Lewis^Y$ antibody complexed to vcMMAE-conjugated secondary antibodies was 1–2 orders of magnitude greater than cytotoxicity measured with the equivalent saporin-conjugated secondary antibodies. In addition, a vcMMAE-conjugated secondary antibody conferred nearly identical cytotoxicity to that of the direct vcMMAE conjugate, indicating that a high-throughput screening with the secondary antibody may be predictive of the activity of the final conjugate. This high-sensitivity approach has recently been facilitated by the commercial availability of secondary reagents conjugated with representative payloads such as Monomethyl auristatin E (MMAE), Monomethyl auristatin F (MMAF), duocarmycin, and mertansine (DM1) (Moradec, LLC, San Diego, CA).

Direct conjugation to a linker payload is frequently a late step in screening for antibodies, carried out on only a small number of candidate antibodies. However, small-scale methods for direct conjugation of toxin to antibodies are coming into increasingly widespread use, allowing early-stage screening of large panels of

candidate antibodies. Lyon et al. (2012) describe a method for small-scale thiol-based conjugation of mcMMAF (Fig. 3.2c), capturing antibodies on Protein G resin and reducing interchain disulfides with tris(2-carboxyethyl)phosphine (TCEP) to expose free thiols. The process uses less than 5 ml of conditioned medium in a 96-well block format, allowing for early-phase assessment of antibodies as ADC carriers prior to hybridoma subcloning, and by inclusion of both a maleimide linker payload and a capping agent is designed to minimize variations in drug–antibody ratios (DARs) despite the variable concentrations of antibody expected in crude supernatants. This critical step allows direct comparison of antibodies, since they carry similar levels of toxin. Lyon et al. describe preparation of 120 antibodies in parallel with a narrow range of (DARs) slightly under 4. Other methods have been published for thiol-based conjugation at the 5-mg scale (Stefano et al. 2013). Site-specific conjugation via several alternative strategies (see below; reviewed in Behrens and Liu (2013)) also hold the potential for application at early screening phases.

3.6 Payload Conjugation Strategies

The classes of cytotoxins currently being utilized to design ADCs fall into two categories: Those that target microtubules and those that target DNA. The method by which a payload is conjugated to an antibody is of critical importance to the overall efficacy of an ADC. The chemistry involved and placement of the payload linkage on the antibody can have a profound effect on binding, tolerability, pharmacokinetic (PK) properties and potency of an ADC.

Conventional conjugation strategies involve random conjugation to either lysine amines (as for Mylotarg; gemtuzumab ozogamicin), CMC-544, and Kadcyla; T-DM1) or sulfhydryl groups on cysteines (for SGN-35 and CDX-011) of the antibody. Due to the high availability of multiple exposed lysines on the antibody surface (~70–90 per IgG1), lysine-based conjugation methods can produce a high degree of conjugation heterogeneity. For example, analysis of the recently approved ADC Kadcyla showed the presence of various drug-to-Ab ratio species ranging from 0 to 7 payloads, with an average of 3.5 payloads per antibody (Junutula et al. 2010), whereas Mylotarg possessed a large mixture of unconjugated species and conjugates with an average loading of 2.5 (Hamann et al. 2002). Alternatively, conventional cysteine-based random conjugation methods produce a more uniform degree of antibody loading. The presence of four reducible disulfide bonds within the IgG structure that covalently link the heavy and light chains together is an attractive feature that allows for selective attachment of the drug to up to eight distinct sites using a thiol-reactive handle. The cysteine-conjugated Adcetris (SGN-35; brentuximab vedotin) contains a lower level heterogeneous mixture of drug-loaded variants with an average of four payloads per antibody (Wakankar et al. 2011; Okeley et al. 2010). In theory, each of the loaded species in an ADC can represent a unique conjugate and thereby exhibits distinct PK and other biological properties.

In fact, for anti-CD30–vcMMAE decreasing drug loading from eight to four or two drug molecules per Ab leads to slower ADC clearance and improvements in therapeutic index (Hamblett et al. 2004). In addition, batch-to-batch consistency in ADC production can be challenging and may require diligent manufacturing capabilities. Site-specific conjugation, in which a known number of linker drugs are consistently conjugated to defined sites, is one way to overcome these challenges.

One of the first site-specific conjugation processes was established by Junutula et al. at Genentech who developed a cysteine-based site-specific conjugation approach called "THIOMAB drug conjugates" (TDCs; Junutula et al. 2008). The authors engineered anti-Mucin-16 antibodies with additional cysteines on light and heavy chains that provide reactive thiol groups and did not perturb immunoglobulin folding and assembly, or alter antigen binding. The engineered Abs (ThiomAbs) were then reacted with maleimide functionalized toxins, thereby yielding nearly homogeneous conjugates, TDCs (Junutula et al. 2008). TDCs possessed comparable antitumor activity as the conventionally conjugated ADCs, despite having a lower DAR (Junutula et al. 2008). Furthermore, the TDCs were tolerated at higher doses in Sprague Dawley rats and cynomolgus monkeys, and had an improved PK profile compared to conventional conjugates (Junutula et al. 2008). However, this method includes a reduction–reoxidation step that can potentially lead to unpaired, reactive sulfhydryl groups. Furthermore, depending on the location of the engineered cysteines, maleimide exchange processes could lead to drug loss and thus decrease the potency of the ADC.

Recently, the use of nonnatural amino acids has also been employed for site-specific conjugation. In one study, a redundant stop codon was introduced at specific locations within the coding sequence of an anti-HER2 antibody, such that an amber suppressor, tRNA/aminoacyl-tRNA synthetase gene pair can incorporate p-acetylphenylalanine into these locations. This allowed for the site-specific conjugation of an auristatin derivative through a stable oxime linkage. This ADC possessed excellent in vitro and in vivo potency with similar clearance rates as the parental IgG in rats (Axup et al. 2012). In a separate study, this technology was also used to produce an anti-5T4 and an anti-HER2 site-specific ADC, containing two monomethyl auristatin D (MMAD) payloads per antibody (Sapra et al. 2013; Tian et al. 2014). These particular site-specific ADCs (NDCs) also possessed improved in vitro cytotoxicity, superior in vivo efficacy, increased PK stability relative to the conventional conjugates, and were well tolerated in preclinical toxicology studies (Sapra et al. 2013; Tian et al. 2014). Additionally, when NDCs were produced with protease-cleavable linkers, the site of conjugation on the antibody had a considerable effect on the stability of these rationally designed prodrug linkers, highlighting the importance of selecting suitable conjugation sites within the antibody (Tian et al. 2014).

In a different methodology to produce site-specific conjugates, the enzyme transglutaminase (mTG) from *Streptoverticillium mobaraense* was used to enzymatically conjugate payloads to antibodies in a site-specific manner and subsequently determine whether the site of conjugation affects the characteristics of the ADC (Strop et al. 2013). mTG does not recognize any of the naturally occurring glutamine residues in the Fc region of glycosylated antibodies, but does recognize

a "glutamine tag" that can be engineered into an antibody (Strop et al. 2013). The glutamine tag, LLQG, was engineered into different sites in the constant domain of an antibody targeting the epidermal growth factor receptor. mTG was then used to conjugate these sites with fluorophores or monomethyl dolastatin 10 (MMAD), and several sites were found to have good biophysical properties and a high degree of conjugation (Strop et al. 2013). Additionally, mTG was able to conjugate to glutamine tags present on anti-Her2 and anti-M1S1 antibodies (Strop et al. 2013). An anti-M1S1-vc-MMAD site-specific conjugate displayed strong in vitro and in vivo activity, suggesting that conjugation using this method does not alter antibody binding or affinity and demonstrates the utility of this approach in the site-specific conjugation of ADCs. The authors also showed that the site of conjugation has a significant impact on ADC stability and PKs in a species-dependent manner (Strop et al. 2013). These differences were attributed to the position of the linkage rather than the chemical instability, as was observed with a maleimide linkage. The authors conclude that this method provides a conjugation strategy that produces homogeneous ADCs and allows for the adjustment of ADC properties in order to maximize the therapeutic window.

3.7 Conclusions and Perspectives

Current linker chemistry has enabled the development of ADCs that are highly stable in the circulation but are amenable to intracellular drug release after entry into the endosome or lysosomal compartment of the cell. While this approach may yield reduced toxicity, as the toxic drug is liberated from the antibody within the tumor cell, the advancement of linker chemistry may make it feasible to devise next-generation linkers that release the drug on the cell surface of cancer cells, but not in plasma. The successful implementation of such linker chemistry would open new avenues in target identification and discovery of noninternalizing antigens that are uniquely present or highly overexpressed in tumors. The notion of noninternalizing antigens has been explored since the early period of radioimmunotherapeutics development. Correspondingly, localized extracellular drug release by the ADCs is a direction being actively explored by several groups. However, it remains to be seen whether this approach will provide sufficient potency in preclinical settings and later in clinics.

Unique, tumor-activating antibody applications are also emerging as a popular strategy to enhance the potency and reduce the toxicity of monoclonal antibodies. In one such example, the authors developed a probody technology, in which antibody binding remains masked against antigen binding until becoming activated locally by proteases commonly active in the tumor microenvironment (Desnoyers et al. 2013). Hypothetically, this technology could help in developing ADCs against targets which have high normal tissue expression. Furthermore, the technology could enable the development of safer ADCs and/or ADCs with an increased serum half-life. However, the utility of such technologies in the clinic remains to be evaluated.

In general, antibody-based therapies (i.e., ADCs, immunotoxins, or immunoliposomes) have performed better in hematological tumor settings compared to solid tumors, despite the fact that the overall expression level of target antigens are frequently expressed at lower levels in liquid tumors. However, in liquid tumors, antigens are expressed more homogeneously and the entire tumor is more accessible for antibody-based therapy. Additionally, liquid tumors are usually more sensitive to cytotoxic compounds and have a more restricted antigen expression pattern on normal tissues. Indeed, a solid tumor environment poses unique challenges with regard to the number of antibody-based molecules that extravasate the tumor blood vessels and translocate through the tumor interstitium toward the surface of tumor cells (Rybak et al. 2007). Further, solid tumors have a heterogeneous blood supply and high interstitial pressures within tumor tissue, especially in necrotic zones, which may limit the diffusion of drugs or ADCs to poorly perfused areas (Stohrer et al. 2000). Additionally, the binding-site barrier hypothesis suggests that antibodies bind to the first target cells they encounter, impairing their penetration through the entire tumor, and limiting their therapeutic effects (Weinstein and van Osdol 1992; Rudnick et al. 2011). Therefore, the development of novel vehicles that allow increased penetration through the solid tumor mass may offer improved efficacy of therapeutic antibodies and ADCs.

The development of companion diagnostic tests will likely become an essential component of future ADC development programs. These diagnostic tests may help translate effective antibody-based therapeutics to successful, targeted treatments for populations that will best respond to the treatment, especially if the drug is being developed against an antigen that has heterogeneous distribution. Designing this companion diagnostics during the early stages of an ADC program may provide valuable information about the best indications to target, but will also allow for the use of novel antibody tools being generated during the antibody isolation stage of the ADC program. It will be interesting to follow whether the field will continue to use the "gold standard" immunohistochemistry-based approaches to identify patient populations or move to less invasive procedures, such as circulating tumor cells or imaging modalities.

It remains to be determined whether amino acid alterations introduced to specific sites within an antibody will present any challenges for immunogenicity or stability of corresponding ADCs in the clinic. Although the site-specific ADCs appear to improve upon conventional conjugation strategies currently used in the clinic, these conclusions are based on fairly limited preclinical data and require a more rigorous analysis in the clinic. Further improvements in site-specific conjugation strategies are still possible, such as homogeneous conjugates with greater than two drugs per antibody. While higher drug loading has been correlated with an increased rate of clearance, those conclusions have been drawn from ADCs constructed using nonspecific cysteine modification methods, and may not be valid for site-specific ADCs. It will also be interesting to follow whether site-specific conjugates possess different off-target toxicity profiles compared to conventional conjugates. Furthermore, site-specific conjugates also promise increased drug exposure, and while higher exposure may be advantageous to achieve higher efficacy, it remains to

be determined whether higher exposure results in more on-target toxicities. To our knowledge, none of the ADCs currently being evaluated in trials in humans used site-specific conjugation approaches; however, emerging preclinical data continue to build on the promise of site-specific conjugation technology to produce safer ADCs with controlled manufacturing and improved analytics.

ADCs have emerged as therapeutic modalities with great promise for the treatment of cancer. However, more may be needed to ensure the clinical success of this therapeutic class. Advances in antigen identification, antibody engineering, and linker and payload conjugation chemistry may help to design more efficacious and safer ADCs.

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Chapter 4
Linker Design for Antibody-Drug Conjugates

E. Erica Hong and Ravi Chari

4.1 Introduction

Antibody-drug conjugates (ADCs) are composed of a cytotoxic agent conjugated to a monoclonal antibody (mAb) raised against an antigen that is preferentially expressed on cancer cells relative to normal tissues (Fig. 4.1; Lambert 2013). A linker provides a covalent bridge between the cytotoxic agent and the antibody, which serves to deliver the cytotoxic agent specifically to cancer cells. The function of a linker is to keep the cytotoxic molecule stably attached to the antibody during formulation and storage of the drug product, and during circulation in plasma following administration. However, a linker must also allow rapid and efficient release of the cytotoxic agent upon internalization of the ADC within cancer cells. This fine balance of extracellular stability and intracellular release is a necessary starting point for the design of a linker for ADCs.

Contemporary linker technologies have taken the linker beyond its basic role as a physical bridge between the antibody and a cytotoxic moiety. Linkers have been modified to improve the activation of ADCs, thereby allowing ADCs to release the cytotoxic agents inside the cells at a faster rate with higher efficiency utilizing different mechanisms for cytotoxin release. Modulation of the linker to effect a change in the polarity or charge of the final metabolite has also allowed improved activity toward multidrug resistant (MDR) cells owing to better retention of the cytotoxic agent inside the cells. Some linker designs have facilitated the generation of catabolites that are capable of diffusing into proximal tumor cells, inducing bystander killing that results in greater in vivo antitumor activity. This chapter reviews various linkers that have been designed for ADCs and the impact of linkers on the activity and safety of ADCs as cancer therapeutics.

E. E. Hong (✉) · R. Chari
Chemistry and Biochemistry, ImmunoGen, Waltham, MA, USA
e-mail: erica.hong@immunogen.com

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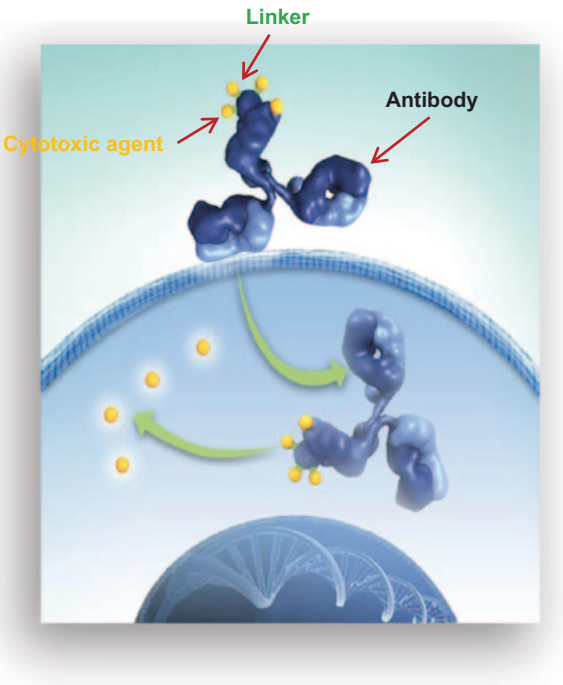

Fig. 4.1 Antibody–drug conjugate

4.2 Sites of Attachment and Reactive Chemical Moieties on Linkers

Sites of attachment on the antibody and the nature of the chemical groups on linkers to effect such attachment are important considerations in ADC design. They not only affect the conjugation efficiency and the ease of production of ADCs, but also the integrity and stability of the conjugate during production/storage as well as in patients. In addition, the linker often remains as an integral part of the metabolites and plays a key role in the activity and safety of ADCs in patients.

Lysine and cysteine residues have been utilized as sites of attachment on antibodies. Lysines are abundantly present on the antibody (80–100 lysines per antibody), and their primary amine readily reacts with *N*-hydroxysuccinimide esters, a chemical group often incorporated into linkers, to form stable amide bonds. Cysteines are present in antibodies in the form of disulfide bonds, which mediate intrastrand and interstrand bridges connecting light and heavy chains. For cysteines to be used as sites for conjugation, typically the interstrand disulfide bonds need to be reduced to generate reactive thiols, which can undergo the Michael reaction with a maleimide group commonly utilized in linkers to form thioether bonds. Both conjugation sites/methods allow efficient reactions and generate conjugates as a heterogeneous mixture with varying number of cytotoxic molecules per antibody. The distribution of the cytotoxic molecules per antibody can be highly reproducible

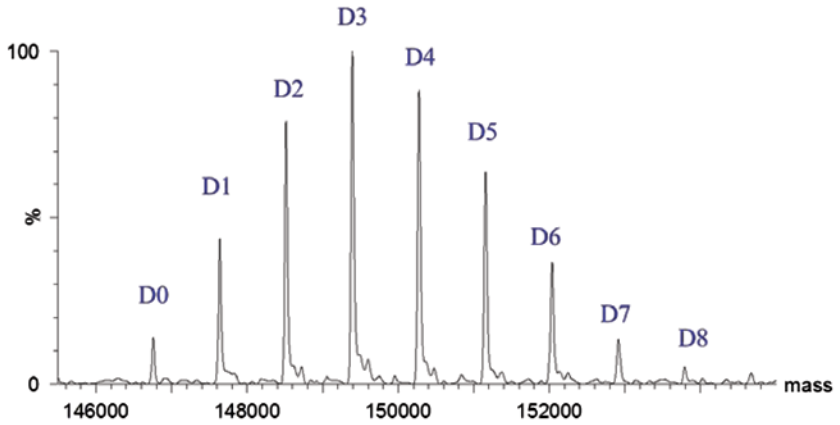

Fig. 4.2 Mass spectrometry profile of an antibody-drug conjugates (ADCs). The profile depicts a maytansinoid–ADC made with an average maytansinoid-to-antibody ratio of 3.5. The *n* of *Dn* refers to the number of cytotoxins attached to the antibody

from batch to batch, and typically follows a binomial function (Fig. 4.2; Singh and Erickson 2009; Wang et al. 2005).

Recent efforts to make homogeneous conjugates have explored diverse sites of attachment. An engineered cysteine on heavy and light chains of the antibody has been utilized as a site of attachment via maleimide linker chemistry (Junutula et al. 2008; Kung Sutherland et al. 2013). The reactive thiol of an engineered cysteine is not readily available for conjugation, because during antibody production, the sulfhydryl group of the engineered cysteine forms a mixed disulfide through exchange with cystine in the media. Before conjugation can proceed, a free thiol of the engineered cysteine must be generated by reduction of the antibody. The reduced antibody is then subject to partial oxidation to restore the intrachain and interchain disulfides that are important for antibody integrity, while maintaining the engineered cysteine in its thiol form. The thiol can then be utilized to react with a maleimide group on linkers, a conjugation strategy used to produce ADCs from the Thiomab (Junutula et al. 2008) or a similarly engineered antibody (Kung Sutherland et al. 2013). Alternatively, the free thiol of cysteine within a specific recognition sequence can be modified by a formylglycine-generating enzyme posttranslationally to produce an aldehyde-bearing amino acid, which can be conjugated to a cytotoxic agent via a hydrazine linker in a process called “aldehyde tagging” (Rabuka et al. 2012).

Carbohydrate moieties that are naturally present on antibodies have been used as sites of conjugation, especially for antibodies that tend to lose binding to antigen upon conjugation via lysine residues (Walus et al. 1996). Conjugation via glycans involves oxidation of the carbohydrate using sodium periodate, followed by reaction of the resulting aldehyde with a linker bearing a hydrazide group. Although efficient, this reaction suffered from undesired oxidation of the protein. A new method for glycan modification has emerged in recent years. Mannose or galactose containing an azido substituent is introduced into the endogenous glycosylation at Asp297

of the antibody either by expressing the antibody in the presence of azido-sugar (Ac4ManNAz or Ac4GalNAz) or by chemical remodeling using beta(1,4)-galactosyltransferase (Boeggeman et al. 2009). Use of the azido group ushered in the development of linkers with strained alkynes (e.g., cycloalkynes) that can react with the azide in copper-free click chemistry. The use of click chemistry gained popularity in conjugation to engineered sites, as the chemistry provides specificity without affecting the reactivity of the endogenous amino acids on antibodies.

Recently, nonnatural amino acids have been introduced into specific sites in antibodies either by the use of bacterial strains with orthogonal transfer RNAs (tRNAs) that can be charged with the nonnatural amino acid (Hutchins et al. 2011) or by cell-free extracts containing such tRNA and appropriate aminoacyl tRNA synthetases (Zimmerman et al. 2014). Click chemistry, along with oxime ligation, has been used for conjugation to these engineered nonnatural amino acids. A nonnatural amino acid containing an azido group can react with a linker containing a cycloalkyne, or a nonnatural amino acid containing a hydroxylamine can react with linkers containing an aldehyde. The latter oxime ligation has been used to generate a homogeneous anti-Her2 conjugate (Axup et al. 2012).

Glutamine has been used as an acceptor for conjugation when a bacterial enzyme, transglutaminase, was employed (Dennler et al. 2014; Strop et al. 2013). Transglutaminase catalyzes the formation of a covalent bond between the acyl side chain of glutamine (e.g., endogenous Q295 of human immunoglobulin G (IgG)) and a free amino group (e.g., of lysine) on a linker. An antibody can be engineered by introducing a glutamine tag to scan for sites that favor transglutaminase reaction anywhere on antibodies (Strop et al. 2013), or the native glutamine residue (Q295) can be exposed for conjugation by deglycosylation at N297 that typically hinders the accessibility of the glutamine (Dennler et al. 2014).

Conjugation chemistry has become sophisticated in recent years, and different sites on antibody and various functional groups on linkers have been explored. As a result, many claims have been made regarding the effect of these approaches (sites of conjugation and accompanying conjugation chemistry, as well as the consequence of homogeneous vs. heterogeneous conjugates) on the biological activity and potential safety of these ADCs (Junutula et al. 2008; Zimmerman et al. 2014). Thus far, there are no clinical data to support the various claims, and hence there is no clear understanding of the effect of these strategies on the activity and safety of ADCs.

4.3 Types of Linkers

Linkers are generally categorized into noncleavable or cleavable linkers. Noncleavable linkers represent linkers which remain intact during intracellular metabolism. ADCs with such linkers require lysosomal degradation of the antibody to release the cytotoxic agent. The metabolite retains the amino acid residue that served as the site of attachment to the antibody. Cleavable linkers are linkers that cleave during

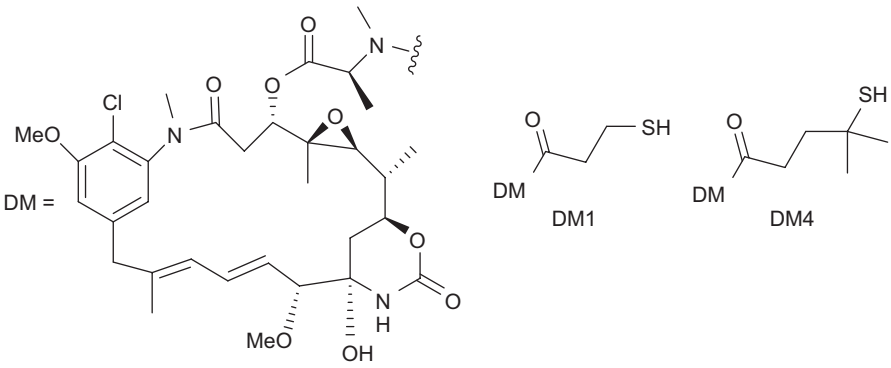

Fig. 4.3 Derivatives of maytansinoid

intracellular metabolism, generating metabolites that contain the cytotoxic agent with or without a portion of the linker. Cleavable linkers may be cleaved by hydrolysis, enzymatic reaction, or reduction, and include acid-labile hydrazone linkers, peptide-based linkers, and disulfide linkers, respectively.

4.3.1 Noncleavable Linkers

Thioethers are a widely used format for noncleavable linkers because of the facile reaction of maleimides with thiols under mild, neutral aqueous conditions. Thioethers can also be generated by reacting thiols with haloacetamido groups, although this reaction needs harsher conditions (e.g., higher pH and excess haloacetamido reagents; Alley et al. 2008). Thioether linkage has been used for microtubule-targeting agents, e.g., thiol derivatives of maytansine (Fig. 4.3; Singh and Erickson 2009) and auristatin (monomethyl auristatin F or MMAF; Alley et al. 2008).

Ado-trastuzumab emtansine (T-DM1 or Kadcyla®) is a conjugate approved by the Food and Drug Administration (FDA) for the treatment of human epidermal growth factor receptor 2 (HER2)-positive, metastatic breast cancer in patients previously treated with trastuzumab and a taxane. T-DM1 is composed of trastuzumab and DM1 linked by a heterobifunctional linker, SMCC (*N*-succinimidyl-4-(maleimidomethyl) cyclohexane-1-carboxylate; Fig. 4.4). The SMCC–DM1 conjugate is prepared by reacting lysine residues on the antibody with the *N*-hydroxysuccinimide ester moiety of SMCC, and linking the thiol of DM1 with the maleimide group on SMCC (Fig. 4.4). The antibody–SMCC–DM1 format is currently used in other ADCs. Epidermal growth factor receptor (EGFR)-targeting IMGN289, CD37-targeting IMGN529, and CD70-targeting AMG172 are being evaluated in the clinic. The sole metabolite of SMCC–DM1 conjugates is Lys–SMCC–DM1 (Fig. 4.5; Erickson et al. 2006).

An analogous thioether linker in which a hydrophilic tetraethylene glycol (PEG_4) replaced the hydrophobic cyclohexyl group of SMCC was developed (Fig. 4.6).

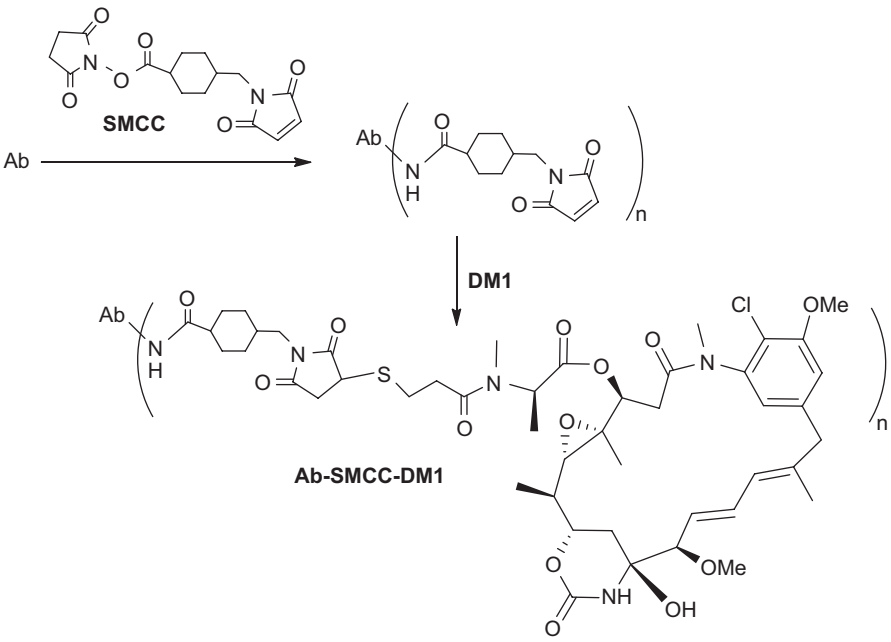

Fig. 4.4 Conjugation scheme for antibody–SMCC–DM1. SMCC *N*-succinimidyl-4-(maleimidomethyl) cyclohexane-1-carboxylate

When conjugated to an anti-epithelial cell adhesion molecule (EpCAM) antibody, the ADC with the hydrophilic linker showed higher potency in cells expressing P-glycoprotein, which is responsible for the MDR phenotype (Kovtun et al. 2010). This PEG_4Mal–DM1 conjugate generates a single metabolite, Lys–PEG_4Mal–DM1 (Kovtun et al. 2010). A similar PEG linker was also used to conjugate DM1 to two engineered cysteines in the heavy chain of trastuzumab (Junutula et al. 2010). The homo-bifunctional BMPEO (bis-maleimido-trioxyethylene glycol) linker used contained a triethylene glycol (PEG_3) spacer with two maleimido groups, used for conjugating to the thiol of DM1 and the thiol of a reduced free cysteine, to generate a homogeneous site-specific conjugate.

A thioether linkage has also been used for conjugation of antibodies to the tubulin inhibitor, auristatin. Anti-CD70–maleimidocaproyl (mc)–MMAF was generated by reaction of the thiol groups of native cysteines on the antibody with a maleimide-containing linker (Fig. 4.7). The anti-CD70–mc–MMAF conjugate was evaluated in the clinic for the treatment of lymphomas and renal cell carcinomas, and contained an average of four MMAF molecules per antibody. The anti-CD70 antibody was evaluated preclinically with another noncleavable linker. When a bromoacetamide-caproyl (bac) group replaced the mc to generate anti-CD70–bac–MMAF, it showed an improved plasma stability and 25% higher intratumoral drug exposure compared with a similar conjugate bearing mc–MMAF. However, despite this improvement, there was no statistically significant difference in the efficacy of these two conjugates in a subcutaneous 786-O renal cell carcinoma xenograft model (Alley et al. 2008).

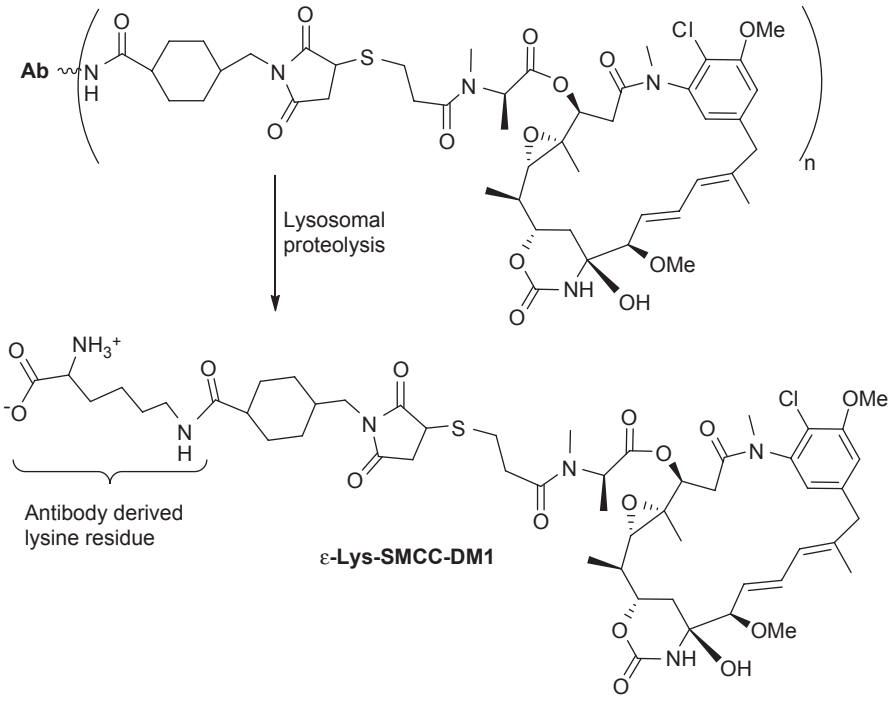

Fig. 4.5 Antibody–SMCC–DM1 generates a single metabolite Lys–SMCC–DM1. SMCC *N*-succinimidyl-4-(maleimidomethyl) cyclohexane-1-carboxylate

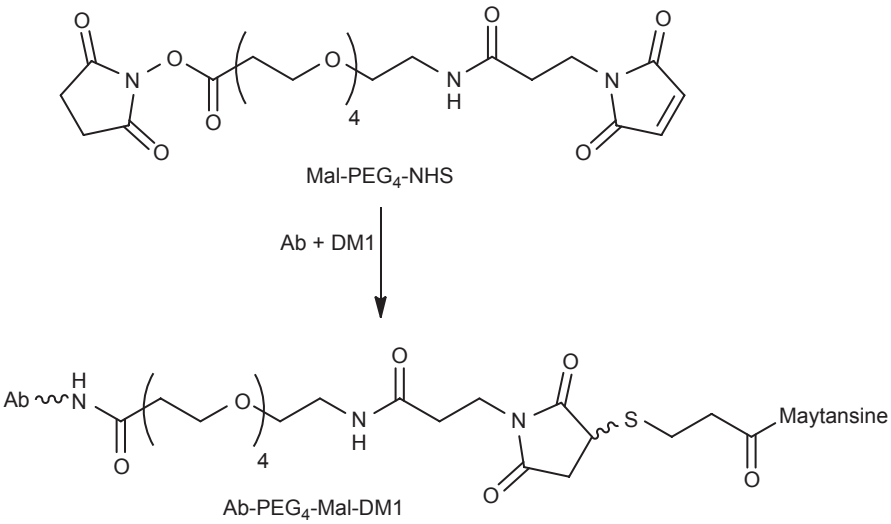

Fig. 4.6 Conjugation scheme for antibody–PEG_4–Mal–DM1. PEG_4 hydrophilic tetraethylene glycol

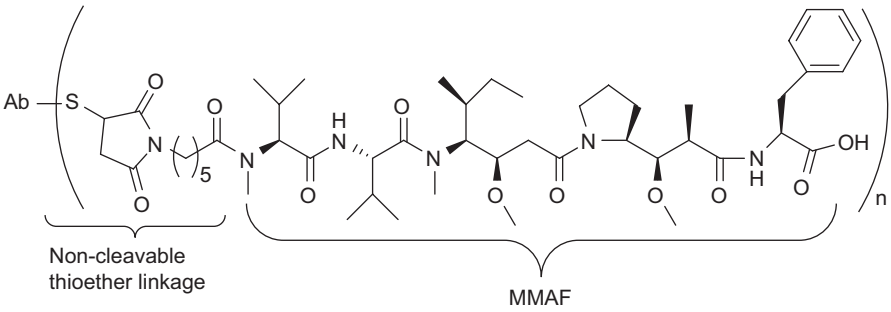

Fig. 4.7 Antibody–mc–MMAF. *MMAF* Monomethyl auristatin, *mc* maleimidocaproyl

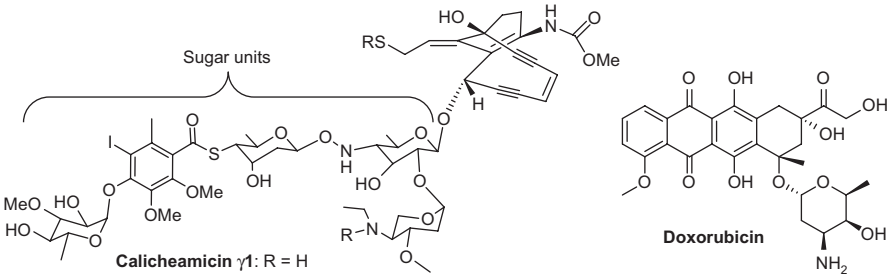

Fig. 4.8 DNA-targeting calicheamicin and doxorubicin

4.3.2 Cleavable Linkers

4.3.2.1 Acid-labile Linkers

Hydrazone linkers are designed to be stable in a neutral pH environment, such as in plasma, but hydrolyzed to release the cytotoxic agent in an acidic pH environment, such as in late endosomes and lysosomes. Potent DNA-targeting cytotoxic agents, e.g., calicheamicin or doxorubicin (Fig. 4.8), have been conjugated to antibodies via hydrazone linkers.

N-acetyl-γ_1^I-calicheamicin is a type of enediyne antibiotics that associates with the minor groove of DNA and causes double-strand breaks in a sequence-specific manner (Greenberg 2014). Calicheamicin has been evaluated in the context of antibodies to CD33, CD22, and Muc1, all of which were conjugated via hydrazone linkers (Ricart 2011; Hamann et al. 2005a).

The first FDA-approved ADC, gemtuzumab ozogamicin (Mylotarg®), was used for the treatment of patients with acute myelogenous leukemia (AML). Gemtuzumab ozogamicin is composed of an anti-CD33 IgG4 conjugated to calicheamicin via a bifunctional linker 4-(4'acetylphenoxy)-butanoic acid. The *N*-hydroxysuccinimide ester of *N*-acetyl-γ-calicheamicin dimethyl hydrazide 4-(4acetylphenoxy)-butanoic acid reacts with lysine residues on the antibody, leading to an average

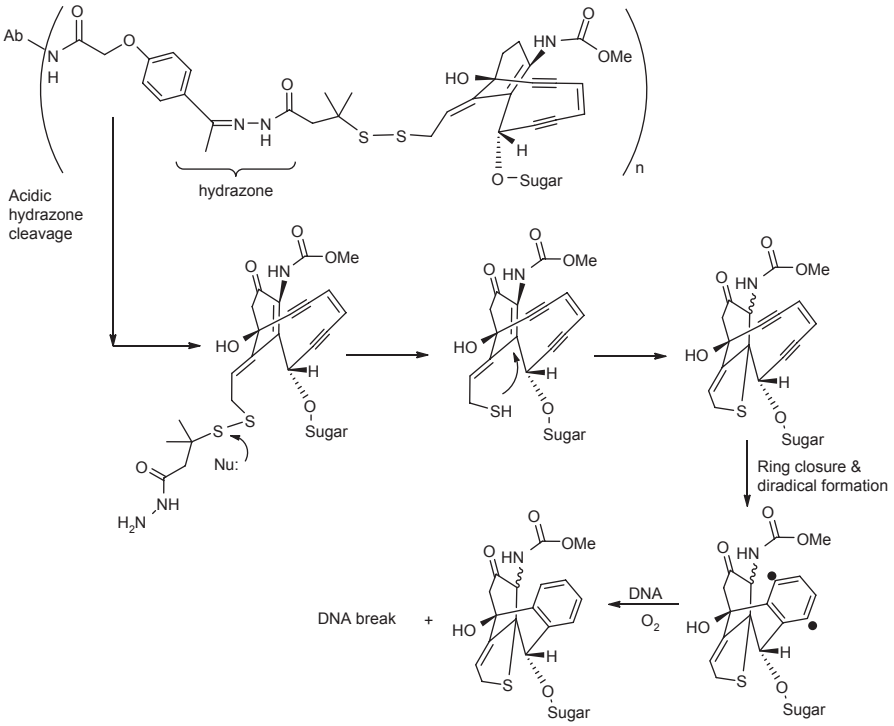

Fig. 4.9 Mechanism of release and action for antibody conjugated to calicheamicin via a hydrazone linker

loading of 4–6 calicheamicins per antibody, but with 50% of the antibody remaining unconjugated (Hinman et al. 1993). In an acidic environment, calicheamicin is released from the ADC upon hydrolysis of the hydrazone linker, and undergoes thiol-mediated reduction, followed by Masume–Bergman cyclization. This reaction generates ρ-benzyne biradicals that abstract hydrogen atoms from the phosphodiester backbone of DNA and leads to single- and double-strand lesions (Fig. 4.9). Mylotarg was voluntarily withdrawn from the market when a confirmatory phase III clinical trial failed to demonstrate clinical benefit. A new regimen of fractionated doses for better efficacy and safety is being evaluated in the clinic (Pilorge et al. 2014). Anti-CD22 antibody was also conjugated to calicheamicin via the same acid-labile 4-(4'acetylphenoxy)-butanoic acid linker, generating inotuzumab ozogamicin (CMC-544). CMC-544 was evaluated in the clinic for treatment of B-lymphoid malignancies (DiJoseph et al. 2004a, 2004b; Jain et al. 2014); however, its evaluation for the treatment of CD22-positive aggressive non-Hodgkin lymphoma was halted due to lack of benefit in overall survival. Another example for the hydrazone linker is highlighted in the anti-Muc1 antibody, CTM01, which was conjugated to calicheamicin via a hydrazone link to periodate oxidized carbohydrates on the antibody (Hamann et al. 2005b).

Similarly, doxorubicin, a DNA intercalator that elicits cytotoxicity by inhibiting topoisomerase II, has been conjugated to antibodies *via* a hydrazone linker. Thiols from the reduced interchain disulfides of the anti-CD74 antibody, IMMU-110, are conjugated to doxorubicin via a 4-[*N*-maleimidomethyl] cyclohexane-1 carboxyl-hydrazide, yielding an average of eight doxorubicin per antibody. This conjugate, milatuzumab–dox, was evaluated for the treatment of patients with multiple myeloma (Sapra et al. 2005), but was recently withdrawn for lack of efficacy.

4.3.2.2 Reducible Linkers

Reducible disulfide linkers take advantage of the difference in reduction potential in plasma versus the intracellular compartment. They are designed to keep conjugates intact during systemic circulation in patients, while efficiently allow reduction/cleavage of the disulfide bond inside cells. Reducible disulfide linkers generate neutral metabolites that can freely diffuse into neighboring cells and elicit bystander killing aiding the tumor penetration of the cytotoxic anticancer agent (see Sect. 4.4.3).

The most abundant low molecular thiol in blood is cysteine, the concentration of which is low at ~5 μM (Mills and Lang 1996). Serum albumin is another source of reducing potential in the blood, and its concentration is higher at ~0.6 mM (Turell et al. 2009). However, despite its relative abundance, the thiols in albumin are buried and inaccessible to thiol–disulfide exchange, thus it is not a major source of disulfide cleavage (for more discussion, see the linker stability section). In contrast to blood, the reduction potential inside cancer cells is much higher. Reduced glutathione is present at 1–10 mM (Wu et al. 2004), and cells also contain enzymes of the protein disulfide isomerase family, which may contribute to reduction of the disulfide bond in cellular compartments. This differential allows stable circulation in plasma, but efficient release inside the cells upon internalization of the ADC.

An important consideration for designing disulfide linkers is the steric hindrance of the disulfide bond and its impact on the stability of the linker. The HuC242 antibody has been used as a model system to evaluate the effect of hindrance on the reactivity of disulfide linkers to thiol–disulfide exchange in vitro and in plasma (Kellogg et al. 2011). The humanized C242 antibody recognizes CanAg, which is abundantly expressed in colorectal carcinoma. Various numbers of methyl groups were introduced on the carbon atoms bearing the disulfide bond (Fig. 4.10), and

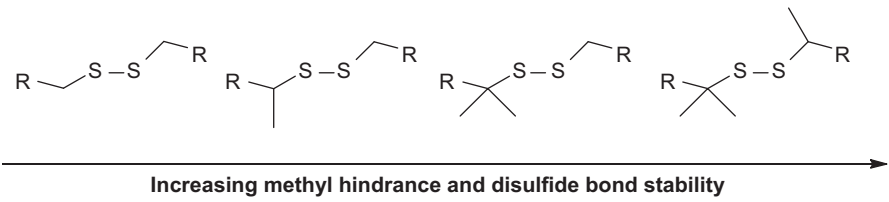

Fig. 4.10 Increasing methyl hindrance and disulfide bond stability

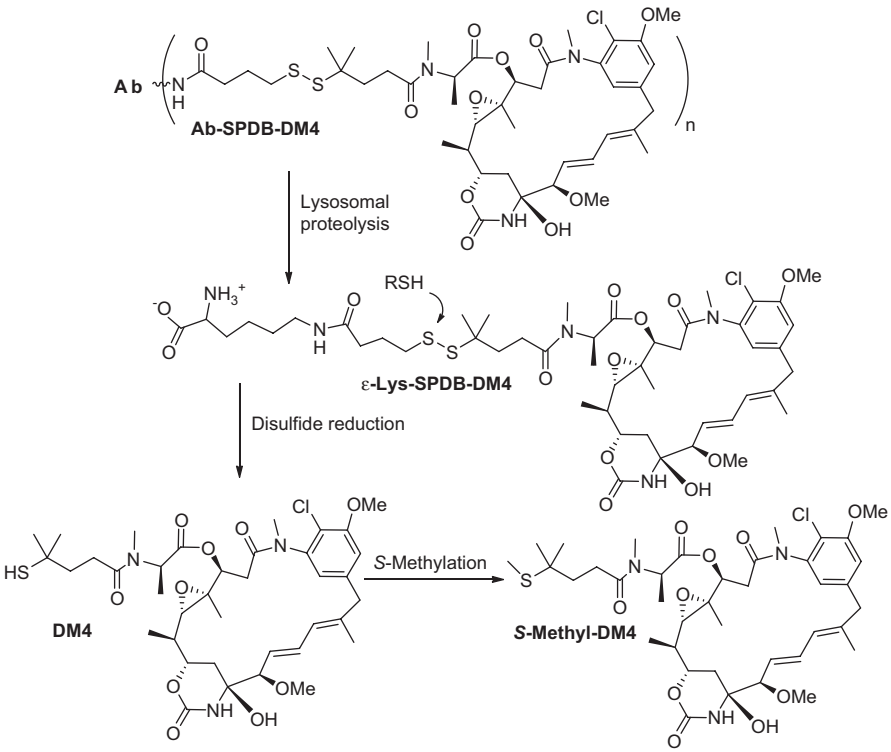

Fig. 4.11 Metabolism of antibody–SPDB–DM4. SPDB *N*-succinimidyl-4-(2-pyridyldithio) butanoate

the rate of reduction of these disulfide bonds in ADCs by dithiothreitol (DTT) was measured. The study led to the conclusion that the stability of the disulfide linker, in the presence of reducing agent, increased with the level of steric hindrance. The introduction of one methyl group on each side (total two methyl groups) of the disulfide bond was shown to confer greater stability than two methyl groups on one side of the disulfide. The difference in stability despite the same total number of methyl groups/hindrance highlights the importance of the position of hindrance (Kellogg et al. 2011).

Where are disulfide linkers reduced? The pKa of the thiol of reduced glutathione is 9.65. Hence, it is expected that the reduction will be inefficient in a low pH environment such as late endosomes and lysosomes (pH ~ 5). By contrast, the reduction is expected to be more efficient in a higher pH environment, as in cytoplasm (pH 7.4; Fig. 4.11). Consistent with this, no significant amount of reduction was reported to occur in lysosomes when probed with a conjugate linked to a pH-sensitive fluorophore via a disulfide linker (Austin et al. 2005; Yang et al. 2006). However, it has been demonstrated that in certain cell lines, there is some degree of reduction of the disulfide bond in endosomes, allowing an alternative pathway of metabolism

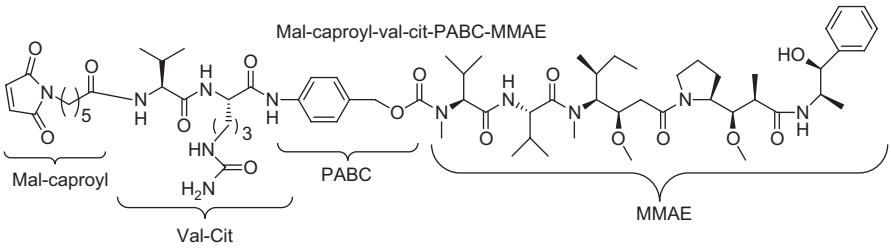

Fig. 4.12 mc–Val–Cit–PABC–MMAE. *mc* maleimidocaproyl, *Val* valine, *Cit* citrulline, *MMAE* monomethyl auristatin E

before lysosomes and consequential advantage in cytotoxic effect (Maloney et al. 2009). Accordingly, significant reduction takes place in the endosomes for folate conjugated to fluorescence resonance energy transfer (FRET) fluorophores via a linker with a disulfide bond (Yang et al. 2006).

4.3.2.3 Peptide Linkers

Peptide linkers are attractive for several reasons. First, peptide linkers may allow easier release of the cytotoxic molecule, as cleavage of one bond within the peptide linker is sufficient to free the metabolite within cells, as opposed to noncleavable linkers that require a cleavage of two bonds, both the N- and C-termini of the conjugated amino acid residue. Second, unlike hydrophobic synthetic linkers, peptide linkers, with the appropriate choice of amino acids, can offer hydrophilicity allowing higher cytotoxin loading per antibody, and/or better solubility in combination with unusually hydrophobic cytotoxins.

Microtubule-targeting MMAE is often conjugated to an antibody via a linker containing a valine–citrulline (Val–Cit) dipeptide and a self-immolative ρ-aminobenzylcarbamate (PABC) spacer (Fig. 4.12). Upon hydrolysis of the Val–Cit peptide by lysosomal proteases such as cathepsin B, PABC–MMAE is released and subsequently undergoes self-immolation at the PABC site to generate MMAE, which can diffuse into neighboring cells and elicit bystander cytotoxicity (Fig. 4.13). When compared with antibodies conjugated to MMAE via the hydrazone of 5-benzoylvaleric acid-AE ester (AEVB), the ADC with the Val–Cit dipeptide linker showed better stability and greater specificity of ADC activity in vitro and in vivo (Doronina et al. 2003). Val–Cit–PABC is the linker used in brentuximab vedotin (SGN-35, Adcetris™), a CD30-targeting ADC (Fig. 4.14; Senter and Sievers 2012). Val–Cit with PABC spacer has also been conjugated to the DNA-interacting cytotoxin, doxorubicin, in the context of mAbs c1F6 (anti-CD70) and cAC10 (anti-CD30) for targeting renal cell carcinoma and anaplastic large cell lymphoma, respectively (Jeffrey et al. 2006).

In addition to Val–Cit, variations of the dipeptide have been evaluated. Antibodies recognizing Lewis Y and CD30 on carcinomas were conjugated to MMAE via a

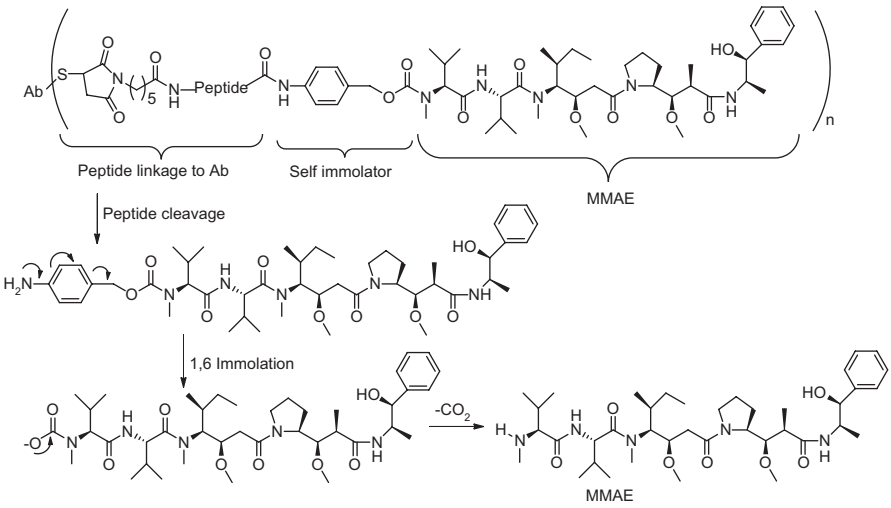

Fig. 4.13 Metabolism and self-immolation of mc–Val–Cit–PABC–MMAE. *mc* maleimidocaproyl, *Val* valine, *Cit* citrulline, *MMAE* monomethyl auristatin E

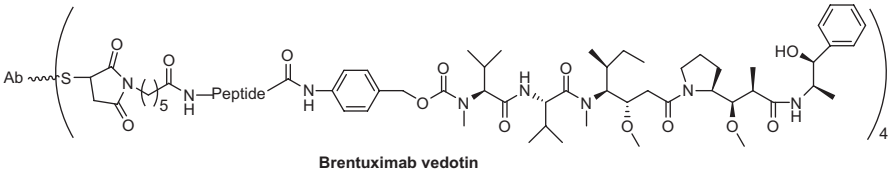

Fig. 4.14 Brentuximab vedotin

linker containing phenylalanine–lysine with a PABC spacer. In a head-to-head comparison using the same antibodies, the ADC with the Phe–Lys linker conferred lower stability in mouse and human plasma compared to that with a Val–Cit linker as measured ex vivo, with projected half-lives of 12.5 days versus 30 days in mouse plasma, and 80 days versus 230 days in human plasma, respectively (Doronina et al. 2003).

Peptide linkers can be modified to confer hydrophilicity for conjugation of hydrophobic drugs such as DNA minor-groove-binding drugs (MGBs). The valine–lysine–tetraethyleneglycol and valine–lysine–para-aminobenzyl ether self-immolative spacer allowed the conjugation of MGBs to antibodies without forming aggregates (Jeffrey et al. 2005).

4.3.2.4 β-Glucuronide Linker

A β-glucuronic acid-based linker was used to conjugate MMAE, MMAF, and doxorubicin propyloxazoline individually to antibodies c1F6 (anti-CD70) and cAC10 (anti-CD30; Jeffrey et al. 2007). β-Glucuronides are hydrolyzed by β-glucuronidase,

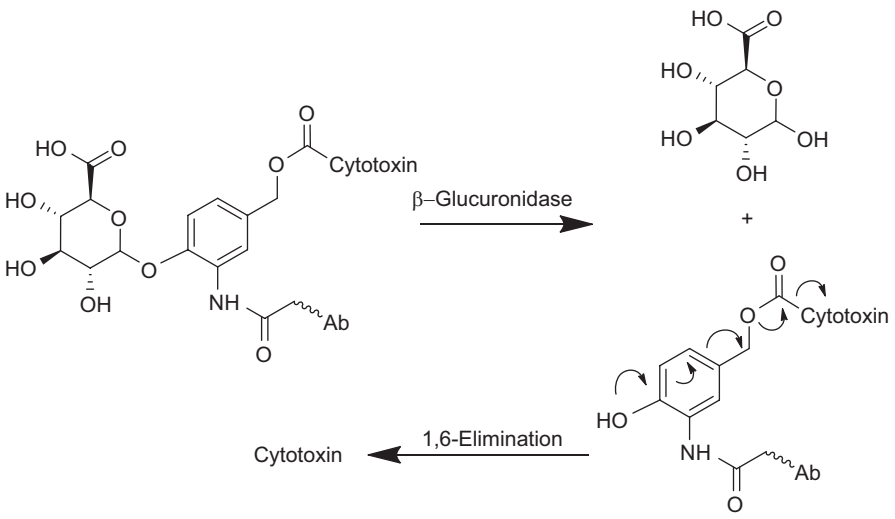

Fig. 4.15 Metabolism of antibody-β glucuronide-cytotoxin

an enzyme that is abundantly present in lysosomes and is overexpressed in some tumors (Fig. 4.15; Albin et al. 1993), and is reported to be the main drug metabolizing enzyme systems in human breast tumors and peritumoral tissues. The hydrophilicity of this linker allows the generation of monomeric ADCs with as many as eight cytotoxins per antibody. In addition, the hydrophilicity of the glucuronide linker afforded conjugation of hydrophobic drugs such as DNA minor-groove binders (Jeffrey et al. 2005, 2007).

4.4 Effect of Linkers on the Activity and Safety of ADC

4.4.1 Linker and Intracellular Processing/Activation

Ideally, ADCs behave as prodrugs: They are designed to be inactive during systemic circulation in plasma, but become activated upon internalization inside cells (Fig. 4.16). In the first step, ADCs bind to antigens on the cell surface, and undergo endocytosis. Upon clathrin-mediated vesicle formation, ADCs are transported to endosomes (pH ~ 5–6). Subsequently, endosomes fuse with lysosomes (pH ~ 4), the compartment that is rich in enzymes responsible for degradation of proteins, e.g., proteases and esterases. Inside lysosomes, ADCs are degraded to generate metabolites that consist of the cytotoxic agent covalently linked to the amino acid site of conjugation (Erickson et al. 2006). For example, T-DM1 generates Lys–SMCC–DM1, as the conjugation occurred between the primary amine of lysine and *N*-hydroxysuccinimide ester of SMCC (Fig. 4.5; Erickson et al. 2012).

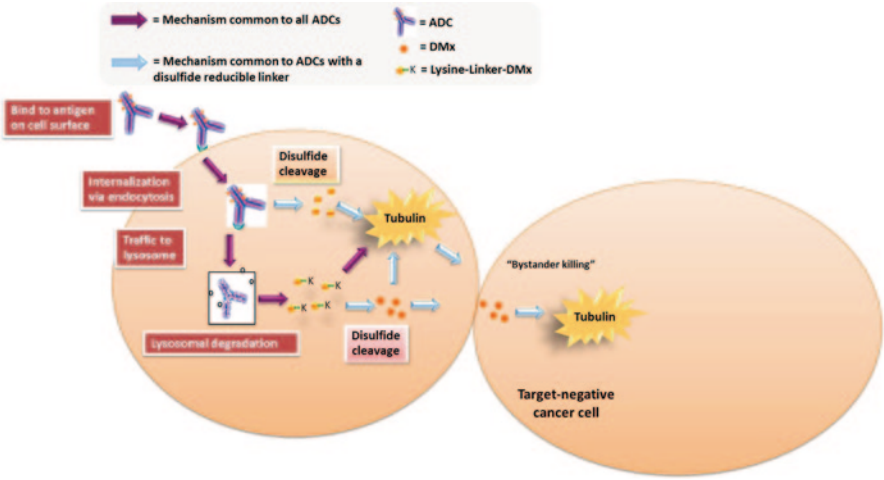

Fig. 4.16 Intracellular processing of antibody-drug conjugates

Linkers can be modified to take advantage of different modes of activation/metabolism. For example, conjugates containing peptide linkers may allow a faster rate of activation, as cleavage of one bond in the peptide linker is sufficient to release the cytotoxic agent, as opposed to ADCs with a noncleavable linker, which necessitates cleavage of two bonds at both N- and C-termini of the amino acid of attachment. Peptide linkers may also be designed so that the conjugates are metabolized in both endosomes and lysosomes, by altering the peptide sequence to match the substrate specificity of enzymes that are present in both compartments. For example, conjugates containing a peptide linker that can be cleaved by cathepsin B may be metabolized in both endosomes and lysosomes since cathepsin B is present in both compartments (Diederich et al. 2012). This may or may not be advantageous for the activity of ADC for the following reasons. Metabolism in both endosomes and lysosomes may allow faster activation that may afford an advantage in activity. However, if the linker is cleaved in the endosomes, it may also be susceptible to cleavage in the endosomal compartment during Fc recycling. Antibodies are recycled via neonatal Fc receptor (FcRN) binding in endosomes and are transported back to the cell surface thereby avoiding degradation in lysosomes. Such recycling of antibodies via FcRN is responsible for their long half-life in plasma (Lencer and Blumberg 2005). If the linker is cleaved in endosomes during recycling, the number of molecules of cytotoxic agent linked per antibody will decrease, which leads to (i) delivery of a lower amount of cytotoxic agent per antibody to cancer cells and (ii) toxicity caused by early release of free cytotoxin in normal tissues, with the degree of toxicity correlating to membrane permeability of the free cytotoxic agent. A similar activity and toxicity concern may apply to the acid-labile hydrazone linker, with an added concern for the lack of specificity; rather than relying on the enzyme specificity and its localized compartments (e.g., endosome), hydrazone linkers can be cleaved in any acidic environment.

4.4.2 Linkers to Overcome MDR

Treatment of cancer patients with chemotherapeutic reagents often leads ultimately to an MDR phenotype. The mechanism of the MDR phenotype varies, but overexpression of multidrug transporter MDR1 (also called P-glycoprotein) is the most commonly observed phenotype in the clinic. MDR1 is a membrane-associated transporter that confers drug resistance by mediating efflux of cytotoxic compounds. Many compounds used for ADCs, including calicheamicin (Matsui et al. 2002; Walter et al. 2003), doxorubicin, taxanes (Szakacs et al. 2006), maytansinoids (Tang et al. 2009), and analogs of dolastatin (Toppmeyer et al. 1994), are substrates of multidrug transporter MDR1, which poses a barrier to effective treatment of cancer patients with an ADC.

Linkers can be designed to evade the MDR1-mediated drug resistance. In a study to understand the effect of linkers on MDR1-dependent drug resistance, cell lines ranging from those naturally expressing a high level of MDR1, e.g., the colon adenocarcinoma HCT-15 and the renal adenocarcinoma UO-31, to an engineered cell line that mimics the high expression of MDR1, COLO 205^{MDR} (MDR positive; parental COLO 205 is MDR negative), were used (Kovtun et al. 2010). The presence of MDR1 led to a 6–18-fold reduction in sensitivity to tubulin inhibitors including maytansine, paclitaxel, and vinblastine. Similarly, cells became resistant to anti-EpCAM–SMCC–DM1 compared to those co-treated with cyclosporin A that inhibits MDR1, indicating that MDR1 is also effective against the metabolite of the ADC, Lys–SMCC–DM1. The replacement of SMCC with PEG_4Mal (Fig. 4.6) in an anti-EpCAM ADC led to enhanced cytotoxic activity in vitro and in HCT-15 and COLO 205^{MDR} xenograft models in vivo. The sole metabolite generated by anti-EpCAM–PEG_4Mal–DM1 was Lys–PEG_4Mal–DM1, suggesting that the evasion of MDR phenotype is due to the hydrophilicity of the linker. The potency of the metabolites is expected to be the same for Lys–SMCC–DM1 and Lys–PEG_4Mal–DM1, as anti-EpCAM–SMCC–DM1 and anti-EpCAM–PEG4Mal–DM1 display similar cytotoxic potency in non-MDR cell lines (Kovtun et al. 2010).

Evasion of MDR1-mediated drug resistance is not limited to noncleavable linkers. Recently, a hydrophilic disulfide linker was generated by modifying *N*-succinimidyl-4-(2-pyridyldithio) butanoate, SPDB, with a sulfonate group positioned distal to the disulfide bond (Fig. 4.17). When evaluated as an anti-EpCAM–sulfo-SPDB–DM4 conjugate, it showed better activity in the COLO 205^{MDR} cell line and xenograft model than anti-EpCAM–SPDB–DM4, suggesting that the sulfo-SPDB linker is effective in overcoming MDR1-mediated drug resistance. A similar enhanced activity in MDR1-positive cell lines was also observed for huC242–sulfo-SPDB–DM4 targeting CanAg, a novel glycoform of Muc1 (Zhao et al. 2011).

In addition to MDR1-resistance, the hydrophilicity of PEG_4Mal and sulfo-SPDB affords conjugation of a higher number of maytansinoid molecules per antibody (8–9 drugs per antibody; (Zhao et al. 2011)). Thus, these linkers may be particularly beneficial in conjugation of hydrophobic cytotoxins that may not have been previously feasible using other linkers. The ADC targeting the folate receptor

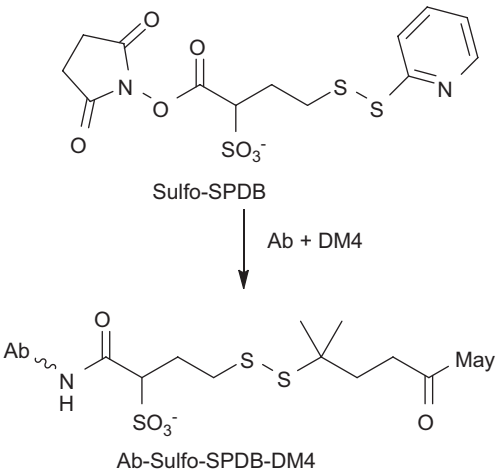

Fig. 4.17 Sulfo-SPDB linker that evades MDR resistance. *SPDB* N-succinimidyl-4-(2-pyridyldithio) butanoate, *MDR* multidrug resistant

(IMGN853) using DM4 linked to the antibody via a sulfo-SPDB linker is currently undergoing clinical evaluation in patients with ovarian carcinoma and nonsmall cell lung cancer.

4.4.3 Linkers to Improve Activity in Solid Tumors

Solid tumors are architecturally complex, often heterogeneous, and composed of different tissue types. These inherent properties of solid tumors can lead to heterogeneous expression of the target antigen, which limits the population of cancer cells that can be targeted by ADC. Even for tumors expressing the target antigen homogeneously, it has been documented that tumor penetration is not efficient due in part to the large molecular size of antibody-based therapies and uneven vasculature within tumors. When anti-Her2 antibody penetration was monitored in a MDA-435/LCC6^{HER2} xenograft model, despite homogeneous expression and immunohistochemical (IHC) staining of Her2, the fraction of Her2 bound by anti-Her2 antibody was patchy and localized (Baker et al. 2008). Thus, even solid tumors with homogeneous expression of the target antigen suffer from incomplete tumor penetration, which could limit the effectiveness of ADCs.

Linkers can be designed to help compensate for this. Thus far, two types of linkers have been designed to release cell-permeable free cytotoxic agents that can diffuse into neighboring cells and cause “bystander killing,” irrespective of whether these neighboring cells express target antigen. ADCs with bystander killing have often shown better activity in vivo compared with those without a bystander effect. This advantage in vivo has not always been apparent in vitro, where the bystander killing cannot be sufficiently recapitulated due to a difference in the architecture of two-dimensional tissue culture versus three-dimensional tumors.

One type of linker that can elicit bystander killing are disulfide linkers (Kellogg et al. 2011). The disulfide bonds in ADCs of the maytansinoid DM4 can be reduced by intracellular thiols to generate DM4 that can freely diffuse into neighboring cells, and if dividing, kill them. Furthermore, it has been demonstrated that DM4 undergoes methylation to form *S*-methyl DM4 in vitro and in vivo (Fig. 4.11; Erickson et al. 2006, 2010). Capping of DM4 through methylation may lead to an improved bystander effect: (i) methylation leads to formation of a noncharged compound that is readily membrane permeable and (ii) capping the free thiol of DM4 by methylation prevents possible disulfide exchange with endogenous disulfides such as cystine, which creates a hydrophilic charged compound, cysteinyl-DM4, that is not as membrane permeable. The ADC, huC242–N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP)–DM1, wherein DM1 is linked via a disulfide bond using the SPP linker, displayed bystander killing as a result of the reduction of the disulfide to release DM1. This ADC showed better efficacy than the conjugate with a noncleavable linker, huC242–SMCC–DM1 in COLO 205 and HT-29 xenograft models expressing the CanAg antigen, suggesting that the bystander effect plays an important role in the antitumor activity in vivo (Kellogg et al. 2011).

Peptide linkers have been utilized to generate free cytotoxins that can elicit bystander killing. Conjugates containing Val–Cit–PABC–MMAE are cleaved at Val–Cit dipeptide and release *p*-aminobenzyloxycarbonyl (PABC)–MMAE, which can undergoes self-immolation to generate a noncharged MMAE molecule that can penetrate neighboring cells (Fig. 4.13; Sievers and Senter 2013).

4.4.4 Linker Stability in Plasma

Noncleavable linkers often employ a thioether bond formed by a Michael reaction between free sulfhydryl and maleimide groups, the examples of which are evident in maytansinoid and auristatin conjugates. Noncleavable linkers are considered relatively stable during circulation in plasma. However, recent data suggest that the stability of the thioether bond may vary for different linkages.

1F6-C4v2-mc–MMAF, an ADC targeting the CD70 antigen on lymphomas and renal cell carcinoma, is generated by reacting a maleimido group of mc–MMAF with the reduced thiol of cysteines that normally form interchain disulfides in IgG. When compared with IF6-C4v2–bac–MMAF, an ADC that uses a haloacetamido group instead of a maleimide, the IF6–C4v2–mc–MMAF conjugate showed a reduced serum concentration and drug exposure, and it was found that a portion of mc–MMAF becomes conjugated to cysteine 34 of serum albumin during incubation in plasma. It is speculated that IF6–C4v2–mc–MMAF undergoes a retro-Michael reaction, which releases the maleimide drug that subsequently reacts with cysteine of serum albumin (Alley et al. 2008).

A similar retro-Michael reaction has been implicated for the instability of site-specific conjugate with a Thiomab that utilizes engineered cysteine as a reaction site for maleimide. Interestingly, a conjugation site-dependent instability of the thiol–maleimide bond was observed. When conjugated to a solvent-exposed cysteine

residue S396C of Fc, mc–vc–MMAE or mc-Alexa488 was released from the antibody at a higher rate than the same chemical moiety conjugated to V205C on a light chain that is located in a positively charged environment. This site-dependent loss of conjugated moiety from the antibody was accompanied by conjugation of the released "payload" to serum albumin, as modeled utilizing mc–Alexa488 conjugates, suggesting that a maleimide exchange has occurred between antibody and albumin. It was speculated that the thiol–maleimide bond in a solvent-exposed environment readily undergoes maleimide exchange in plasma, whereas maleimide in a positively charged environment undergoes succinimidyl ring opening, which prevents maleimide exchange (Shen et al. 2012b).

Maytansinoid conjugates have proven to be an exception. Although the thiol-containing maytansinoid, DM1, utilizes the same thiol–maleimide chemistry, it is much more resistant to the retro-Michael reaction, likely owing to the higher pKa of the thiol donor, compared to cysteine residues on the antibody. A study to further understand the stability of the SMCC–DM1 thioether linkage led to the observation that free DM1 is released only by β-elimination following oxidation of the thioether bond to sulfoxide, which likely occurs in ex vivo conditions (Fishkin et al. 2011). It is suggested that the thioether oxidation is a potential ex vivo artifact that is less likely to occur in vivo where the redox potential of plasma is more tightly regulated.

The stability of the disulfide linkers depends on hindrance of the disulfide. As discussed previously (see Sect. 4.3.2.2.), the greater the degree of hindrance around the disulfide bond, the lower the propensity to reduction in vitro by DTT, and this stability correlates well with pharmacokinetics (PK) of the ADCs in vivo. HuC242–SPDB–DM4 (two methyl groups at the carbon next to the disulfide bond) shows a longer half-life in mouse plasma than huC242–SPDB–DM3 or huC242–SPP–DM1 (both contain one methyl group at the carbon adjoining the disulfide bond; Kellogg et al. 2011).

4.4.5 Linker Stability and Activity of ADCs

Do stable linkers provide better activity for ADCs? Preclinical findings suggest that although stable linkers may increase exposure of tumors to ADC, it is the careful balance between the resistance to extracellular cleavage (e.g., in plasma) and facility of intracellular cleavage (upon cellular internalization) of linkers that provides the maximal activity.

The study of reducible linkers demonstrates this point elegantly. First, an ADC with SPP–DM4 with three methyl groups around the disulfide bond shows better stability against the thiol–disulfide exchange in vitro and longer half-life in plasma of 218 h compared with a SPP–DM1 conjugate, which has only one methyl group on the carbon atom adjacent to the disulfide bond (half-life at 47 h). Accordingly, the exposure for SPP–DM4 conjugate is greater than that of SPP–DM1, with AUC being 22,712 and 5186 h μg/mL, respectively. Yet, the huC242–SPP–DM1 shows better efficacy compared with huC242 antibody conjugated to SPP–DM4 in subcutaneous COLO 205 and HT-29 xenograft models. It is hypothesized that SPP–DM1 releases catabolites more readily than SPP–DM4 inside cells, such that the higher

exposure of SPP–DM4 cannot compensate for the faster intracellular activation of SPP–DM1 (Kellogg et al. 2011).

Similar results were observed for α_v integrin-targeting conjugates. CNTO365, using SPDB–DM4 with two methyl groups hindering thiol–disulfide exchange, showed better efficacy than CNTO366, using SPP–DM4 with three methyl groups hindering thiol–disulfide exchange, in HT-29 colon cancer and A-549 human lung cancer xenograft models (Chen et al. 2007).

Recent studies with T-DM1 illustrates that the linker stability alone does not predict efficacy. When compared against trastuzumab–SPP–DM1 (or T–SPP–DM1), T-DM1 (T–SMCC–DM1) showed better stability in plasma and longer half-life (Fig. 4.18). However, despite faster clearance and less total conjugate localization to tumors, T–SPP–DM1 showed a similar amount of metabolites generated at tumors (Fig. 4.18). As such, T-SPP-DM1 demonstrated similar efficacy as T–SMCC–DM1 in the BT474-EEI xenograft model (Erickson et al. 2012). It is clear that rate of activation inside the tumors and the total amount of metabolites are important predictive factors in antitumor activity. Moreover, different mechanisms of action for cell killing, i.e., bystander activity for T–SPP–DM1, but not for T-DM1, should also be considered in interpreting the efficacy data.

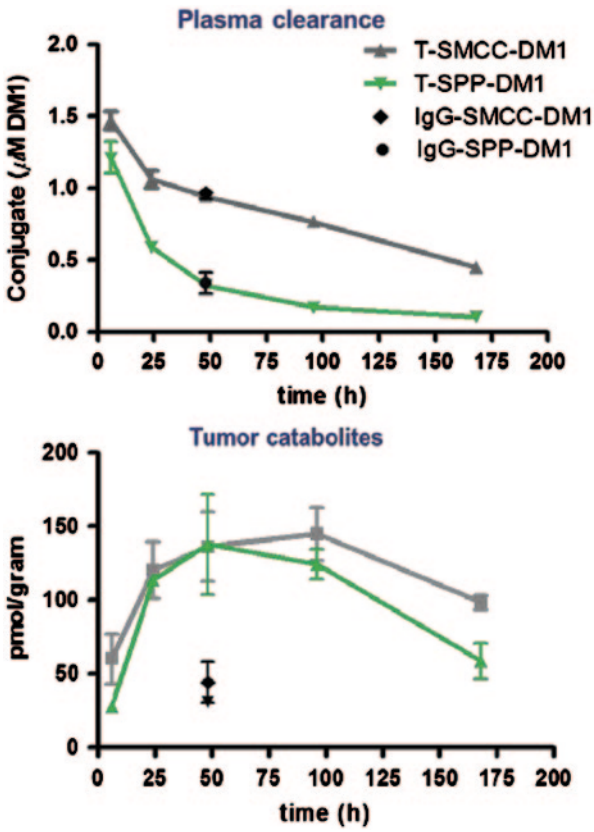

Fig. 4.18 Pharmacokinetics of trastuzumab-SPP-DM1 (T-SPP-DM1) and T-DM1 (T-SMCC-DM1) and accumulation of the metabolites in the BT474 EEI-derived tumor xenograft model. *SMCC N*-succinimidyl-4-(maleimidomethyl) cyclohexane-1-carboxylate

In conclusion, efficacy conferred by the linker cannot be predicted based on one aspect of ADC behavior, such as PK, as a number of different factors can contribute to the activity of ADC. Thus, each ADC with different linkers must be tested empirically to determine the combined effect of PK, exposure, rate of intracellular activation, and mode of killing (e.g., bystander, etc.) in the context of each target.

4.4.6 Linker Stability and Safety

4.4.6.1 Effect of Linker on Liver Detoxification of ADC and Biodistribution

The liver is the primary site of antibody metabolism, and indeed, a significant amount of metabolites from ADCs are found in the liver. The anti-CD56 antibody, huN901, conjugated to maytansinoid via various linkers was used to study the effect of a linker on detoxification of ADC (Sun et al. 2011). A radioactive tracer, tritium, was incorporated at the C-20 methoxy group of maytansinoid to allow for the detection of metabolites. HuN901-SMCC-[^{3}H]DM1 with a noncleavable linker was metabolized in liver to Lys–SMCC–[^{3}H]DM1, which is more than 50-fold less cytotoxic than the parental compound due to poor cell penetration. Both huN901–SPP–[^{3}H]DM1 and huN901–SPDB–[^{3}H]DM4 containing disulfide linkers also generate initially the analogous lysine-linked maytansinoid. However, subsequent reduction, *S*-methylation, and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidation in the liver leads to the formation of *S*-methyl sulfoxide and *S*-methyl sulfone derivatives of maytansinoid (Fig. 4.19). When tested in vitro, these oxidized maytansinoids were found to be 5- to 50-fold less cytotoxic than parental maytansine in many human cancer cell lines, illustrating efficient detoxification of ADCs in liver (Sun et al. 2011).

Biodistribution of ADC and the effect of linkers on the tissue distribution was assessed using the huC242 antibody targeting CanAg antigen. The unconjugated antibody, antibody–SPP–DM1, and antibody–SPDB–DM4 were labeled with ^{125}I on the antibody backbone to track the localization of ADC to various tissues. Following a single bolus injection of 4.16 mg/kg, it was found that the biodistribution profile is similar among all conjugates and the unconjugated antibody, demonstrating that ADC distribution is dictated by the antibody component (Xie et al. 2004; Xie and Blattler 2006). A closer look at huC242–SPDB–DM4 with a tritium label at the C-20 methoxy group of maytansinoid to follow the drug portion of ADC led to the finding that 30–50 % of injected dose/gram was recovered in the gall bladder from 2 h to 2 days post dosing, which is consistent with hepatobiliary elimination of maytansinoid (Erickson and Lambert 2012). Similar observations were made when rats were administered with a single bolus injection of T-[^{3}H]DM1; up to 80 % of the radioactivity was recovered in the feces over 7 days, consistent with hepatobiliary elimination of maytansinoid (Shen et al. 2012a). The biodistribution profile of huC242–SMCC–[^{3}H]DM1 resembled the profile of huC242–SPDB–[^{3}H]DM4 (Erickson and Lambert 2012), suggesting a lack of significant contribution of linkers on biodistribution for the linkers tested. Thus, different degrees of toxicity

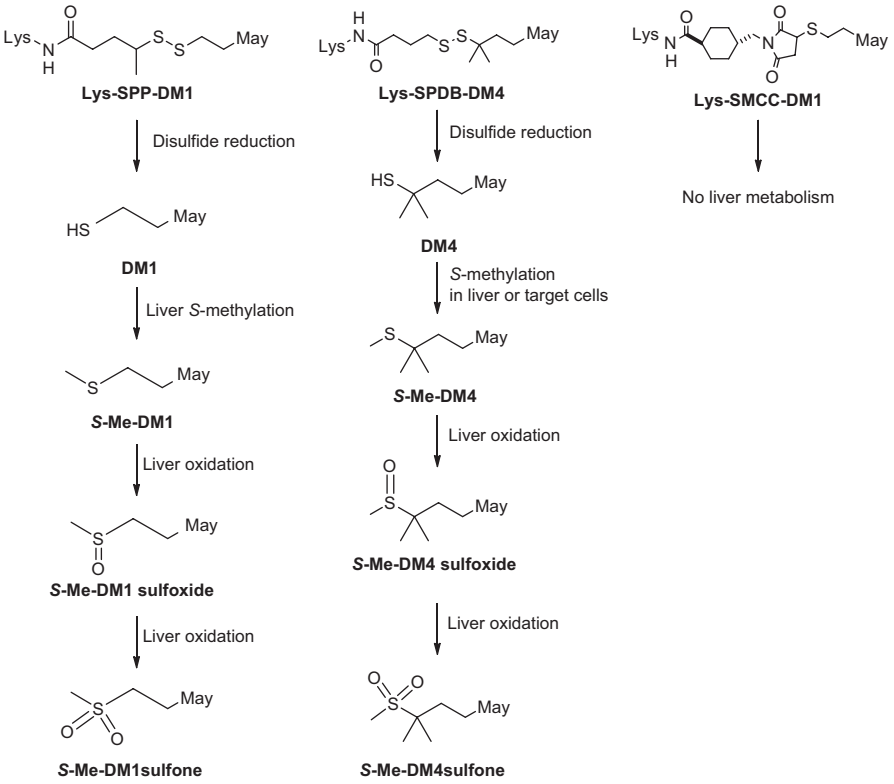

Fig. 4.19 Liver detoxification of antibody–maytansinoid conjugates

conferred by SPP, SPDB, and SMCC linkers in mice may be due to the cell permeability and subsequent potency of the metabolites, rather than linker-dependent distribution of the ADCs.

4.4.6.2 Effect of Linkers on Safety in Clinic: Inverse Correlation of Stability and Safety

There are many ADCs with different linkers in the clinic today. The PK of the ADC in humans has been a reflection of both stability of the linker and antigen-mediated clearance. For maytansinoid conjugates containing disulfide linkers, the PK in humans has been consistent with the susceptibility of the linker to reduction by DTT and the PK observed in preclinical animals. Cantuzumab or anti-huC242 targeting CanAg antigen provides an ideal example, in which the same antibody has been conjugated with two linkers and the resulting conjugates have been evaluated in phase I clinical trials. Cantuzumab mertansine (huC242–SPP–DM1), which contains mildly hindered disulfide, has a half-life of 2 days in human plasma (Rodon et al. 2008). Cantuzumab ravtansine (huC242–SPDB–DM4), which contains a

highly hindered disulfide, shows a half-life of 4.6 days (Qin et al. 2008). The PK of these conjugates in humans reflects the difference in the linker stability of SPP–DM1 and SPDB–DM4 in the context of CanAg. SAR3419 (huB4–SPDB–DM4) targeting CD19 shows 7.9 days of half-life in human plasma (Younes et al. 2009; Ribrag et al. 2014), indicating that the faster clearance of huC242–SPDB–DM4 (half-life of 4.6 days) is likely due to some contribution of antigen-mediated clearance rather than the inherent instability of the SPDB–DM4 linker–drug combination. Ado-trastuzumab emtansine has a half-life of 4.4 days in human plasma (Krop et al. 2010), reflecting largely the antigen-mediated clearance.

The maximum tolerated dose (MTD) can be affected by target-dependent toxicity, i.e., target expression on normal tissues could contribute to the final tolerable level of dose. However, different ADCs with the same linker-cytotoxic agent pairing directed against unrelated targets with diverse expression in tissues demonstrate that pairings can create an upper limit for the highest administered dose. For example, the MTD for auristatin conjugates containing dipeptide Val–Cit linker is typically close to 2 mg/kg (Younes et al. 2010) due to neutropenia and/or peripheral neuropathy. Maytansinoid conjugates also show a strong linker impact on tolerability. T-DM1 and AMG595, which use the SMCC–DM1 pairing, both have dose-limiting toxicity (DLT) of reversible thrombocytopenia; the MTD for T-DM1 is 3.6 mg/kg (Q3W; Krop et al. 2010) and AMG595 has been dosed to 3.0 mg/kg (Q3W). SAR3419 or huB4–SPDB–DM4 with a cleavable disulfide linker shows an MTD of 4.3 mg/kg (Q3W) with the DLT of reversible ocular toxicity (Younes et al. 2009). Cantuzumab mertansine or anti-huC242–SPP–DM1 with the most readily cleavable disulfide linker had an MTD of 6.4 mg/kg (Q3W) with the DLT of reversible elevation of liver transaminases (Rodon et al. 2008). Interestingly, there is an inverse correlation of the tolerability and the stability of linkers (Fig. 4.20). The chemical stability of the linkers in plasma (in vivo) can be ranked as SMCC > SPDB > SPP, with SMCC being the most stable linker and SPP being the least stable linker. In contrast to the chemical stability in plasma, the tolerability as demonstrated by the MTDs in the clinic for the maytansinoid conjugates listed above can be ranked as SPP > SPDB > SMCC. More clinical data are needed to determine whether (i) this stands true for all maytansinoid conjugates that may yield different small molecular weight metabolites during their eventual elimination and (ii) whether a similar trend

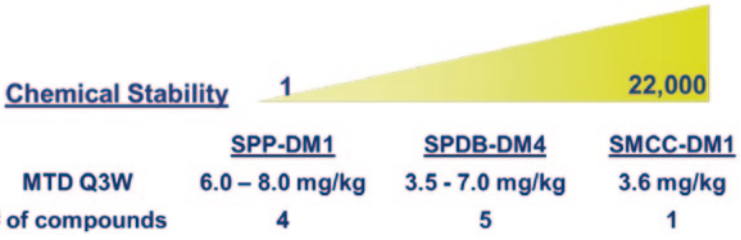

Fig. 4.20 Inverse correlation of the linker stability and MTD in human. Number of compounds refers to those that have been evaluated in the clinic. *MTD* maximum tolerated dose

can be found for other cytotoxin payloads. These findings suggest that it should not be hastily concluded that the most stable linker is the best linker for clinical development, and as has discussed above, stability, efficacy, and safety must be all factored in for consideration of the optimal linker for ADCs.

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Part III
Development Issues

Chapter 5
Formulation Development for Antibody-Drug Conjugates

Junyan A. Ji, Jun Liu and Y. John Wang

5.1 Introduction

Antibody-drug conjugates (ADCs) offer a promise of delivering more powerful tumor-killing activity while resulting in diminished side effects for cancer patients. Delivery of ADCs is precise and selective by combining a targeted monoclonal antibody (or antibody fragment) linked to a potent anticancer therapeutic. A variety of cytotoxins such as auristatins, maytansinoids, calicheamicin, duocarmycin (Tse et al. 2006; Phillip et al. 2008; Naito et al. 2000; Terrett et al. 2007), and other small molecules (Kim 2009) have been conjugated to monoclonal antibodies to generate ADCs. A variety of linker chemistries have been developed in the past 20 years, including acid labile hydrazones, sterically hindered *N*-succinimidyl-4-(2-pyridyldithio) butanoate (SPDB) and unhindered N-succinimidyl 4-(2-pyridyldithio)-pentanoate (SPP) disulfides, cleavable (vc) and noncleavable peptides, noncleavable thioethers N-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), etc. (Nolting 2013). Cysteine and lysine are the two most common naturally occurring amino acids that are used to attach the drugs through the linker to the antibody. Due to its specific reactivity, fast conjugation reaction, and lack of by-products, thiol-based Michael-type addition reactions have been widely employed for covalent conjugation of proteins, peptides, and drugs by the reaction of free cysteine or thiols with maleimides (Mather et al. 2006). In the case of brentuximab vedotin (ADCETRIS®) which was approved by Food and Drug Administration (FDA) in 2011), brentuximab is conjugated to maleimide-vc-PAB-MMAE with the sulfhydryl groups of cysteine from the reduced interchain disulfides of the antibody (structure refer to Chap. 4, Fig. 4.14, and Chap. 11, Fig. 11.1 Lyon et al. (2012)). The same reaction is also used in the newly developed THIOMAB®–drug conjugates (TDCs) in which the engineered free cysteine residues at specific sites of the antibody are conjugated with cytotoxins (Junutula et al. 2008, 2010). For

J. A. Ji (✉) · J. Liu · Y. J. Wang
Late Stage Pharmaceutical Development, Genentech, South San Francisco, CA, USA
e-mail: jji@gene.com

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trastuzumab emtansine (KADCYLA®) which was approved in 2013, the lysine residues on trastuzumab (Tmab) are first modified with the linker species SMCC to form the intermediate Tmab-MCC which is then reacted with the cytotoxin DM1 to form the ADC T-DM1 (structure refer to Chap. 4, Fig. 4.4, and Chap. 12, Fig. 12.1 Wakankar et al. (2010)). Although the synthetic routes vary, the linker of these conjugates contains the same thio-succinimide moiety of which the stability is critical for the product quality, safety, and efficacy (Alley et al. 2008). While the TDCs exhibit a more homogeneous distribution of linker drugs, both lysine-linked and cysteine-linked ADCs often lead to heterogeneous distribution of small-molecule drugs conjugated to antibodies.

Linker stability, drug-to-antibody ratio (DAR), and drug load distribution are critical to product quality, safety, and efficacy. This chapter focuses on the analytical methods and formulation strategies to address these critical quality attributes. It discusses degradation pathways of the thio-succinimide linker, the effect of DAR on ADC aggregation propensity, and formulation development considerations, including choice of pH and buffers, antioxidants, surfactants, and issues regarding liquid and lyophilized formulations.

5.2 Stability of Thio-Succinimide Linker

5.2.1 Mechanism of Degradation Pathways

Studying linker stability alone in the presence of a myriad of other degradation pathways in the antibody is difficult due to the complexity of the ADC structure and degradation products. The degradation of the thio-succinimide linker using a synthetic small-molecule model compound *S-(N-ethylsuccinimido)-N-acetyl-L-cysteine* (NEM-NACys; Fig. 5.1) was studied (Baldwin and Kiick 2011). Two primary degradation pathways were identified: (i) hydrolysis of succinimide to form an open ring structure and (ii) retro-Michael reaction in the presence of thiol compounds.

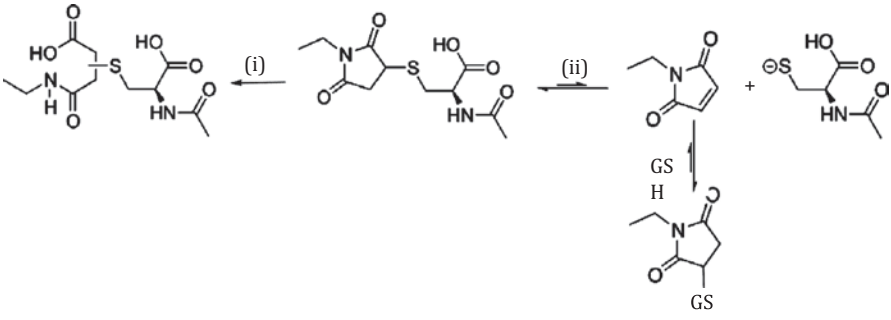

Fig. 5.1 Degradation pathways of a model compound *S-(N-ethylsuccinimido)-N-acetyl-L-cysteine* (NEM-NACys): **a** succinimide hydrolysis and **b** retro-Michael reaction in the presence of Michael donor (GSH = glutathione)

Succinimide is the key intermediate for deamidation of asparagine (Asn) and isomerization of aspartic acid (Asp) residues in a protein or peptide, either in vivo or in vitro (Wakankar and Borchardt 2006). Formation of succinimide is the rate-determining step in these reactions. Deamidation of Asn is dependent on hydroxide ion [OH^-] concentration, even as low as pH 3–4, and it increases rapidly under neutral and basic pH as the rate of succinimide hydrolysis accelerates. Unlike succinimide hydrolysis, the thioether bond is relatively stable in the absence of Michael donors such as thiol-containing compounds. The kinetics of the retro-Michael reaction and extent of thiol exchange is modulated by the reactivity of the Michael donor. It should be noted that a thioether bond is stabilized upon succinimide ring opening, and thus no further thiol exchange is possible even in the presence of a Michael donor (Baldwin and Kiick 2011).

Studying the degradation mechanism of the thio-succinimide moiety in the model compound facilitates an understanding of thio-succinimide linker stability in ADCs. The free drug release from a cysteine-linked ADC involving a retro-Michael exchange reaction to form an albumin adduct was reported (Alley et al. 2008). Using radioisotopes and liquid chromatography–mass spectrometry (LC–MS), it was determined that the first step involves a reversible dissociation reaction, in which the thio-succinimide-linked cytotoxic drug is cleaved from the antibody. Albumin, the predominant thiol-containing protein in plasma, has a reactive cysteine residue at position 34. Albumin reacts with the maleimide attached with the cytotoxin to form the albumin–drug conjugate. However, the retro-Michael reaction was not observed in phosphate-buffered saline because of the absence of a Michael donor.

Linker stability is critical to the safety and efficacy of ADCs. Using TDC as an example, three variants of monoclonal antibody are engineered, each having cysteine placed at a different site: light chain (Lc), heavy chain (Hc), and Fc region (Fc), differing in local charge environment and solvent accessibility to form conjugates via Michael addition reaction (Shen et al. 2012). The environment of the Lc conjugation site is the most positively charged and the Fc conjugation site has the most solvent accessibility. The extent of succinimide hydrolysis and albumin adduct formation was compared among these three variants. Due to the high local concentration of hydroxide ion for the positive charge environment in the Lc domain, succinimide hydrolysis appears to be much faster in the Lc TDC variant than in the Hc and the Fc TDC variants. Consequently, the Lc TDC variant has the least amount of albumin adduct formation because the thioether bond is stabilized upon the ring opening of succinimide, thereby preventing further thio-maleimide exchange. On the other hand, the Fc TDC variant has the most solvent accessibility and thus the most albumin adducts form via the retro-Michael reaction. Bioactivity loss and toxicity of protein conjugate have been observed in studies using cyno monkeys (*Macaca Fascicularis*) involving the retro-Michael reaction, which is due to the solvent-accessible sites undergoing maleimide exchange in the presence of albumin or another Michael donor, such as glutathione (GSH; Lin et al. 2008).

Both in vitro and in vivo stability and potentially therapeutic activity of an ADC could be greatly improved with a stable succinimide ring-opened linker, which would not result in a retro-Michael exchange reaction. To fulfill this need, a modified thio-succinimide linker was developed by incorporating a basic amino group

adjacent to the maleimide to introduce a rapid intramolecular base catalysis of the succinimide hydrolysis at neutral pH and room temperature (Lyon et al. 2013a; Challener 2014). The hydrolysis was completed in less than 2 h, which is suitable for manufacturing processes. The antitumor activity and safety were greatly improved with this type of self-stabilized ADC, which represents a potential advancement over the current ADC technology (Lyon et al. 2013b).

5.2.2 Characterization of Thio-Succinimide Hydrolysis of ADC

Linker stability is one of the critical quality attributes for ADCs. For thio-succinimide linker, succinimide hydrolysis creates additional charges from the ring-opened form, changing its charge state and hydrophobicity. Analytical methods that have been widely used for the identification and quantification of succinimide in proteins are also applicable to ADCs. Reverse-phase liquid chromatography with mass spectrometric (rpHPLC–MS) detection has been used for the analysis of thio-succinimide linker hydrolysis of intact ADCs in serum plasma (Xu et al. 2011). A fast FabRICATOR®(Genovis AB) rpHPLC-mapping method was recently developed utilizing the unique specificity of a novel proteolytic enzyme that cleaves at the hinge region creating pure F(ab′)2 fragments and Fc without any further degradation at mild physiological pH conditions within just a few minutes (Schneiderheinze 2011; Wagner-Rousset et al. 2014). With mass spectrometry detection, the FabRICATOR®rpHPLC method can also monitor site-specific thio-succinimide hydrolysis by separating and quantifying the ring-opened and ring-closed forms. Another alternative analytical method is imaged capillary isoelectric focusing (iCIEF), a common high-resolution separation method for charge variants of proteins. To accurately determine the level of succinimide hydrolysis of thio-succinimide linker by iCIEF, the contribution from the deamidation of the antibody moiety of an ADC must be subtracted. Figure 5.2 shows an overlay of iCIEF profiles of a cysteine-linked ADC and its unconjugated monoclonal antibody precursor. The heterogeneity of the iCIEF profile in the acidic region is due to various negative charges of the ADC gained from both deamidation and succinimide hydrolysis of the linker. Assuming that (i) charge variants are only caused by deamidation and thio-succinimide linker hydrolysis and (ii) each peak shift to the acidic region from the main peak represents a gain of one charge, an approach using weighted peak area which considers the charge state and drug load to quantify the thio-succinimide hydrolysis of ADCs was utilized (Zheng et al. 2013).

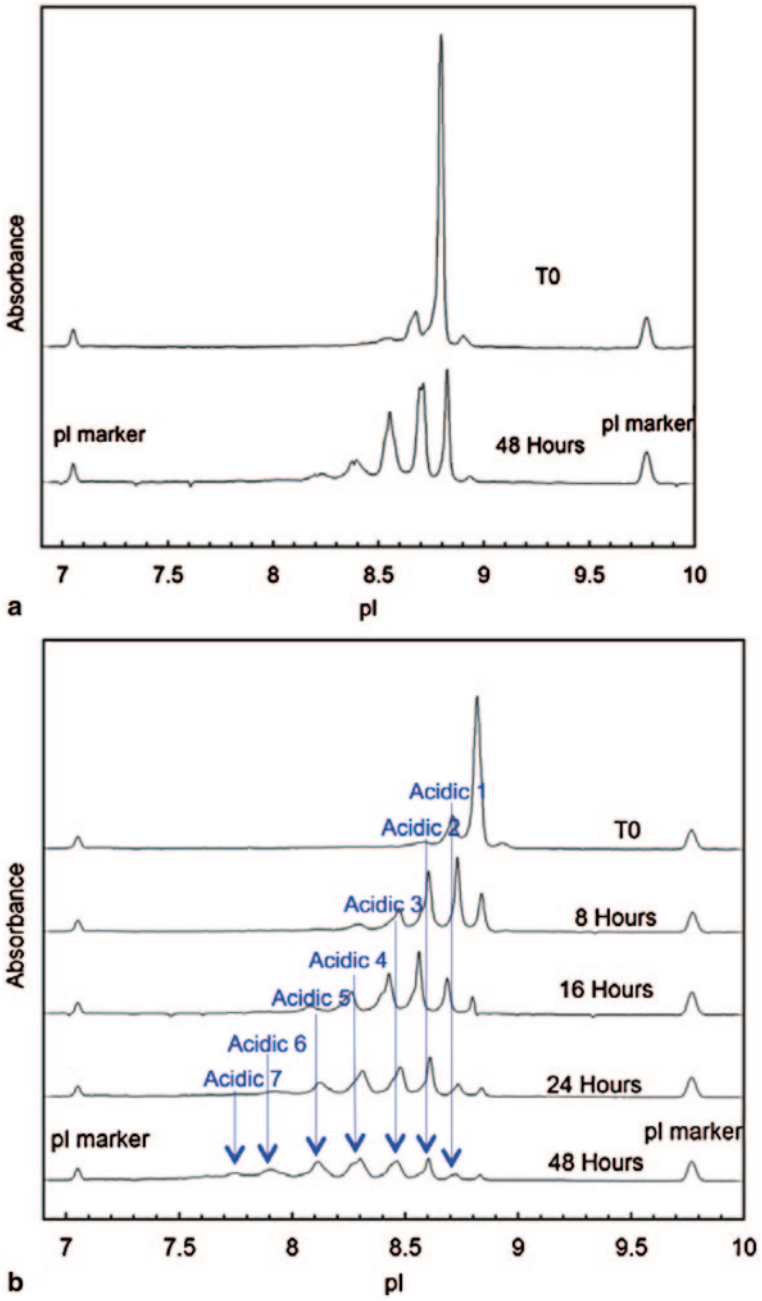

Fig. 5.2 iCIEF profiles of (**a**) an unconjugated Mab solution incubated at pH 9 for 48 h: negative charged variants generated from deamidation and (**b**) a cysteine-linked ADC incubated at pH 9 for 8, 16, 24, and 48 h: negative charged variants generated from both deamidation and succinimide hydrolysis (Zheng et al. 2012)

5.3 Effect of DAR on ADC Stability

5.3.1 Determination of DAR and Drug Load Distribution

Various analytical methods have been used to determine DAR (Ducry 2013). A simple and convenient ultraviolet–visible (UV/Vis) spectrometric method can be used, provided that the spectra of the antibody and the drug have distinct maximum absorbance wavelength so that the concentration of both can be separately determined, and thus allow the calculation of an average DAR. The method works for both cysteine-linked and lysine-linked ADCs. However, it is important to note that the absorptivity of the antibody and the drug may be affected in different buffers, ionic strengths, or pH. Hydrophobic interaction chromatography (HIC) has also been used for the determination of DAR for cysteine-linked ADCs, based on the increasing hydrophobicity with the least hydrophobic, unconjugated form eluting first and the most hydrophobic species with the highest drug load eluting last. The relatively mild and non-denaturing conditions of HIC, which do not interrupt the structure of an ADC, enable the measurement of the drug load distribution of an intact ADCs with different DARs. The average DAR can be calculated based on the percentage of each peak area and its respective drug load. Similar results can be obtained by using rpHPLC; it is an alternative method to provide better resolution than HIC for determination of DAR. However, the presence of organic solvent and small amount of organic acid present in the mobile phase of rpHPLC can be disruptive to the intact cysteine-linked ADC. Treatment with a reductant, such as dithiothreitol (DTT), can result in fully reduced interchain disulfides and yield a stable light chain, a light chain loaded with one drug, a heavy chain, and a heavy chain with one to three drugs attached. The average DAR is calculated based on the percentage of each peak area and its associated drug load of each peak. The rpHPLC method has been recently improved by the FabRICATOR® rpHPLC method (Schneiderheinze 2011; Wagner-Rousset et al. 2014). Another fast and direct method for the determination of DAR is liquid chromatography–electron spray ionization–mass spectrometry (LC-ESI–MS), which identifies the drug load distribution by intact mass measurement and determines the DAR using the peak area of each species. Deglycosylation and removal of the C-terminal lysine heterogeneity are often needed to reduce the complexity of the mass spectra (Rosati et al. 2014).

5.3.2 Aggregation

Both cysteine- and lysine-linked ADCs often lead to the heterogeneous distribution of drug molecules conjugated to antibodies. The cysteine-linked ADC with the conjugation sites at the sulfhydryl groups generated from nonspecific reduction of interchain disulfide bonds of the antibody such as brentuximab vedotin typically generates a mixture of ADCs with zero to eight drugs per antibody (Ducry 2013;

Valliere-Douglass et al. 2012). The DARs of lysine-linked ADC such as trastuzumab emtansine being conjugated through the lysine residues of the antibody ranges from DAR 0 to DAR 9 (Flygare et al. 2013). Studies have shown that both types of ADCs are more prone to aggregation compared with the unconjugated monoclonal antibodies, because of changes of the surface property resulting from the hydrophobic cytotoxic drug attachment or an altered higher-order structure of the antibody due to the drug load inducing new modes of inter- or intramolecular interactions (Wakankar et al. 2010; Adem et al. 2014; Acchione et al. 2012). For instance, higher DAR species such as DAR 6 and DAR 8 of an ADC are the major contributors to the formation of high molecular weight species (HMWS) under thermal stress for a cysteine-linked ADC with an average DAR of 3.5 (Beckley et al. 2013). These high DAR species are found to be irreversible, and noncovalent, and structurally altered forms of the ADC. Based on differential scanning calorimetry (DSC), it is deduced that destabilization of the hinge region/CH2 domain of the ADC molecule occurs upon thermal stress (Fig. 5.3). The melting temperature onset of the CH2 domain decreases with the increase of the average DAR value of the antibody, resulting in multiple CH2 melting transitions. Similar observations were reported for trastuzumab emtansine in which modification and conjugation of the antibody significantly impacted the thermal stability, primarily the CH2 domain (Wakankar et al. 2010).

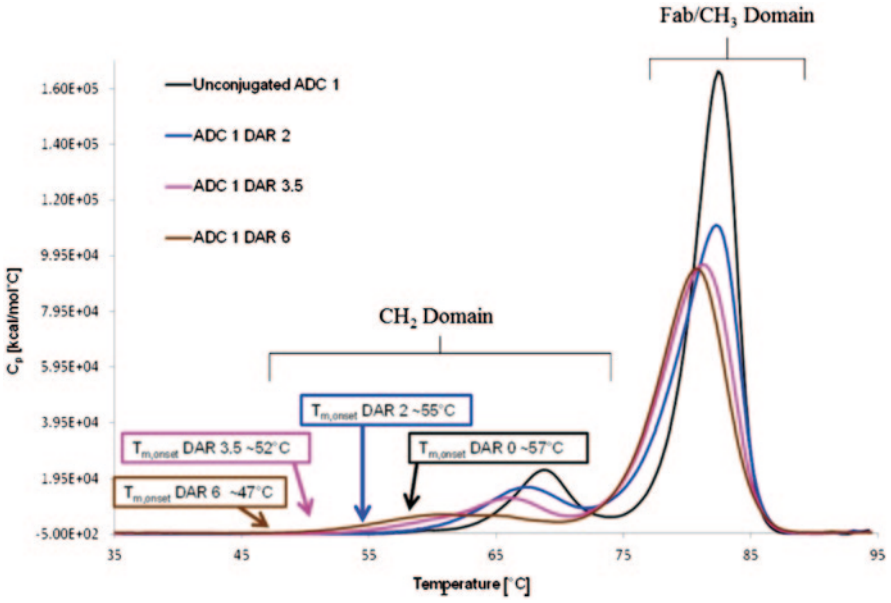

Fig. 5.3 DSC thermograms of an unconjugated ADC 1 and ADC 1 batches with average DAR 2, 3.5, and 6, respectively. (Reprinted from Beckley et al. (2013) with permission from the American Chemical Society)

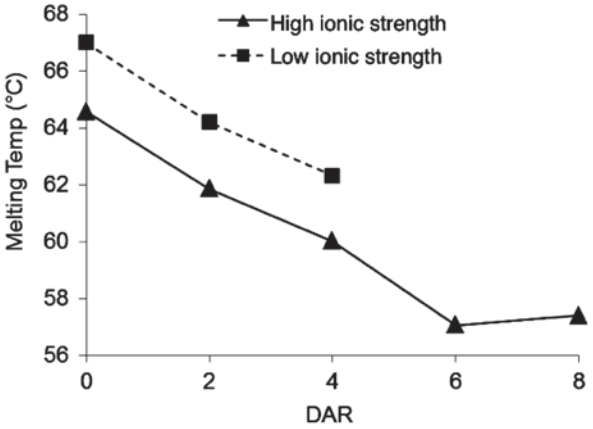

Fig. 5.4 Effect of ionic strength on the thermal stability of a cysteine-linked ADC in low ionic strength solution (20 mM histidine acetate pH 5.5) and high ionic strength solution (20 mM histidine acetate pH 5.5, 100 mM NaCl). (Reprinted from Adem et al. (2014) with permission from the American Chemical Society)

Ionic strength may also affect the stability of ADC, making it more prone to aggregation than unconjugated antibodies. A recent example of an ADC showed that a significant amount of aggregates formed with an increase of ionic strength, while the thermal stability of the monoclonal antibody was not affected in solutions up to 500 mM NaCl solution (Adem et al. 2014). The effect of DAR on the physical stability of ADCs can be exacerbated with the increase of the ionic strength of the solution. The correlation between thermal-induced unfolding and drug payload by DSC showed that the melting point of the same DAR species was significantly lower in the solution with high ionic strength (20 mM histidine acetate pH 5.5, 100 mM NaCl) than in that with low ionic strength (20 mM histidine acetate pH 5.5; Fig. 5.4). Other forms of stress can also induce aggregation such as agitation, light exposure, oxidative stress, or dilution into saline during intravenous (IV) administration, which is similar to how most of the monoclonal antibodies respond to the these stresses (Kumru et al. 2012; Nakajima and Suzuki 2013; Zheng et al. 2012).

Aggregation could potentially affect the safety and efficacy profile of the ADC. Controlling DAR and drug load distribution may facilitate the analytical characterization and formulation development. Antibodies with engineered free cysteine residues at specific sites, such as the TDCs, yielding predominantly homogenous 2-DAR species present a potential approach to the development of novel ADCs with improved quality and stability (Panowksi et al. 2014).

5.4 Formulation Considerations

The formulation development of ADCs is similar to that of antibodies. Additional critical quality attributes of an ADC product such as DAR and the release of free drug need to be controlled to provide a stable, safe, and efficacious product to patients. A balanced consideration among the stability of antibody, cytotoxic drug, linker, and the specific attributes of conjugate is required for ADC formulation development.

5.4.1 pH and Buffer

Similar to the controlling degradation pathways of deamidation, fragmentation, isomerization, etc. of the monoclonal antibodies, the choice of formulation pH and buffer plays a critical role in controlling the rate of succinimide hydrolysis of thio-succinimide ether linker of ADCs. The pH-rate profile of deamidation, isomerization, and succinimide hydrolysis was established using a model tetrapeptide, as shown in Fig. 5.5 (Gieger and Clarke 1987; Capasso et al. 1993, 1995), which illustrates the dependency of the reactions on hydroxide ion concentration. Similar observation can be found in the thio-succinimide reaction. Examples of deamidation occurring at lower pH have been reported, however, primarily through a mechanism

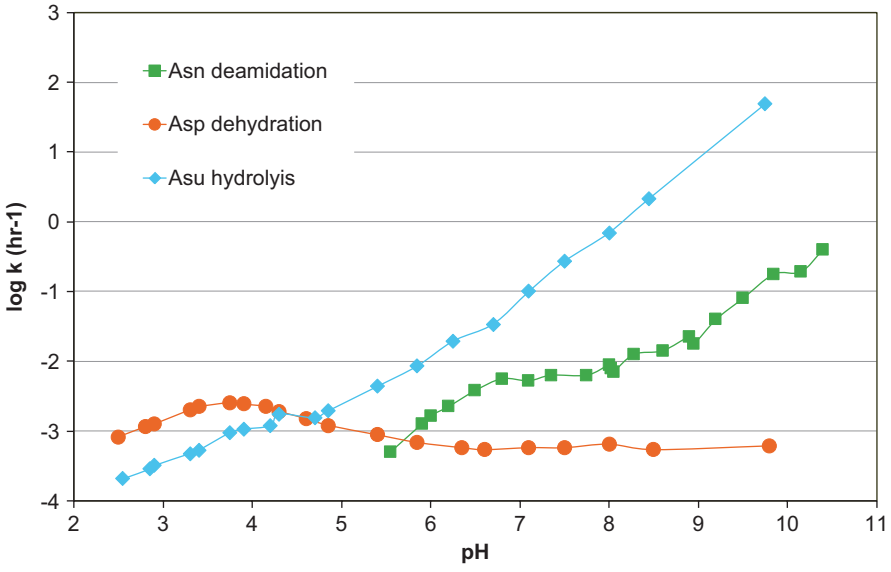

Fig. 5.5 pH-rate profiles of a tetrapeptide (Ac–Gly–x–Gly–NHMe): x = asparagine (Asn) or aspartic acid (Asp) or succinimide (Asu). (Data reconstructed from Gieger and Clarke (1987), Capasso et al. (1993, 1995))

independent of succinimide formation (Goswami et al. 2013). While the rate of deamidation increases rapidly with the increase of pH, the pH-rate profiles of Asp show its pH dependency in the range of pH 3–6 and then no effect above pH 7 with a sharp maximum at a pH close to the apparent pKa of the corresponding carboxyl group. Although the increased alkalinity increases the intramolecular nucleophilic attack by the deprotonated peptide nitrogen to facilitate succinimide formation in Asp residue, the OH− or O− moiety needs to leave in order to complete the ring closure reaction. A faster rate of attack by nitrogen at higher pH is canceled out because O−, formed readily at high pH, is a poor leaving group. As a result, the ring closure reaction of Asp is pH independent from pH 5 or above (Capasso et al. 1995). Factors other than pH such as sequence and steric effect of the side chain of neighboring amino acids may also influences the rate of deamidation and isomerization in proteins (Tyler-Cross and Schirch 1991; Son and Kwon 1995). As the rate-determining step is the formation of succinimide intermediate for deamidation of Asn and isomerization of Asp, succinimide hydrolysis of thio-succinimide linker is expected to be much faster. As illustrated in Fig. 5.5, the rate of succinimide hydrolysis of the tetrapeptide can be up to 10–100 times faster than that its deamidation of Asn or isomerization of Asp rate. Therefore, the choice of pH range for ADC formulation is limited due to its rapid rate of succinimide hydrolysis even in the range of pH 5–7 which is typical for most of the monoclonal antibody formulations. For this reason, it is recommended that a slightly acidic pH be used to minimize deamidation and succinimide hydrolysis; however, the pH should not be so low that the Asp isomerization, if present, becomes noticeable. Alternatively, to avoid these hydrolytic degradations altogether, the formulation of a lyophilized product could be considered.

Besides pH, buffer components and ionic strength also play an important role in ADC formulation as they do in monoclonal antibody formulations. Due to the dependency upon hydroxide ion concentration as in the deamidation of Asn, isomerization of Asp, or hydrolysis of succinimide, one needs to be cognizant of the temperature effect on the hydroxide ion concentration, [OH^-], in different buffer solutions. At different temperatures, the buffering species will affect degradation rate trends because of the changing [OH^-] in the solution. At an elevated temperature, base-type buffer such as histidine, Tris, etc. will generate less hydroxide ion than the acid-type buffer such as acetate. A buffer species such as acetate that may not seem advantageous in reducing degradation under accelerated temperature could be a better choice at the long-term storage temperature (Pace et al. 2013). Histidine buffer, one of the most commonly used buffer components in commercial formulations of monoclonal antibodies, may also be perceived to provide better protection against aggregation of ADCs. However, caution needs to be practiced when considering histidine buffer because of potential oxidative degradation (Stroop et al. 2011; Qi et al. 2009). Proper choice of a buffering agent can potentially achieve both pH control and stabilization of antibodies.

5.4.2 Antioxidants

Oxidation is a major concern in the development of stable formulations for the biopharmaceuticals (Nguyen 1994; Li et al. 1995; Hovorka and Schoneich 2001; Teh et al. 1987; Pearlman and Bewley 1993; Zhao et al. 1997; Sasaoki et al. 1989). The oxidizable amino acid residues in proteins include Met, Tyr, Trp, His, and Cys. More recently, Leu was also found to be subject to oxidation via free radical reactions (Steimann et al. 2012). H_2O_2, *t*-butyl hydroperoxide (tBHP), ozone, or UV radiation have been used to stress protein to predict oxidative potential of Met or Trp residues (Wei et al. 2007; Liu et al. 2008a, b; Harmon et al. 2006). A specific and reproducible stress model using a free radical generator 2, 2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) was developed to assess the propensity of both Met and Trp oxidation (Ji 2009). The same approach can be applied to investigate oxidative potential of ADCs since AAPH stress can be applied to both protein and cytotoxic drug (Ji 2009). To protect protein against oxidation, methionine is commonly used as a stabilizer in protein formulations (Lam et al. 1997; Oeswein et al. 1998). Several other antioxidants have been reviewed (Nema et al. 1997). However, some are unsuitable for protein formulation because of the following reasons: butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are limited to lipid- or surfactant-containing formulations, thio compounds (thioglycerol, Cys, *N*-acetyl cysteine, and GSH) may cause disulfide exchange, and reducing agents (sulfites) and ascorbic acid have been shown to cause undesirable reactions. Chelators such as ethylenediaminetetraacetic acid (EDTA) or diethylene triamine pentaacetic acid (DTPA) have been used to prevent metal ion-induced oxidation, although EDTA has been found to be ineffective at preventing metal-induced oxidation in a couple of incidents (Ji et al. 2009a). Other pharmaceutical excipients such as mannitol or sucrose also show relative antioxidant effect (Ji et al. 2009b). Polyols are considered a universal stabilizer against both physical and chemical degradation. Mannitol is a well-known hydroxyl free radical scavenger but not for alkyl peroxides or H_2O_2. Sucrose has generated similar results as mannitol, except it is less effective. The utility of antioxidants as stabilizers should be further explored in various dosage forms with drug candidates, including ADC molecules.

5.4.3 Surfactants

Nonionic surfactants, such as polysorbate (PS) 20 and PS 80, have been widely used in the biopharmaceutical formulations as a stabilizer to prevent surface adsorption and protein aggregation during manufacturing processes, shipping, storage, and even during administration, due to their effectiveness at low concentrations, inert nature, and long history of safety record when mixed with proteins (Kumru et al. 2012; Carpenter et al. 1999; Wang 1999; Kreilgaard et al. 1998; Chang et al. 1996). The higher propensity of the ADC towards aggregation as compared with the antibody, as a result of heterogeneous DAR distribution and hydrophobicity of the

cytotoxic drug in nature, may require more PS in the ADC formulation. It is also important to evaluate the in-use stability for administration. For IV delivery, the volume, material, headspace of the IV bag, diluent and dilution factor, dosing strength, dosing duration, and simulation of shipping post-dilution all need to be assessed to determine the appropriate PS content in the drug product (Alavattam et al. 2012).

Although highly effective in stabilizing proteins from aggregation and adsorption, PS is not innocuous. One needs to be cognizant of the oxidation potential when PS is used. Significant amount of peroxides, which cause PS and protein oxidation, can form under the stress of light, elevation of temperature, or catalyst upon storage (Ha et al. 2002). There have been concerns about PS degradation recently (Kerwin 2008). The detailed degradation mechanisms of PS were studied by Mahler et al. (Kishore et al. 2011; Borisov et al. 2011). PS cleaves at both the polyethylene oxide ether bond and fatty acid ester bond. The degradants from PS can cause aggregation and visible and subvisible particle formation (Maggio 2012). A new mechanism of hydrolytic degradation of PS by enzymatic catalysis was reported by LaBrenz in 2014 (Labrenz 2014). A lipase-like enzyme present in antibody bulk solution was alleged to cause significant PS 80 degradation, with a concurrent increase in particle counts. When PS cannot be used as an effective stabilizer, alternative surfactants can be explored (Patel et al. 2008; Ji et al. 2013).

5.4.4 Liquid Versus Lyophilized Formulation

A successful formulation ensures the delivery of a manufacturable, stable, safe, efficacious, and elegant as well as marketable product to the patients. The selection of a formulation for clinical and commercial applications is dictated by many factors. Product stability is one of the key drivers. In addition, it is also important to consider the manufacturing capability, cost, convenience of dosing, and competitive landscape. For both liquid and lyophilized products, it is essential to understand the chemistry and level of the major degradation products during the early and late stage of product development. To this end, one needs to design appropriate preclinical and clinical studies to address the potential impact of product-related degradants and impurity.

Due to the complexity of ADC, formulation development requires an understanding of not only the physicochemical stability of the monoclonal antibody but also that of small-molecule cytotoxic drug, linker moiety, as well as the intrinsic instability of the ADC itself. In addition to the high aggregation propensity of an ADC, one of the major concerns for efficacy, safety, and stability is the presence of excessive levels of free drug in an ADC product that could potentially produce significant adverse effects (Chih et al. 2011). Although a liquid formulation is desirable for manufacturing and convenience of drug delivery, the lyophilized formulation is often chosen due to the concerns for product stability in aqueous solution, despite complexity of the manufacturing process, as evidenced by the many clinical and commercial products, including both brentuximab vedotin and trastuzumab

emtansine (Goswami et al. 2013). However, lyophilization is not a panacea for all stability problems (Roy and Gupta 2004). Rational design of stable lyophilization formulations has been extensively reviewed (Carpenter and Manning 2002; Rey andMay 2010; Nireesha et al. 2013; Patel et al. 2013; Johnson and Lewis 2011). While it is typically assumed that enhanced stability can be achieved at low moisture level, work has also been conducted on the effect of surface area on the physical and chemical stability of the proteins (Johnson and Lewis 2011; Abdul-Fattah et al. 2007a, b, c, 2008;Luthra et al. 2008; Chang et al. 2005). Surface-exposed proteins are more susceptible to damage and improved stability appears to correlate with lower amounts of surface exposure, particularly in lyophilized product with low level of proteins. In some cases, product having equivalent residual moisture contents could display markedly different stability behaviors during storage due to the different surface areas (Yu and Anchordoquy 2009). In addition to the critical quality attributes such as soluble aggregates, visible and subvisible particles, moisture, cake appearance, reconstitution time and free drug which are commonly evaluated during stability testing, formulation and lyophilization cycle development, specific surface area measurement of a lyo-product using Brunauer–Emmett–Teller (BET) analysis, based on nitrogen multilayer adsorption as a function of relative pressure, could be recommended for characterization purpose. These studies are essential for the development of a robust lyophilization cycle to achieve a successful lyophilized formulation

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Chapter 6
Bioanalytical Assay for Characterization of Antibody-Drug Conjugates (ADCs)

Chen Xie and Zhijun Wang

6.1 Introduction

Antibody-drug conjugates (ADCs) are typically heterogeneous molecules composed of a cytotoxic drug linked to an antibody (a whole monoclonal antibody or its fragment) via a stable chemical linker (Polakis 2005; Teicher and Chari 2011). ADCs are becoming increasingly important for the treatment of cancer. The drug is considered to be stably associated with the antibody in the systemic circulation. When the antibody binds to the antigen-expressing tumor cells, the ADC is able to be internalized and trafficked to the lysosomes. The linker can be hydrolyzed by the low intralysosomal pH (pH 4.5–5.0) or digested by proteases, resulting in the release of the free drugs. ADCs have complex molecular structures that can incorporate the features of both large and small molecules. The small-molecule drugs are conjugated to an antibody via a variety of amino acid residue conjugation sites (Carter and Senter 2008; Junutula et al. 2008) which will result in heterogeneity of ADCs. These include conjugation of the linker drug at lysine residues and cysteine residues through reduction of the interchain disulfide bonds or at engineered cysteine residues (Fig. 6.1**).** The conjugation reaction can result in heterogeneous mixtures of ADC molecules with various drug-to-antibody ratios (DARs). If the conjugation is at the lysine residue, the distribution of DARs can range from 0 to 9 drugs, based on previous reports (Stephan et al. 2011). A range of drug numbers such as 0, 2, 4, 6, 8 will be conjugated at different disulfide bond where the cysteine residues are located. ADCs with mainly DAR 2 have been reported for conjugation at engineered cysteines (Stephan et al. 2011; Kaur et al. 2013).

Z. Wang (✉) · C. Xie
Center for Advancement of Drug Research and Evaluation, Western University of Health Sciences, Pomona, CA, USA
e-mail: zwang@westernu.edu

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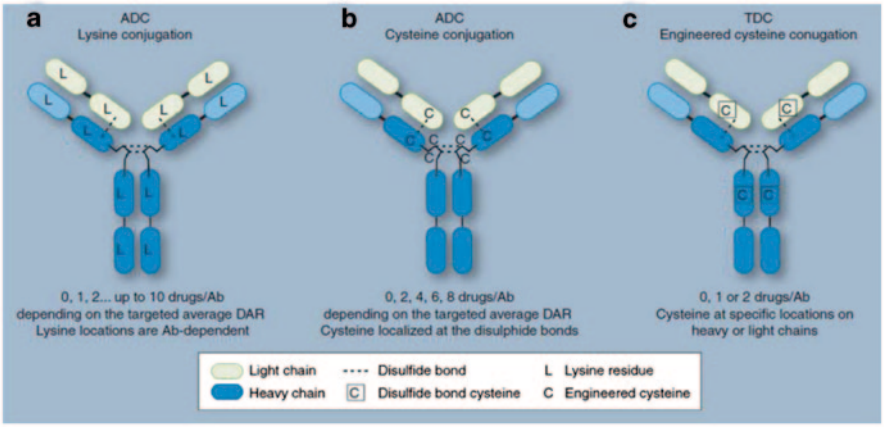

Fig. 6.1 Antibody–drug conjugate (ADCs) conjugation sites and drug load characteristics. **a** Conjugation through lysines, **b** Conjugation through reduced interchain disulfide bonds, **c** Conjugation through engineered cysteines. *Ab* Antibody, *ADC* Antibody–drug conjugate, *DAR* Drug-to-antibody ratio, *TDC* ThiomAb-drug conjugate. (Adapted by permission from Future Science Ltd: Stephan et al. 2011)

The complexity of ADCs may increase in vivo due to the biotransformation resulting from catabolism and metabolism. In addition to drug deconjugation by chemical or enzymatic cleavage, other biotransformation, such as adduct formation, generation of peptide fragments or linker drug fragments can also lead to increased complexity (Kaur et al. 2013; Shen et al. 2012). Due to the presence of mixtures with different DARs and the potential for biotransformation, it is important to develop an appropriate bioanalytical assay to accurately measure ADCs and all the analytes in the plasma or serum (Gorovits et al. 2013).

The species measured for ADCs generally include a combination of the total antibody, the conjugated antibody, the antibody-conjugated drug, and free (unconjugated) drug. The amount of total antibody can be used as an assessment of the protein component of the ADC. The content of conjugated antibody and antibody-conjugated drug can provide an assessment of the conjugate efficiency. Free drug could be used for evaluation of the safety (Gorovits et al. 2013). DAR is one of the most important markers for quality evaluation of an ADC in terms of potency and toxicity. Thus, reliable in vitro and in vivo methods to measure DAR distribution are highly required. In addition, a variety of methods have been utilized to analyze the distribution of drug-linked forms (e.g., fraction of antibodies containing different number of drugs) which is an important characteristic of ADC, because different forms may lead to different pharmacokinetic and toxicological properties (Hamblett et al. 2004; Wakankar et al. 2011). The information and typical bioanalytical methods for quantification and qualification of ADC are listed in Table 6.1. A single assay may serve multiple purposes and provide different information. However, a set of assay methods is required to comprehensively describe the concentration and composition of heterogeneous ADCs. The commonly used approaches and methods for bioanalytical characterization of ADCs are summarized in this chapter.

Table 6.1 Analytes and related parameters commonly assessed for antibody–drug conjugate (ADCs) bioanalysis

Analyte types and parameters	Details	Typical bioanalytical method
Total antibody	Conjugated and unconjugated antibodies (DAR ≥ 0)	LBA
Conjugated antibody	Antibody with drugs (minimum of DAR ≥ 1)	LBA
Antibody-conjugated drug	Total drug conjugated to antibody	MS/LBA
Free drug	Drug fallen off the antibody	MS/LBA
DAR	Average number of conjugated drug	HPLC/UV/Vis/HIC/MS
Drug distribution	Location of drug on the antibody	MS (LC-ESI-MS/MALDI-TOF-MS)/ HIC/ HPLC/IEC/SEC

LBA ligand-binding assay, *DAR* drug to antibody ratio, *MS* mass spectrometry, *HIC* hydrophobic interaction chromatography, *HPLC* high-performance liquid chromatography

6.2 Typical Bioanalytical Methods for ADCs

There are a variety of assay methods that have been used to analyze ADCs. These include ligand-binding assay (LBA), ultraviolet–visible spectrophotometry (UV/Vis), mass spectrometry (MS), and chromatography-based assays.

6.2.1 LBAs

LBA refers to an assay method based on the binding of a ligand to its specific receptor (Luckey et al. 1993). Large molecules have well-defined tertiary structures that are suitable for LBAs. LBA has been commonly used to determine the concentrations of ADC, total antibody (unconjugated and conjugated), and the free or released small-molecule drug. LBAs are generally performed using enzyme-linked immunosorbent assays (ELISAs), cell-based binding assays, or other types of binding assay.

6.2.1.1 ELISA-Based Binding Assays

ELISA is a test using the basic immunology concept of an antigen binding to its specific antibody, which allows detection of various substances including both small molecules (such as chemical drugs) and large molecules (such as peptides, proteins, or antibodies). It is commonly used to determine the ADC concentration based on the specific antigen which can capture the antibody. ELISA can be used to measure the total antibody including fully conjugated antibody, partially deconjugated antibody, and fully deconjugated antibody, using specific reagents which can bind to either the antibody or the small chemical drugs conjugated to the antibody. In

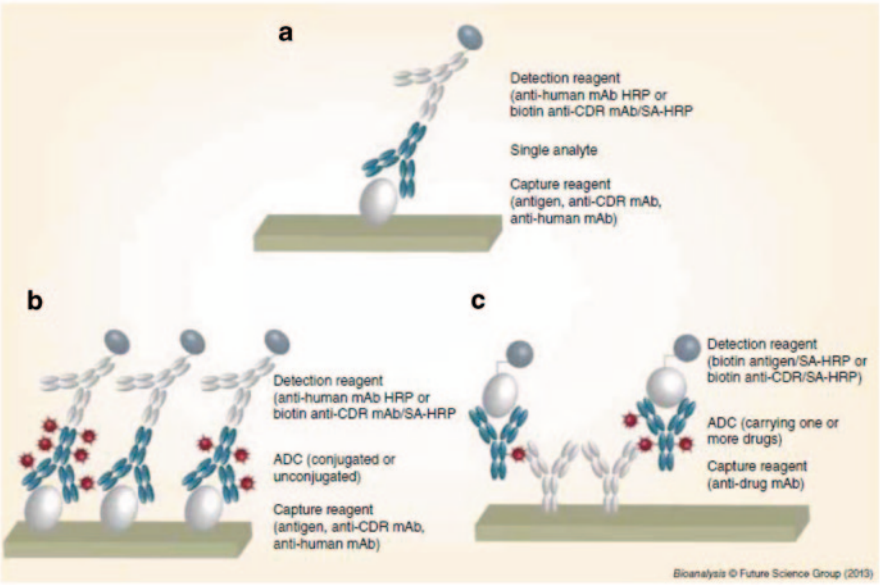

Fig. 6.2 ELISA-based binding assays. **a** Typical ELISA, **b** ELISA for ADC total-antibody measurement, **c** ELISA for ADC conjugated-antibody measurement. *ADC* antibody–drug conjugate, *Anti-CDR* anti-complementarity determining region, *HRP* horseradish peroxidase, *mAb* monoclonal antibody, *SA–HRP* streptavidin–horseradish peroxidase. (Adapted by permission from Future Science Ltd: Kaur et al. 2013)

addition, ELISA can been used to measure the concentration of the free drugs released from the ADC.

ELISA is the most common assay for measurement of total antibody. Figure 6.2a shows a typical ELISA binding assay that is used for large-molecule analysis. The capture reagent (antigen, anti-complementarity determining region monoclonal antibody (mAB), or antidrug mAB) is attached in a solid matrix. The target analytes can be recognized and bond to the capture reagent, while other molecules will be washed out. Then the amount of analytes can be quantified by a detection reagent. Figure 6.2b shows an example of a measurement of total antibody using reagents that bind to the antibody (Kaur et al. 2013). This format uses the specific antigen to capture either monoclonal or humanized antibody followed by detection of captured antibody with enzyme-conjugated anti-murine or human IgG (Stephan et al. 2011). This method has been applied to quantification of several ADCs, including huC242-DM1 (Tolcher et al. 2003), trastruzumab-DM1 (Lewis Phillips et al. 2008), anti-CD33-Calicheamicin (gemtuzumab ozogamicin) (Dowell et al. 2001), anti-MUC16-vc-MMAE (Junutula et al. 2008), and CR011-vc-MMAE (Pollack et al. 2007). When using this type of direct antigen coat approach, the purified protein as capture reagent must be available.

The antidrug antibody can be used as the capture reagent (Fig. 6.2c; Lewis Phillips et al. 2008; Advani et al. 2010**;** Xie et al. 2004). In addition, the antidrug antibody can also be utilized as a detection reagent (Junutula et al. 2008; Tolcher et al.

2003; Stephan et al. 2008; DiJoseph et al. 2004; Sanderson et al. 2005). The major difference is the assay sensitivity to drug load. The antidrug antibody is supposed to capture every ADC binding at least one drug when it is used as capture reagent and thus the assay should be insensitive to the drug load, while the signal intensity will be proportional to the amount of drug conjugated to the antibody if antidrug antibody is used as the detection reagent. (Stephan et al. 2011). As exemplified by the study performed by Kovtun et al. (2006), the concentration of cantazumab mertansine maytansinoid conjugate (huC242-DM1) is measured by using a murine anti-maytansinoid monoclonal antibody as the capture reagent. Then the conjugate from either the standard or the test samples are detected using the horseradish-peroxidase-labeled donkey antihuman IgG. The challenges to the assay using antidrug antibody as the capture antibody is that they may not be able to measure all the ADCs with different drug loads. As discussed by Xie et al. (2004), such assay may underestimate the loss of drug of molecules from the conjugate when the clearance rate for the huC242-DM1 is identified in a PK study. The antidrug antibodies have also been used as detection reagents for ADC quantification. For example, Stephan et al. (2008) have used a biotinylated anti-DM1 antibody or a biotinylated anti-MMAF antibody to detect anti-CD22-MCC-DM1, or MC-MMAF ADCs, respectively. The conjugated assays described above can provide signals dependent on the drug load with a signal proportional to the number of DM1or MMAF. Sanderson et al. (2005) have used an anti-idiotype cAC10 mAb as capture and an biotinylated anti-MMAE mAb antibody to detect the anti-CD30 ADC (cAC10-Val-Cit-MMAE) with 2, 4, and 8 val-cit-MMAE drug linkers per antibody (DAR 2, 4, 8). It has been found that the cAC10-Val-Cit-MMAE, with a DAR of 2, can generate lower conjugated antibody assay signal intensity than with a DAR of 4. This suggests that the ADC conjugate circulating concentration might be higher than what has been measured considering the potential drug loss in the circulation versus the original ADC used as the standard material. It is noteworthy that ADC ELISA is not capable of providing measurement of the DAR or the overall drug loading (Stephan et al. 2011).

ELISA can also be used to measure the concentration of the free drugs released from ADCs, although mass spectrometry is considered to be the most common method (Tolcher et al. 2003; Sanderson et al. 2005). After determining the amount of total mAb in plasma by the cAC10 mAb ELISA, Sanderson et al. have subsequently used an MMAE competition ELISA to detect the amount of MMAE released from ADC (cAC10-Val-Cit-MMAE) following in vitro incubation with cathepsin (Sanderson et al. 2005). For free MMAE competition ELISA, an anti-MMAE mAb (clone SG3.218) has been found to be the most sensitive reagent for the detection of free MMAE. To determine the sensitivity of the assay using mAb SG3.218 for capturing the free and horseradish peroxidase (HRP)-conjugated MMAE, a series of dilutions of free MMAE standards is mixed with HRP–MMAE conjugate using known concentrations to compete for binding to anti-MMAE mAb SG3.218-coated microtiter plates. The binding of the HRP–MMAE reporter is effectively competed by free drug in a dose-dependent manner.

ELISAs are high throughput and relatively inexpensive. However, this kind of measurement has some limitations (Polakis 2005). The reagents are susceptible to various interferences (Teicher and Chari 2011). It is not able to distinguish ADCs

with different DARs. Such information is very important because efficacy and safety of ADCs can potentially be compromised due to the release of conjugated drugs from the ADC in systemic circulation over time, which may result in a change in the DAR.

6.2.1.2 Cell-Based Binding Assays

Although ADC ELISA is very commonly used, it is not applicable when the antigen is not available. In this case, another common approach to characterize ADC-binding activity is the use of cells expressing either the endogenous or transfected antigen which could be recognized by the ADCs. In vitro cell binding experiments have been performed to determine the targeting effectiveness of ADC to antigen-positive cells. The cells need to express sufficient amount of target protein while the nonspecific binding to the cells has to be as low as possible to provide sufficient assay sensitivity. In general, the cells are placed in a 96-well plate and subsequently incubated for 30 min to several hours with serial dilutions of ADC. After incubation, the plate is washed and detected with a specific antispecies secondary antibody radiolabeled or conjugated to a fluorescent dye. In order to reduce possible antigen internalization upon ADC binding, the cell-based binding assays are performed at the temperature between 0 and 4 °C. When no purified antigen is available, the cell-based binding assay will have its own advantage although this approach seems to be a little cumbersome. At present, there are various types of cell-binding assays that can evaluate ADCs such as direct cell-binding assay (Sapra et al. 2005) and competitive cell-bind assay (McDonagh et al. 2006). Flow cytometry-based analysis can also be used in cell- binding assays (Junutula et al. 2008; Chen et al. 2007). Cell surface binding is usually analyzed by incubation of whole cells with either radiolabeled or fluorescent-labeled ligands followed by detection of cell-binding label. To decrease nonspecific binding to the cell, extensive washing is imperative with this strategy. Since the cell surface binding assay cannot exhibit sufficient reproducibility and accuracy due to variable cell loss from the plate during the multiple washing steps, an alternative procedure for measuring antibody binding to cell surface antigens using an immobilized plasma membrane fraction has been developed (Vater et al. 1995). In this method, isolated plasma membrane fraction exhibiting cell surface antigen is bound to a 96-well plate and incubated with antibodies that can recognize a certain cell surface protein. These approaches can provide the alternatives to mitigate the limitation of conventional cell-based assay.

6.2.1.3 Other Types of Binding Assays

Surface plasmon resonance (SPR) binding analysis methodology is another type of binding assay for detecting the interaction of two different molecules (Ramakrishnan et al. 2006; Schuck 1997), in which the antigen is immobilized onto the chip and has been used for various ADCs, including inotuzumab ozogamon (CMC-544) (Boghaert et al. 2008) and gemtuzumab ozogamicin (CMA-676) (DiJoseph et al.

2004). In principle, the detection system relies on the measurement of the changes in refractive index caused by the interaction of macromolecules on the biosensor chips. As SPR directly detects mass (concentration) with no need for special radio or fluorescent labeling of interacting components before measurement, it presents a great advantage in eliminating possible changes of their molecular properties. This method is very sensitive; however, these instruments need to be dedicated to handle toxic materials which can limit their use for ADC analysis. In order to prevent contamination of the instrument itself, a convenient alternative is the implementation of biolayer interferometry-based platforms. This optical technique analyzes the interference pattern of white light reflected from two surfaces: one is a layer of immobilized protein on the biosensor tip and the other is an internal reference layer (Stephan et al. 2011). The antigen immobilized on the biosensor tip surface can specifically capture ADC in the solution. This type of binding will result in a wavelength shift. Regardless of the format of the assay, the ADC binding should be performed along with the unmodified antibody control.

6.2.2 UV/Vis Spectroscopy

One of the most important attributes of an ADC is the average number of drugs that are conjugated to a single antibody (DAR). Various methods have been used to measure DAR, depending on the properties of the drug and how it is linked to the protein (i.e., lysine-linked or cysteine-linked). Ultraviolet–visible spectrophotometry (UV/Vis) is the simplest analytical method to determine DAR and ADC concentrations. Examples include the characterization of calicheamicin analogues (Hinman et al. 1993), cA10-Val-Cit-MMAE (Hamblett et al. 2004), and maytansinoid DM1 (Chari et al. 1992). The measure absorbance of the ADC and extinction coefficients of the antibody are used to determine the average DAR; however, the calculation based on UV absorption can be complicated by similarities in extinction coefficients of the antibody and drugs (Chen 2013).

6.2.3 MS Based Assays

Mass spectrometry (MS) is an analytical technique which can be used to determine the masses of molecules such as peptides and other chemical compounds by their mass-to-charge (m/z) ratios. MS-based assay has been utilized to characterize DAR fractions, determine the relative ratios of ADCs with different DARs, analyze free drug and metabolites, and monitor various ADC molecular entities (Kozak and Raab 2013). The process involves ionizing molecules to generate charged species or molecular fragment followed by measuring their m/z ratios. DARs are typically assessed by electrospray ionization mass spectrometry (ESI-MS). Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique to profile and monitor biomolecules. Matrix-assisted laser desorption-ionization time-of-flight

mass spectrometry (MALDI TOF-MS) is another reported new approach for determining drug loading (Quiles et al. 2009; Safavy et al. 2003). The observed mass shifts of the peak centroids are used to calculate the average drug loading and the peak profiles are used to mathematically model the drug distribution. IR MALDI has been used to evaluate the analysis of calicheamicin conjugates (Siegel et al. 1997). MALDI in combination with UV (UV MALDI-TOF MS) has been used to analyze the average DAR of lysine-linked conjugates prepared using activated paclitaxel (Safavy et al. 2003).

Although the MS-based assay can be used alone to characterize the ADCs without any additional step, such as separation and purification, it is usually coupled with chromatography technology, e.g., it is used in combination with reverse-phase high-performance liquid chromatography (RP-HPLC), liquid chromatography–mass spectrometry (LC–MS), and liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) for determination of the DAR which it is suitable for lysine-linked ADC. First, the ADC samples are desalted using RP-HPLC with a reversed-phase LC column and then the MS spectrum is processed, deconvoluted, and converted to a series of zero charge state masses that corresponds to the increasing number of drugs in the ADC (Beck 2014). The quantification of small molecules such as free drug or its metabolites is commonly analyzed by LC-MS or LC-MS/MS following extraction from tissues or plasma/serum, while both LBA (Buckwalter et al. 2004) and MS (Kaur et al. 2013; Alley et al. 2008) analytical platform can be applied for the unconjugated drug assay. The cAC10-vc-MMAE incubated in human, mouse, or dog plasma can be analyzed by LC-–MS/MS for determination of the release of free MMAE (Francisco et al. 2003). High-resolution accurate mass system such as affinity capture capillary LC-–MS can characterize drug release by quantifying DAR distributions of the ADC in plasma/serum in a semi-quantitative manner (Clark et al. 2013; Xu et al. 2011).

Recently, LC-MS with electrospray ionization coupled to time-of flight (TOF) or triple–quadrupole mass detectors (Wakankar et al. 2011) has been used to analyze ADC entities such as huN901-DM1(Wang et al. 2005), T-DM1(trastruzumab-MCC-DM1) (Junutula et al. 2010), and C242-DM4 (Lazar et al. 2005). Wang L et al. studied the drug distribution profile of huN901-DM1 using size-exclusion chromatography (SEC) coupled with ESI-TOF-MS (Wang et al. 2005). Figure 6.3 shows an example of the deconvoluted spectrum of deglycosylated huN901-DM1 using this method. The seven major peaks can be assigned to naked dghuN901 (0D) and dghuN901 with 1–6 convalently linked DM1 drugs (1D−6D) with the expected mass difference (852 Da) between drug-containing forms. For the reduced conjugate, the light and heavy chains can be separated by RP-HPLC. The number of linker attached to the light and heavy chains can be obtained directly from the deconvoluted MS spectrum. The three prominent peaks in the light-chain spectrum are for species with zero, one, and two linkers, while the four prominent peaks in the heavy chain spectrum correspond to chains with zero, one, two, and three attached linkers (Fig. 6.4). Therefore, both chains of the antibody are modified with attached linker and conjugated with DM1 drugs.

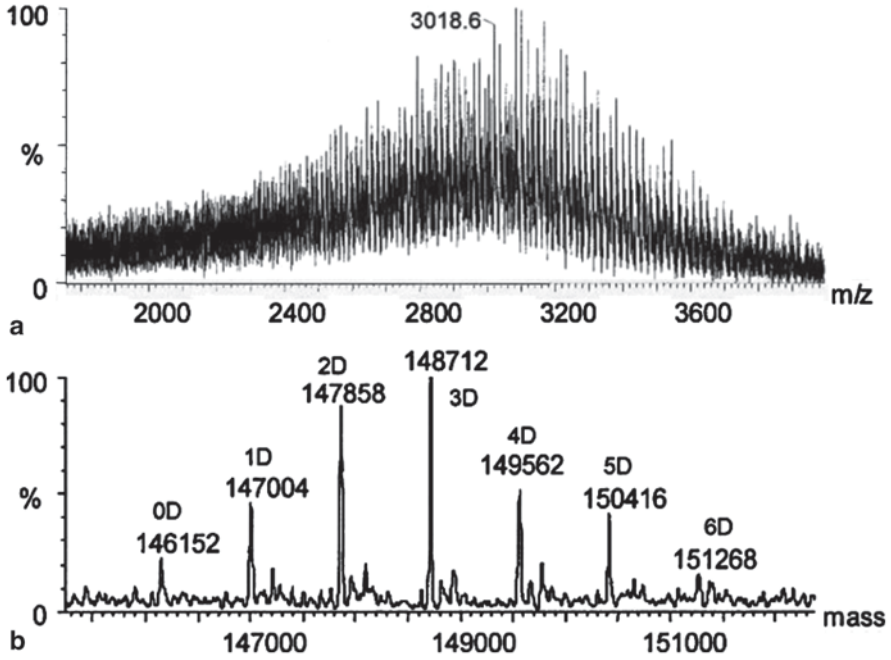

Fig. 6.3 ESI-TOF MS analysis of deglycosylated huN901-DM1. **a** Raw ESI-MS spectrum spanned an *m/z* range of 2000–4000, **b** Deconvoluted MS spectrum of seven prominent peaks which were assigned to naked dghuN901(0D) and dghuN901 with 1–6 covalently linked DM1 drugs (1D–6D). "D" in B designates DM1 drug. (Adapted by permission from John Wiley & Sons: Wang et al. 2005)

One of the challenges of LC-MS is that extra consideration is needed when preparing ADC samples. For example, protease inhibitor should be added to the homogenization buffer for preventing any degradation of antibody and link-drug when tissues are homogenized. Any pH alteration of the matrix, such as that used for protein precipitation by organic solvent or solid-phase extraction (SPE) along with LC conditions, may lead to a change in DAR or loss of the drug (Clark et al. 2013). Another issue is that conventional LC-MS for small-molecule drug measurement is to quantify a priori postulated forms of the drug released by the ADC from catabolism. However, it is theoretically possible that the released drugs may bind to the plasma peptides/protein or contain part of ADCs linker. Therefore, the putative released drug analyte may not be the major form of the released drug. The acidic conditions during electrospray ionization of the ADC which may cause the dissociation of drug-derivatized chains have limited the application of this technique to ADCs produced by conjugation with only cysteine residues (Clark et al. 2013; Wang et al. 2005; Lazar et al. 2005).

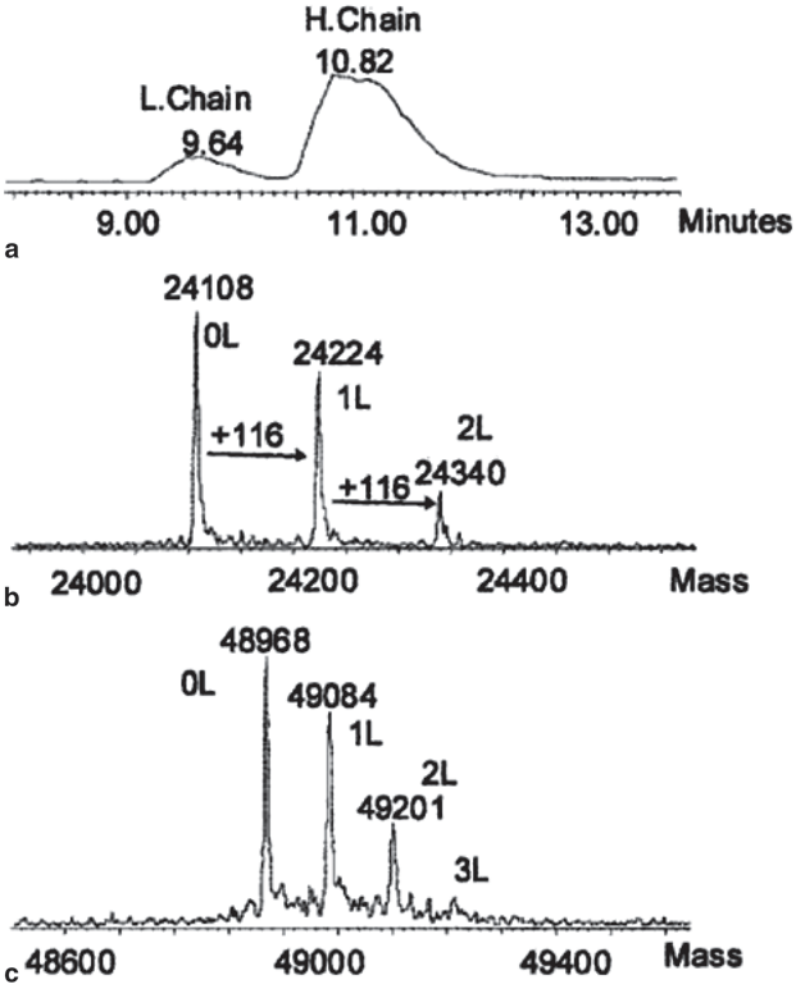

Fig. 6.4 RP-HPLC coupled with ESI-TOF MS analysis of deglycosylated and reduced huN901-DM1. **a** RP-HPLC separation of Light (*L*) chain and heavy (*H*) chain, **b** deconvoluted MS spectra of conjugated light chains with zero, one and two linkers (0L–2L), **c** deconvoluted MS spectra of conjugate heavy chains with zero, one, two, and three attached linkers (0L–3L). (Adapted by permission from John Wiley & Sons: Wang et al. 2005)

6.2.4 Chromatography-Based Assay

Although various types of binding assays have been implemented to characterize the ADC binding and biological activity, they do not allow a detailed evaluation of the ADCs as multicomponent mixtures. Therefore, additional analytical methods should be applied to profile and monitor various ADC molecular entities. Chromatography is a useful approach to separate ADCs followed by providing important

information such as the number and location of conjugation sites, amount of drug, and average DAR. Chromatographic methods generally capitalize on the increase of hydrophobicity imparted to the antibody by conjugation with the drug-linker and it is the most common methods to characterize drug-load distribution. The most frequently published methods are RP-HPLC, hydrophobic interaction chromatography (HIC), ion-exchange chromatography (IEC), and size-exclusion chromatography (SEC).

6.2.4.1 RP-HPLC

Reverse-phase chromatography can be used to separate, identify, quantify, and purify the individual components of a mixture. RP-HPLC is the most widely used analytical technique to separate the free drug released from ADC. The components are then monitored with a UV/Vis detector with the wavelength at the absorption maxima of the drug. The amount of free drug can be calculated based on the HPLC peak area with a standard curve constructed by plotting the peaks areas versus the corresponding nominal concentrations. RP-HPLC in combination with mass spectrometry (MS) has also been reported to monitor the free drug species (Francisco et al. 2003).

In addition, reverse-phase chromatography has been used to assess the drug-linker distribution on antibody heavy and light chains for cys-linked ADCs (McDonagh et al. 2006; Sun et al. 2005), which allows calculation of the average drug-load distribution of ADCs. The method involves a reduction reaction to completely dissociate the heavy and light chains of the ADC. Then the light and heavy chains and their corresponding drug-loaded forms can be separated by an RP column. The weighted average DAR is calculated by the percentage peak area from integration of the light and heavy chain peaks and the drug number (Beck 2014; Ouyang 2013). For example, the location of drug monomethyl auristatin E (MMAE) attachment to the anti-CD30 monoclonal antibody heavy and light chains can be determined by the RP-HPLC methods (Hamblett et al. 2004**;** McDonagh et al. 2006). It is important to mention that direct injection of protein-containing ADC samples onto RP-HPLC column may result in column deterioration due to the irreversible binding of proteins to the stationary phase. Therefore, using guard columns and various sample cleanup procedures to separate the free drug from the protein–drug conjugate in the sample prior to the RP-HPLC analysis is required. (Wakankar et al. 2011).

6.2.4.2 HIC

HIC is another powerful technique that has been used to separate ADC fractions containing various drug loads and to determine DAR and drug load distribution for Cys-linked ADCs. Separation is performed using a gradient elution with decreasing ionic strength and detection is achieved by monitoring the UV absorbance of the eluting species (Alley and Anderson 2013). The drug-loaded species can be resolved

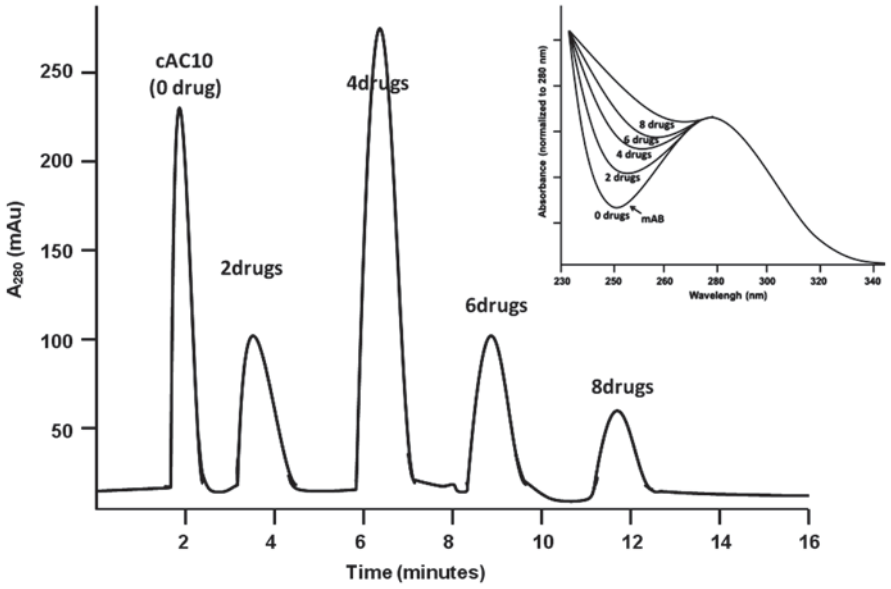

Fig. 6.5 HIC analysis of a cAC10-Val-Cit-MMAE yields five predominant peaks that correspond to mAb (cAC10) conjugated to zero, two, four, six, and eight drugs per antibody. Insert: an overlay of the UV spectra of the starting mAb and the HIC peaks showing the increase in absorbance at 248 nm relative to 280 nm as the level of conjugated drug-linker per mAb increases. (See also Hamblett et al. 2004; Sanderson et al. 2005)

based on their hydrophobicity. The hydrophobicity increases with the increasing number of conjugated drugs. For example, an ADC can bind up to eight drug molecules. The unconjugated form is the least hydrophobic, while the eight-drug form is the most hydrophobic. Thus, the unconjugated form will be eluted firstly and the eight-drug conjugation eluted last. The eluted components can be detected using a UV detector and the peak areas represent the relative percentage distribution of drug-loaded ADCs. Then the weighted average DAR can be calculated using the information of percentage peak area and the drug number (Beck 2014; Ouyang 2013). As shown in Fig. 6.5, Hamblett et al. used an HIC-HPLC method to purify cAC10-val-cit-MMAE into two, four, and eight drugs per antibody along with the corresponding UV spectra of the individual peaks (Hamblett et al. 2004). As attachment of drug MMAE results in greater absorbance at ~248 nm (λmax for MMAE) relative to 280 nm (λmax for cAC10), the five major peaks can be specifically identified. The separation by HIC allows isolation and purification of chromatographically pure species which can be further analyzed by ELISA and cell-based assay. In addition to profile the drug-load distribution, the average drug load for the ADC can be determined by the mean weighted peak area. HIC possesses a great advantage in preserving the integrity of the ADC. However, high salt is usually presented in the mobile phase initially which is necessary for protein binding and the compounds can be eluted with decreasing salt gradients. Thus, this technique cannot be directly coupled to mass spectrometer (Wakankar et al. 2011).

6.2.4.3 Other Types of Chromatography

Charge-based chromatography such as IEC has been used to determine the distribution of ADCs, while size-based chromatography such as SEC can also been used to analyze the drug distribution as well as characterize ADCs for protein fractionation, aggregation, and degradation during storage in liquid formulation (Kozak and Raab 2013; Cordoba et al. 2005; King et al. 2002).

6.3 General Assay Validation Considerations

The ADC is a complex molecule composed of a large molecule (antibody) and multiple molecule drugs. Therefore, an appropriate assay should be validated with the capability to detect both molecules precisely and accurately. Large molecules have well defined tertiary structure and are often more hydrophilic. In contrast, small molecules lack a tertiary structure and are often more hydrophobic (Dere et al. 2013). Due to the significant differences in physicochemical properties between small and large molecule, the combination of different approaches, such as LBAs for large molecule and LC-MS for small molecules, is usually employed for quantification of ADCs. Currently, there are no regulatory guidelines and best standard industry practices for bioanalytical methods of ADCs. However, the current widely accepted guidelines for large and small molecules can be a good reference. For example, the FDA guidance on bioanalytical method validation (US FDA 2013) have described the basic elements of assay validation (see Table 6.2)**.** Based on these guidelines, additional requirements unique to ADCs can be added.

Current guidelines used for validation of chromatographic assays can be applied when LC-MS methodology is used to measure unconjugated cytotoxic drug concentrations. As mentioned previously, ELISA is designed to measure the total antibody analyte, including conjugated, partially unconjugated, and fully unconjugated antibodies. Therefore, additional experiments should be performed during validation to demonstrate the ability of the assay to quantify both conjugated and completely unconjugated antibody with acceptable accuracy and precision. Another aspect unique to ADCs includes an assessment of the stability of free drug in the presence of ADC, because free drug concentration may increase due to the release of additional drug from the ADC under the storage conditions. Assessment of chemical stability is required for small molecules, the stability assessment for large molecule is more complicated and needs to be evaluated not only the physicochemical property but also biological integrity.

When the assay is applied to analyze the real sample (such as samples from a pharmacokinetic study), it should be noted that the calibration curve for LBA assays such as ELISA is usually prepared using the product reference standard that has a fixed DAR distribution. Such standards are only similar to the samples of initial time points. Moreover, most LBA calibration curves are inherently nonlinear over the nominal concentration range and a nonlinear regression is needed to achieve a

Table 6.2 Assay validation parameters for ligand binding and chromatographic assay

Validation process	Ligand-binding assays (LBA)	Chromatographic assays
Selectivity	Matrix effect	
	Cross-reactivity and interference (When possible, LBA should be compared with a validated reference method (i.e., LC-MS)	
Accuracy	A minimum of five determinations per concentration; a minimum of three concentrations in the range of study	
	Within 20% of actual value (except LLOQ) LLOQ should not deviate > 25%	Within 15% of actual value (except LLOQ) LLOQ should not deviate > 20%
Precision	A minimum of five replicates per concentration; a minimum of three concentrations	
	<20% CV (LLOQ <25% CV)	<15% CV (LLOQ<20% CV)
Recovery	Evaluation of samples at three concentrations	
Acceptance criteria for calibration curve	At least 75% of nonzero standards (at least six nonzero calibrator concentrations) should be within the below limits for the analytical run to qualify, including the LLOQ	
	Inherently nonlinear, have response–error relationship, more concentration points than for chromatographic assay LLOQ < 25%, ULOQ < 20%	LLOQ < 20% All other standards < 15%
Number of QC samples in a batch	The minimum number of QCs should be at least 5% of the number of unknown samples or six total QCs QC samples at the following three concentrations (within the calibration range) in duplicate should be added Low QC: 3 × LLOQ Medium QC: midrange of calibration curve High QC: near high end range	
Acceptance criteria for QC samples	At least 67% (four out of six) of the QC concentration results should be within 20% of nominal (theoretical) values. At least 50% of QCs at each level should be within 20% of their nominal concentrations Total error (accuracy and precision) < 30%	At least 67% (four out of six) of the QCs concentration results should be within 15% of nominal (theoretical) values. At least 50% of QCs at each level should be within 15% of their nominal concentrations
Reproducibility	Assessed by replicate measurements including QC and possibly incurred samples. Reinjection reproducibility should be evaluated	
Stability	a. Freeze and thaw stability; b. Bench-top Stability; c. Long-term Stability; d. Stock solution stability; e. Processed sample stability	

Table 6.2 (continued)

Validation process	Ligand-binding assays (LBA)	Chromatographic assays
Additional issues	A. Endogenous compounds: The biological matrix used to prepare calibration standards should be the same as the study samples and free of the endogenous analyte. The matrix should be demonstrated to have (1) no measurable endogenous analyte, (2) no matrix effect or interference when compared to the biological matrix. The endogenous concentrations of the analyte in the biological matrix should be evaluated prior to QC preparation B. Biomarkers: For validation of assays to measure in vivo biomarker concentrations in biological matrices such as blood or urine. To address the same questions as method validation for PK assays. The accuracy, precision, selectivity, range, reproducibility, and stability of a biomarker assay are important characteristics that define the method	
	Diagnostic kits: The LBA kits suitability for using in PK or PD studies should be demonstrated	

QC quality control, *LLOQ* lower limit of quantification, *ULOQ* upper limit of quantification, *PK* pharmacokinetics, *PD* pharmacodynamics

good fitting. Thus, it is important to verify that the calibration curve is appropriate for quantifying the dynamically changing mixture of ADC for PK evaluation (Dere et al. 2013).

The revised draft FDA guidance for bioanalytical method validation contains an additional section on endogenous compounds. Small-molecule assays often include a pre-assay extraction to alleviate problems from individual matrix variability. In contrast, assays to quantify large molecules are often developed to measure analyte in complex matrices without pre-extraction where endogenous protein may be present. Therefore, special considerations must be taken regarding matrix effect (DeSilva et al. 2003).

6.4 Challenges and Future Perspective

ADC bioanalysis is complicated compared with conventional large- or small- molecule bioanalysis. The multicomponent nature of ADCs which arises from the heterogeneity of the conjugation and the biotransformation in vivo represents a significant challenge for the development of reliable and accurate bioanalytical analysis. The DAR composition undergoes continuous change in the circulation due to drug deconjugation and degradation of the antibody, so the ADC reference standard used for assay quantification may not be identical to the ADCs mixture from in vivo samples (Gorovits et al. 2013). Therefore, additional attention for assay reagents is required to ensure that it can detect all DAR species formed in vivo without sacrificing selectivity. As described above, all the bioanalytical assays currently used to characterize ADCs have their own limitations, so developing a comprehensive

bioanalytical strategy is highly recommended for the ADCs. Meanwhile, as the special regulatory guidelines for validation of bioanalytical methods for characterizing ADCs are still being drafted, validation studies should be performed to improve the interpretation of the data so generated.

6.5 Conclusion

We have summarized the common assays that are commonly used to characterize the ADCs and highlighted some examples. Multiple methods are required to fully characterize physiochemical and pharmacokinetic properties of ADCs. The complexity of ADCs presents challenges in the development of bioanalytical methods, so new bioanalytical techniques will need to be developed to ensure assays' accuracy.

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Chapter 7
Pharmacokinetics/Pharmacodynamics and Disposition of Antibody-Drug Conjugates

Siddharth Sukumaran and Kedan Lin

7.1 Introduction

There is a growing need for evaluating and developing new therapeutic modalities in our battle against cancer. In the past few decades, the development of targeted therapies resulted in drugs with improved efficacy and safety (Gerber 2008). Monoclonal antibodies, as a class of highly targeted therapeutics, have been used for the treatment of a variety of cancers. Recently, antibody-drug conjugates (ADCs) have shown impressive potential in advancing cancer treatment to the next level (Sievers and Senter 2013). Conjugation of potent cytotoxic drugs to an antibody can increase potency of the antibody itself, which usually acts by blocking/activating signal transduction, antibody-dependent cellular cytotoxicity (ADCC), and/or complement-dependent cytotoxicity (CDC) (Waldmann 2003). By combining the high target specificity of monoclonal antibodies with potent tumor-killing properties of cytotoxic agents, ADCs have demonstrated convincing antitumor effect in both animal models and patients (Sievers and Senter 2013).

ADCs consist of a potent cytotoxic agent conjugated through a linker molecule to an antibody that can bind with high specificity to a target antigen. The expression of target antigen is selected for its overexpression in tumor compared to normal cells with the exception of B cell targets, such as CD22 and CD79 (Bander 2013). The antibody part of the ADC is designed to bind with high affinity and selectivity to its target antigen. Cytotoxic drugs that are attached to the antibody are usually potent antimitotic or DNA-modifying agents (Ducry and Stump 2010). Administration of these drugs by themselves may have minimal therapeutic index, but conjugation to an antibody renders an acceptable window through enhanced delivery to tumor, reduction of systemic exposure, and minimizing distribution and uptake of drug to nontarget tissues. The third component of an ADC is the linker that conjugates

K. Lin (✉) · S. Sukumaran
Preclinical and Translational Pharmacokinetics, Genentech Research and Early Development, South San Francisco, CA, USA
e-mail: lin.kedan@gene.com

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the cytotoxic agent with that of the antibody usually through the cysteine or lysine amino acid residues (Nolting 2013).

There are two ADCs that are currently approved for marketing. Adcetris (brentuximabvedotin) is an anti-CD30 antibody conjugated to an antimitotic agent monomethylauristatin E (MMAE) through a cathepsin cleavable linker and was approved for the treatment of patients with Hodgkin's lymphoma after failure of autologous stem cell transplant (Cao et al. 2013). Kadcyla (ado-trastuzumabemtansine) consists of transtuzumab (targeted against HER2 antigen) conjugated to mertansine (DM1) and was approved for the treatment of HER2-positive metastatic breast cancer (Ballantyneand Dhillon 2013). In addition, more than 30 ADCs with different cytotoxic drug and linker combinations are in various stages of clinical development against a variety of targets (Mullard 2013). Furthermore, there is tremendous breakthrough in developing next-generation ADCs through antibody engineering, site-specific conjugation, and novel linker/cytotoxic drug combination (Flemming 2014).

However, the inherent complexity of ADCs with their multiple components often makes their development challenging. Pharmacokinetic and absorption, distribution, metabolism, and excretion (ADME) characterization of ADCs reflects the dynamic interactions between the biological system and ADC, and provides critical assessments in lead selection, optimization, and clinical development. A rational strategy integrating the mechanistic understanding of pharmacokinetics/pharmacodynamics and ADC disposition helps to inform target selection, drug selection, linker design, and ultimately helps to maximize the therapeutic window.

Target, antibody, linker, cytotoxic drug, and their interactions make distinct contributions to the mechanism of action for a given ADC, and the disposition of an ADC defines its therapeutic window (Sievers and Senter 2013). Two pharmacologically distinct components, the antibody and the cytotoxic small-molecule drug, necessitate the understanding of the behavior and fate of both components in vivo, and monitoring of their PK/disposition requires comprehensive analytical strategy.

7.2 Analytical Strategy and Its Application in ADC Selection and Optimization

A typical ADC assay strategy evolves with the stage of development with the goal of selecting the right analyte to inform the safety and efficacy, and describe the exposure–response relationship (Kaur et al. 2013). Multiple analytes help to capture the many facets of the behavior of these complex molecules, such as the rate of drug loss from an ADC (i.e., linker stability), the effect of conjugation on ADC clearance, and ultimately the exposure–response relationship. However, the desire to be comprehensive must be balanced by the practicality, the availability of the technology and reagents, and ultimately by the purpose of each study. In discovery stage, many optimization factors are being explored, such as different linkers, drug, drug to antibody ratio (DAR), site of conjugation, and a diverse array of assays are often used to understand the stability and disposition of ADCs, and its initial inter-

actions with biological systems. At clinical stage, with confirmation of preclinical prediction and availability of human data, the assay strategy may be streamlined to increase efficiency and patient compliance (Dere et al. 2013). In general, a panel of assays is required to measure these disparate components: total antibody (conjugated and unconjugated antibody), conjugated antibody, conjugated drug, unconjugated antibody, and unconjugated (free) drug (Kaur et al. 2013). For example, in the development of T-DM1, a comprehensive assay strategy consisting of three validated assays was developed. These validated assays included an enzyme-linked immunosorbent assay (ELISA) designed to measure total trastuzumab, an ELISA to measure conjugated trastuzumab, and a small-molecule LC–MS/MS assay to measure the amount of free DM1 catabolite. These assays are capable of quantifying DAR analytes in circulation to characterize the PK and stability of T-DM1 in nonclinical and clinical studies (Dere et al. 2013).

The total antibody (Tab) concentration captures both the conjugated and unconjugated forms of an ADC and is usually determined using an ELISA-based format. It provides the best assessment of the in vivo stability and integrity of the antibody over time and serves a key role in ADC optimization, particularly in evaluating the impact of conjugation and selecting a drug load.

Conjugated antibody concentrations in systemic circulation are usually determined using an ELISA assay format that measures a mixture of ADC species bearing at least one conjugated cytotoxic drug. Since the detection in this assay requires the presence of both, intact antibody and cytotoxic drug components of the ADC, conjugated antibody concentration is commonly used as an estimate of the active ADC concentration, and is the basis for most ADC PK analyses. The limitation of this assay is its inability to differentiate ADCs with varying numbers of conjugated cytotoxic drugs (DARs). Given ADC species with different DARs may have different potencies, the measured concentrations may not accurately reflect the associated pharmacologic activity (Stephan et al. 2008).

On the other hand, measuring conjugated drug provides a measure of the total amount of cytotoxic drug covalently bound to the antibody (Sanderson et al. 2005; Xu et al. 2013), and changes in conjugated drug concentration could reflect both elimination of ADC from systemic circulation and loss of cytotoxic drug from the antibody. Affinity capture LC-MS is a method of choice by which the ADC is specifically extracted from plasma, then analyzed using LC-MS/MS (Xu et al. 2013). The power of this method lies in its ability to provide direct measurement of average concentrations of drug associated with antibody. Xu et al. used affinity capture LC-MS to assess the site-specific loss of cytotoxic drug from a thiomab ADC, providing valuable insights into the impact of conjugation site on linker stability, a critical factor in ADC safety and efficacy (Xu et al. 2011). These examples illustrate the value of evolving analytical technologies in exploring the behavior of ADCs, which in turn can lead to improvements in ADC design and development.

Unconjugated cytotoxic drug concentrations are used to infer the systemic exposure to the cytotoxic drug released from the ADC and are often associated with loss of efficacy and increased toxicity. Assays for drug-containing products usually employ LC-MS or ELISA methods (Wang et al. 2005; Xie et al. 2004).

LC-MS methods are highly specific for the measured analyte, while ELISA methods may be less specific and able to quantitate multiple analytes of similar structure. A critical consideration of assay selection ensures that the analyte(s) selected for measurement is relevant for efficacy or toxicity and able to provide meaningful PKPD relationship.

7.3 Disposition of an ADC and Its Implication on PKPD

The systemic PK profiles of an ADC provide only partial narratives of its fate. By design, an ADC is a prodrug. Its activity depends on the interaction with target, subsequent internalization of antigen-ADC complex and final releasing of active drug inside the cells. The full ADME properties of ADCs are crucial for the therapeutic window rendered by ADCs. Biologically, the disposition of ADCs is strongly influenced by the underlying antibody backbone conferring properties such as target-specific binding, neonatal Fc receptor (FcRn)-dependent recycling, and Fc (fragment, crystallizable) effector functions. Similarly, the ADME properties of ADCs possess similar attributes associated with unconjugated antibodies (Lobo et al. 2004; Deng et al. 2012; Boswell et al. 2011; Linand and Tibbitts 2012).

7.3.1 Absorption

All ADCs that are currently on the market or in clinical development are dosed intravenously and hence absorption is not considered when assessing PK properties of ADCs. However, for convenience intraperitoneal dosing of ADCs are sometimes used in early preclinical studies and in the future, there is always a possibility for the development of subcutaneous dosing strategies for ADC similar to that of biologics treatment (Sharkey et al. 2011, 2012). In both cases, the absorption properties of ADCs in general will be similar to that of any monoclonal antibody. Although the mechanism is still unclear, it is generally accepted that lymphatic drainage is the major route for absorption from the site of subcutaneous administration to systemic circulation (Zhao et al. 2013;Wang et al. 2008). In addition, it is also acknowledged that diffusion of the molecule across blood vessels can contribute to the absorption kinetics.

7.3.2 Distribution

Distribution characteristics of ADC generally mimic that of monoclonal antibodies. Due to the cytotoxicity of conjugated drug, distribution and accumulation of an ADC to nontarget tissues, via either antigen specific or nonspecific processes, may have profound pharmacologic/toxic effects. Owing to the higher molecular

weight, the initial distribution is limited to the vascular space, with the volume of distribution in the central compartment similar to the total plasma volume in any species (~40 mL/Kg) (Tabrizi et al. 2010; Mould and Green 2010). However, with time, distribution extends to tissue interstitial space. The extent of distribution into tissues is determined by a variety of factors including blood flow, tissue porosity, structure and heterogeneity, and target properties. The movement of the molecules into the tissues occurs by convection, transcytosis, and diffusion across the capillary and studies show that the partitioning of antibodies into tissues ranges between 5 and 15 % of what is observed in circulation, with brain being an exception, having very low penetration of antibody because of the tough blood–brain barrier (Shahand and Betts 2013).

Beyond these similarities, ADCs bring in a different level of complexity as a result of interactions between target antigen and ADCs. Factors such as binding affinity, tissue expression profile, target turnover/internalization rates, target density, and conjugation impact on ADC distribution and influence the therapeutic index of an ADC (Tabrizi et al. 2010). Unlike target for monoclonal antibodies which are developed for blocking specific function/pathway in tumors leading to tumor growth inhibition or killing, targets for ADCs do not need to be causal or implicated in tumor progression. The role of the target antigen for an ADC is to provide high specificity, either expressed only by the tumor or at levels significantly higher in tumor (Bander 2013; Silver et al. 1997). In some cases, low level of target antigen expression in normal tissues and their subsequent uptake of ADC may lead to decreased ADC delivery to tumor and/or increased delivery of cytotoxic drug to normal tissues; a phenomenon that could affect the therapeutic index of the ADC (Boswell et al. 2013). Employing a "predosing" or capping non-tumor expression site with an inactive antibody may be helpful to mitigate the toxicity (Boswell et al. 2013).

The level of target antigen expression and binding affinity of the target to the antibody are critical in determining the amount of ADC delivered to the target. Irrespective of how specific the target expression is, if the target expression levels and/or antibody binding affinity are low, ADCs delivered to tumor compared to normal tissues (nonspecific uptake) will be low resulting in a poor therapeutic ratio. Hence, in addition to being specific, the level of target expression (and binding affinity) should enable adequate accumulation of the drug at the site of interest (Bander 2013).

Most of cytotoxic agents in the ADC act intracellularly and hence internalization and rate of internalization of the target play an important role in determining the drug accumulation inside the tumor cells and its subsequent killing (Sievers and Senter 2013). The linker molecules are designed to be stable in circulation, but are cleaved in endosome/lysosome compartments to effectively release the cytotoxic agents inside the cell. In addition to a rapid internalization process, the recycling or replenishment of the target at the cell surface is important to sustain the delivery of ADCs into the tumor cells. Furthermore, shedding of target antigen from cell surface can play an important role in determining efficacy and toxicity of ADCs (Tolcher et al. 2003). If shed target antigens are present, ADC binding to these shed antigens will affect ADC distribution and can result in higher liver uptake

(as these complexes are cleared by liver) and hence liver-related toxicities (Lovdal et al. 2000).

A few studies have assessed the effects of conjugation on the distribution of ADCs compared to unconjugated antibodies (Erickson and Lambert 2012). Distribution of DM1- and DM4-conjugated ADCs (lysine conjugation) in mice was similar to that of typical unconjugated IgG molecules (Xie et al. 2004**;** Erickson and Lambert 2012). However, auristatin-conjugated ADCs (cysteine conjugation) show modest differences in tissue distribution compared to unconjugated antibodies (Boswell et al. 2011). For example, MMAE-conjugated STEAP1 ADC showed a general trend towards increased hepatic uptake and reduced levels in other highly vascularized organs in rats. Similarly, CMD-193 antibody conjugated with calicheamicin showed increased hepatic uptake compared to the parental huS193 unconjugated antibody, in a phase I biodistribution and PK study in patients with advanced epithelial cancer (Scott et al. 2007). Conjugation likely increases the hydrophobicity of the antibody, which leads to enhanced uptake by the liver (Boswell et al. 2011). Efforts on antibody engineering fronts have also shown promise in altering distribution and mitigating the toxicity. Mutated antibody with deficiency in FcR binding with attenuated nonspecific uptake led to longer circulation time and enhanced efficacy (Sussman et al. 2011).

7.3.3 Metabolism and Elimination

Therapeutic antibodies are eliminated from the body predominantly via target-mediated and nonspecific uptake into cells followed by proteolytic degradation into inert small peptides and amino acids (Lobo et al. 2004). ADCs, bearing cytotoxic drugs, become active following release from, or degradation of, their associated antibodies. Understanding the mechanism of ADC metabolism and elucidating the identity of its active metabolites have practical implications on ADC optimization, clinical monitoring, and PKPD relationship. Metabolism of ADCs includes at least three processes: deconjugation as cytotoxic drug releasing from the ADC via enzymatic or chemical processes; proteolytic metabolism of the antibody, which can result in small peptides or amino acids that are still conjugated to the cytotoxic small-molecule drug (Tabrizi et al. 2006); and the metabolism of the cytotoxic drug (primarily the free drug) by the typical small-molecule phase I and phase II metabolizing enzymes (Lin and Lu 1997).

Nonspecific cleavage of the linker could happen in circulation resulting in the deconjugation of one or more molecule of the cytotoxic drug from the antibody. Once internalized into the cell (by target-mediated cellular uptake in target cells or nonspecific pinocytosis), depending on the type of linker, the linker can be cleaved by specific proteases (like cathepsin B involved in the cleavage of valine-citrulline (vc) dipeptide linkage used for MMAE or MMAF conjugates) or the proteolytic processing in endosomes/lysosomes (Sutherland et al. 2006**;** Chuand and Polson 2013). For maleimide-containing drug linkers which are conjugated to antibody

cysteine residues to form thiosuccinimide bonds, there are two competing reactions: exchange with sulfhydryl-containing molecules with direct consequence of drug loss (Alley et al. 2009) versus hydrolysis of the thiosuccinimide ring resulting in stable bond (Xu et al. 2013**;** Lyon et al. 2012). Site of conjugation and solvent accessibility have direct influence on drug loss and subsequent efficacy (Shen et al. 2012), which led to the selection of LC, HC over FC variants, meanwhile, taking advantage of chemically induced hydrolysis, Lyon et al. showed the improved therapeutic window by making self-stabilizing maleimido-DPR (Lyon et al. 2012).

The other dominant mechanism that results in cytotoxic drug-containing catabolites is ADC catabolism, a process driven by either receptor-mediated endocytosis or fluid-phase pinocytosis with subsequent trafficking to the lysosome, followed by enzymatic degradation (Alley et al. 2009**;** Erickson et al. 2006**;** Okeley et al. 2010). Both deconjugation and proteolytic degradation of the antibody can occur simultaneously and the contribution of each process to the release of the cytotoxic drug (or drug-containing products) depends on multiple factors including target properties, linker stability, drug load per antibody, and conjugation site. For example, apart from the free DM1 (cytotoxic drug), MCC-DM1 (linker conjugated to drug) and lysine-MCC-DM1 were detected in circulation in both preclinical studies and in patients treated with T-DM1 (Shen et al. 2012b). Furthermore, for some ADCs, studies have shown other mechanisms of cytotoxic drug release or deconjugation like the transfer of linkerdrug from the antibody to circulating albumin in the case of MC-MMAF and VC-MMAE (Shen et al. 2012a). Nonspecific uptake and processing of ADCs may be the main source of ADC toxicity, and modulation of this process has potential benefit in expanding TI. For example, by mutating Fc residues that are in contact with FcγRIII, Sussman et al. have shown that S239°C variant does not bind FcγRIII, blocks localization to nontarget tissues and decreases off-target toxicity (Sussman et al. 2011**;** Jeffrey et al. 2013).

Once the cytotoxic drug is released from the antibody, they are subject to the metabolism and elimination processes associated with small-molecule drugs, including CYPs and drug transporters, and possesses the theoretical potential for drug–drug interactions (DDIs; Lu et al. 2013). In vitro studies in human liver microsome preparations suggest that DM1 is metabolized mainly by CYP3A4 and to a lesser extent by CYP3A5 (Shen et al. 2012b)**;** Davis et al. 2012). The elimination of DM1-containing products and its metabolites was primarily through the biliary/fecal route in rats with 80% recovery of the radio-labeled drug. Similar observations were made with VC-MMAE-conjugated ADCs, with ~80% of the radio-labeled MMAE observed in feces and only ~6% of MMAE was recovered from urine (Pastusovas et al. 2005).

It has been recognized that one of the key factors that modulates ADC disposition is heterogeneity of an ADC, the consequence of both its conjugation process during manufacturing and its deconjugation in vivo. Traditional conjugation through either cysteine and lysine residues on the monoclonal antibody results in a mixture of ADC species differing not only in the number of drugs attached to the antibody but also in the sites of drug linkage (Hamblett et al. 2004**;** Singhand and Erickson 2009). For instance, ADCs conjugated by cysteines are mixtures of conjugated

antibodies with a DAR ranging from 0 to8, representing drugs conjugated to some or all of the cysteines that in unconjugated antibodies form the interchain disulfide bonds (Singhand and Erickson 2009). ADCs conjugated via lysine residues have the potential for even greater variability in the number of conjugated drugs and their locations (Wang et al. 2005**;** Hamblett et al. 2004). Heterogeneity from ADC mixtures potentially could diminish the therapeutic window with higher DAR species contributing to significant toxicity while lower DAR species compromise on efficacy. In addition, it presents a major challenge in describing the collective ADC PKPD profile with multiple species with different pharmacological potency and PK behavior. One particular concern has been the presence of higher DAR species. For the reasons that have been touched upon before, multiple site conjugation tends to increase hydrophobicity and decrease the overall stability on two fronts: faster loss of drug or deconjugation and accelerated antibody clearance (Sun et al. 2011).

Several studies helped assess the heterogeneity on PK, efficacy and toxicity, and guided the field toward more homogenous ADC production. McDonagh et al. engineered the cysteines associated with the interchain disulfides, resulting in an antibody with fewer cysteines for conjugation and potentially resulting in less heterogeneity (McDonagh et al. 2008). ThioMab-drug conjugates (TDC) with site-specifically engineered cysteines provided a defined DAR of 2 per mAb with minimized heterogeneity and increased therapeutic index (Junutula et al. 2008). Alternatively, biosynthetically incorporating nonnative amino acids into a given Ab scaffold provides homogeneous ADCs with precise control over the site and stoichiometry of drug conjugation (Tian et al. 2014). Minimizing heterogeneity and optimizing DARs also help to establish a more defined PKPD relationship.

7.4 Application of PK in ADC Optimization

Integrating information from multiple assays is critical in characterizing the in vivo behavior of these complex molecules, interpreting ADC pharmacologic effects and ultimately building exposure–response relationship. Accumulated knowledge on ADC PK helps to shed lights on mechanism of its stability, such as the effect of conjugation on ADC clearance, the rate of drug loss from an ADC (i.e., linker stability), and has profound impact on ADC optimization.

It has been observed that conjugation causes an increase in the clearance of the antibody for several ADCs (Boswell et al. 2011**;** Hamblett et al. 2004**;** Herbertson et al. 2009), which may result in more rapid delivery of cytotoxic drug-bearing ADC to the normal organs or tissues with potentially toxic consequences (Hamblett et al. 2004). Comparison of Tab PK of the ADC with the Tab PK of unconjugated antibody (administered as unconjugated antibody) provides information regarding the effect of conjugation on antibody clearance (Xie et al. 2004)**;** (Sapra et al. 2005). There are several mechanisms being postulated ranging from the disruption of tertiary structure, weakening of disulfide bonds, to increase in hydrophobicity from conjugation (Boswell et al. 2011**;** Hamblett et al. 2004). Among them, the

hydrophobicity charge seems to be plausible. Conjugation with higher DAR leading to faster clearance seems to corroborate this hypothesis. For example, cAC10-vc MMAE ADCs with high DARs were observed to have a faster Tab clearance than lower DAR ADCs (Hamblett et al. 2004). Adem et al. showed that high-drug-load species leads to thermal unfolding and aggregation and affects the physical stability of ADCs (Jeffrey et al. 2013).

Another key parameter in ADC optimization is the rate of drug loss from ADC. Loss of drug from the ADC can result in decreased efficacy and changes in the toxicity associated with ADC administration. Comparative assessment of Tab PK with either conjugated antibody PK or conjugated drug PK can provide qualitative guidance on the rate of drug loss from the ADC (Linand and Tibbitts 2012). When comparing Tab PK with conjugated antibody PK, it is typically observed that conjugated antibody concentrations decline more rapidly than Tab concentrations, and the degree of divergence of the curves is indicative of the rate of complete drug loss from the ADC (Tolcher et al. 2003; Tijink et al. 2006**;** Burris et al. 2011**;** Kantarjian et al. 2012). Better understanding of mechanism of drug loss from conjugated antibody helps assess the stability of linkers, conjugation sites, and impact of DARs. Illustrative cases for differences in linker stability between disulfide and thioether linkers, and the effect of conjugation site on thiomab-ADC linker stability have been also reported (Shen et al. 2012a**;** Lewis Phillips et al. 2008). One example of different efficacy among anti-Her2 ThioMab Drug Conjugate (TDC) variants (light chain, heavy chain, and Fc site-specific) was evidenced by the differential rate of drug loss or deconjugation despite similar total antibody clearance (Shen et al. 2012a).

Conjugated drug measurements have provided insights when compared with total antibody profiles. Antibody-conjugated drug concentrations decline more rapidly than Tab concentrations because two processes drive the decrease in conjugated drug concentrations: loss of drug from the ADC and elimination of ADC, while Tab concentration changes are driven solely by elimination of ADC and unconjugated antibody. As such, the difference in the concentration decrease can be used to infer the rate of drug loss from the ADC (Kaur et al. 2013**;** Sanderson et al. 2005**;** Xie et al. 2004). Assessment and integration of ADC PK can be valuable not only in understanding a single ADC but also in evaluating multiple ADCs with different structural and pharmacologic characteristics; allowing improved design and development of these complex molecules.

7.5 PKPD Modeling for ADCs

As described earlier, ADC is a heterogeneous mixture of multiple species described by a variety of analytes. Due to this, heterogeneity and dynamics between different species quantitative characterization of ADC pharmacokinetics and pharmacodynamics becomes challenging. Mathematical modeling approaches can provide help in gaining a better understanding of the complex PK and PD behavior of ADCs. In

addition, it can help in the integration of the dynamic changes in different ADC species and provide a way to assess the drivers of efficacy and toxicities.

A variety of PKPD mathematical models ranging from simple data-driven empirical models to semi-mechanistic and physiology-based models have been developed to characterize and describe ADC disposition and actions. For example, for T-DM1, Jumbe et al. described the mouse drug-conjugated antibody pharmacokinetics using a simple two-compartment model with linear drug clearance (Jumbe et al. 2010). In this study, drug-conjugated antibody was assumed to be the driving force for efficacy and the simple model could describe reasonably well the disposition profile. A first-order growth model with tumor transit compartments was used for describing the tumor progression and tumor killing was modeled as nonlinear killing driven by the drug-conjugated antibody (T-DM1). In contrast, Bender et al. developed a mechanism-based multi compartment model that described the pharmacokinetic profile of different heterogeneous species representing T-DM1 ADC (Bender et al. 2012). Preclinical data for individual DAR species were utilized and each DAR species was explicitly modeled with a central and peripheral compartment for distribution. Deconjugation of the cytotoxic drug DM1 from the antibody was modeled with variable rate constants (differentiating deconjugation between higher and lower DAR species). Both in vivo and in vitro measurements were utilized to develop the model. Gibiansky et al. adapted the target-mediated drug disposition model to describe ADC PK and with number of approximations including rapid binding, quasi-steady-state and Michealis–Menten approximations, derived a reduced model that can still describe ADC PK with load-independent properties (Gibiansky and Gibiansky 2014).

Although most of the modeling work has been done with T-DM1, some of the recent efforts have been in modeling other ADCs like that of brentuximab-vedotin which consists of a cleavable linker conjugating the cytotoxic drug with the cysteine residues of the antibody. Shah et al. developed a multiscale-mechanism-based model incorporating a variety of physiological process including ADC and drug payload PK at the cellular level, in circulation, and tumor tissue to characterize brentuximab-vedotin disposition and action and to predict the clinical response to the drug in cancer patients including progression-free survival rates and complete response rates (Fig. 7.1; Shah et al. 2012). Known mechanisms of ADC and payload disposition and tumor growth kinetics were used for the model development and the model utilized invitro, preclinical, and clinical measurements of ADC and payload and tumor-growth inhibition data in xenograft mouse models to develop the model.

7.6 Conclusions

As a hybrid between antibody therapeutics and small-molecule cytotoxic drugs, ADCs exhibit unique pharmacological and PK properties. Among them, the heterogeneity from ADC production and in vivo processing, the necessity to monitor multiple active ADC analytes, and less-understood catabolic and metabolic species,

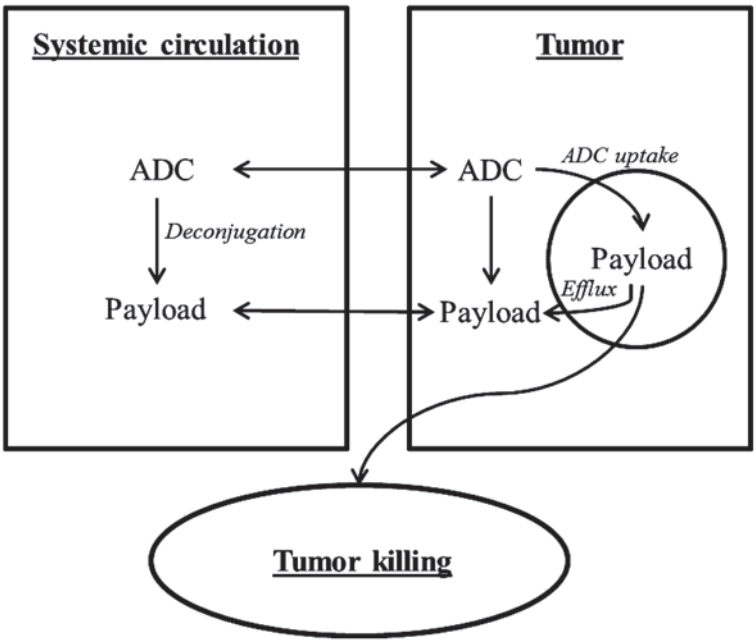

Fig. 7.1 A simplified model representation of disposition and action of brentuximab-vedotin ADC and its payload described by Shah et al (2012). The integrated model takes into account the disposition of ADC and its payload in systemic circulation, tumor tissue, and inside cells and link intracellular payload concentration to tumor killing. *ADC* antibody-drug conjugates

all of which demands meaningful PKPD relationships. As with other therapeutics, PKPD can aid in understanding exposure–response relationships, determining the optimal dose and dose regimen, predicting human PK, facilitating the translation of nonclinical data to clinical outcome, and allowing quantitative understanding of mechanistic pharmacology (Morgan et al. 2012). The growth of the interest in ADCs, the evolvement of powerful analytical tools, and generation of crucial mechanistic data indicate a promising future for ADC development.

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Chapter 8
Regulatory Considerations

Karen J. Cha

8.1 Introduction

Antibody-drug conjugates (ADC) are monoclonal antibodies that are designed to selectively deliver cytotoxic agents to antigen-bearing cells. Most commonly, these have been developed towards tumor-associated antigens, but a number of groups have demonstrated interest in using ADCs to treat immune and neurological disorders.

Since the withdrawal of Mylotarg (gemtuzumab ozogamicin) from the market in 2010, two new ADC products, Adcetris™ (brentuximab vedotin) and Kadcyla® (ado-trastuzumab emtansine), have received marketing approval in the USA and European Union (EU), providing some regulatory precedent (refer to Chaps. 11 and 12); however, the lack of specific guidance on ADCs poses a challenge to sponsors developing products for this promising and attractive area of oncology drug development.

The number of ADCs in development is growing rapidly, with most programs currently in early development. Based on information gathered from publicly available clinical trial databases, company websites, press releases, etc. summarized in Fig. 8.1 below, there are an estimated 22 unique ADCs currently in phase 1 study and nine ADCs in phase 1/2 status. As of February 2014, only three ADCs are in phase 3 clinical trial. At the same time, at least 15 ADCs have been discontinued from development (Fig. 8.1), seemingly after early clinical study according to the current information found in the public domain.

The regulatory expectations of ADC products combine the set of requirements applicable to both biologic and chemical drugs, and given the focus on oncology treatment, the development of ADCs is obviously subject to the regulatory requirements and expectations of developing cancer therapeutics. Given the dual nature of the conjugates which incorporate both a targeting antibody and a highly potent

K. J. Cha (✉)
Regulatory Affairs, Agensys, Inc., Santa Monica, CA, USA
e-mail: kcha@agensys.com

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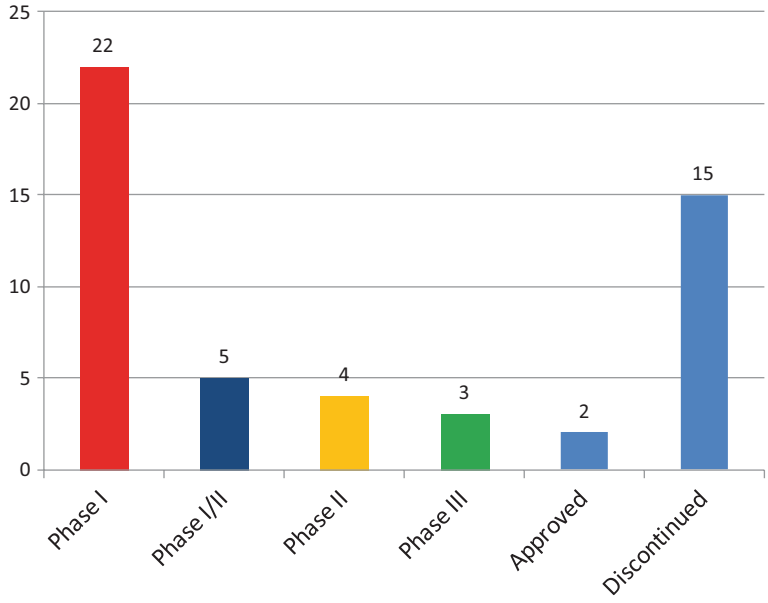

Fig. 8.1 Summary of ADC development activity

chemical cytotoxic agent and linker, the developing sponsor is obligated to consider an extremely wide set of requirements and expectations. This substantially expands the effort required in ADC development, and these challenges are reviewed below. In addition, those areas that are seen as unique to ADC products are discussed where possible.

8.2 Regulatory Review

While not specific to ADC products, it is worth mentioning that in the USA, a reorganization was announced in 2011 in the Food and Drug Administration's (FDA) office responsible for reviewing all drug and biologic applications for cancer therapies. ADC products for cancer will be reviewed in the newly named Office of Hematology and Oncology Products (OHOP, formerly the Office of Oncology Drug Products), based on indication. The FDA website provides the information necessary to determine which division will review a given ADC application based on target indication. Given this format, the FDA has often requested sponsors to submit separate Investigational New Drugs applications (INDs) to the relevant divisions for the same product being evaluated in different cancer indications presenting some challenges with maintaining multiple INDs for a given compound, including the cross-filing of safety information.

8.2.1 Organization of the Application

In July 2003, the Common Technical Document (CTD) became the mandatory format for new drug applications in the EU and Japan and the strongly recommended format of choice for marketing applications submitted to the FDA. The agreement among the regions participating in the International Conference on Harmonization (ICH) to assemble all the quality, safety, and efficacy information in a common format has significantly improved the regulatory review processes and has led to harmonized electronic submissions in these regions. For sponsors, it has eliminated or minimized the need to reformat the information for submission to the different regulatory authorities in the ICH regions and other countries following the harmonized guidance.

The CTD is organized into five modules in which module 1 is region specific and modules 2–5 are intended to be common for all regions (ICH M4, 2004). Guidance for the content of each module of the CTD can be found on the official ICH website.

The CTD format is now also used for applications during the investigational stage, and as is common for the US IND Applications, the granularity of the CTD format is well suited for an application that grows over time as a product advances in development. The quality overall summary (QOS) structure of Module 2 and quality structure of Module 3 are complementary across the regions, and FDA regulations fit this CTD structure well.

ADCs consist of two key intermediates—the monoclonal antibody (mAb) and the drug-linker (DL) which are covalently conjugated to generate the bulk drug substance (BDS). Therefore, regulatory applications for ADC products should have three separate drug substance quality (or chemistry, manufacturing, and controls (CMC)) sections (Module 3.2.S in the CTD format), one each for the mAb, the DL, and the conjugated BDS as well as the required drug product (DP) section (Module 3.2.P in the CTD format). The separate section for each intermediate and the drug substance allows both the sponsor and reviewer to update/review information for specific intermediates and manage more efficiently the life cycle of the application. An example of this Module 3 structure used in the USA is shown below in Fig. 8.2. If a drug master file (DMF) exists for the DL, as is sometimes the case, the DMF can be cross-referenced within the application with a letter of authorization from the DMF holder and the drug substance section for the DL omitted or partially omitted.

In some countries outside the USA, QOS of Module 2.3 is required during development to provide the overview of the quality information, and Module 3 is used only to place supplemental data or those sections containing a significant amount of information. Similarly, the information should be provided in three separate drug substance documents—one QOS each for the mAb, DL, and BDS.

From a review perspective, this structure also supports the fact that regulatory review of CMC information involves multidisciplinary divisions within the US FDA. The mAb will be reviewed by the Division of Monoclonal Antibodies (DMA) in the Office of Biotechnology Products (OBP), the DL will be reviewed by the Office of New Drug Quality Assessment (ONDQA), and the BDS and DP information by

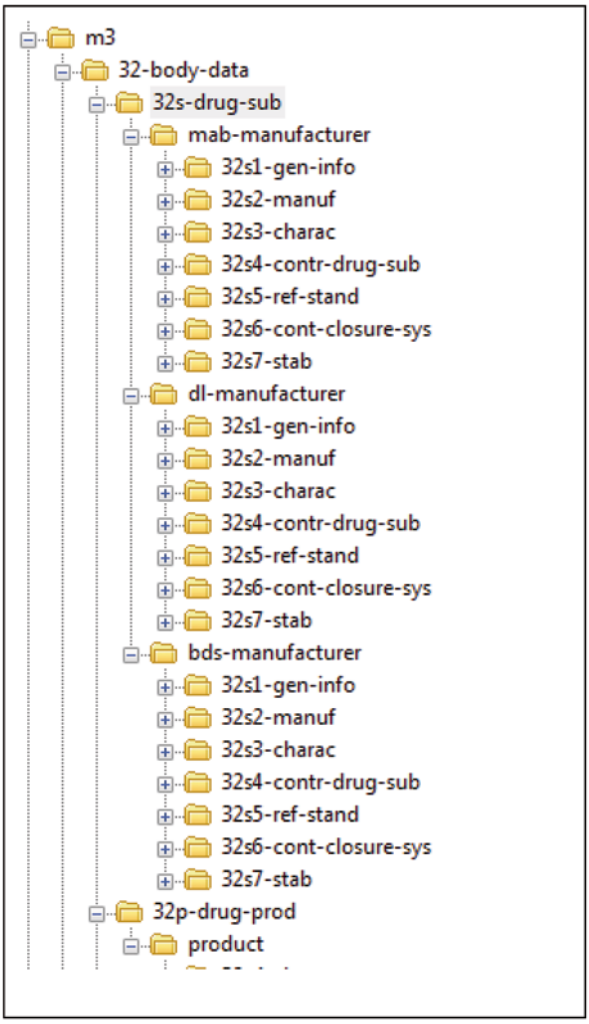

Fig. 8.2 Example of Module 3 Quality eCTD Backbone (Partial) for an ADC product application. *mAb* monoclonal antibody, *DL* drug-linker, *BDS* bulk drug substance

both groups. Each of these intermediates and the drug substance and their corresponding sections have particular features—different manufacturing sites, different manufacturing processes, different analytical methods and method validations, different stability protocols, etc., further supporting the separate drug substance sections.

The OBP perspective includes characterization information for the mAb, BDS, and DP; comparability of the mAb, BDS, and DP; impurities, and testing/specifications. The ONDQA perspective considers starting materials and intermediates of the DL; characterization of the DL, BDS, and DP; and testing/specifications (Miksinski and Shapiro 2012). This high-level list alone affirms that the tasks at hand for fulfilling the expectations of regulatory review for CMC information

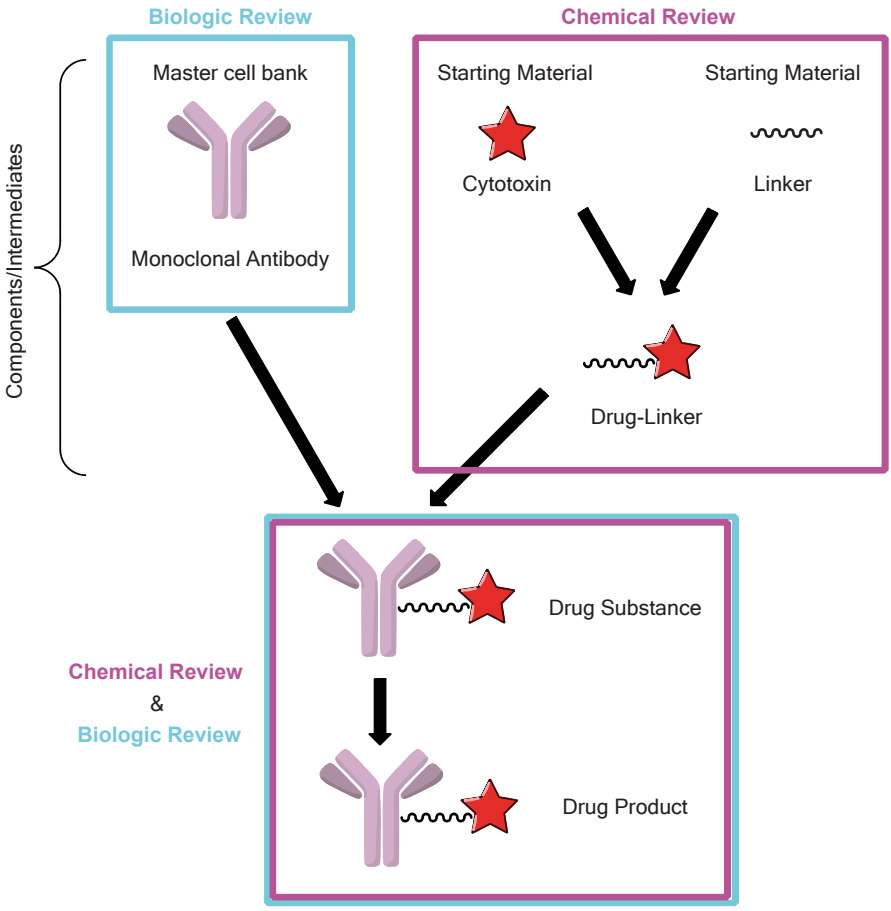

Fig. 8.3 ADC review responsibility

are much greater for an ADC product because the data requirements for the mAb and DL intermediates are the same as those for drug substance and DP. Figure 8.3 depicts the various components/intermediates in ADC product manufacture and the collaborative review responsibility by both biologic and chemistry disciplines common within regulatory agencies.

8.2.2 Meetings with FDA

It is highly recommended that sponsors entering into any new therapeutic class meet with the FDA review division in a pre-IND (type B) meeting, and ADCs are no exception. The FDA continues to support its position of promoting communication with sponsors under the fifth authorization of the Prescription Drug User Fee Act

(PDUFA V: Fiscal Years 2013/2017) which includes meeting management goals as well as a general goal for "Promoting Innovation Through Enhanced Communication Between FDA and Sponsors During Drug Development" among many other enhancements (PDUFA Reauthorization Performance Goals and Procedures Fiscal Years 2013/2017 www.fda.gov).

The timing of the pre-IND meeting is often a question for new sponsors, and the standard regulatory advice relates to the questions to be discussed with the agency. Some sponsors prefer to meet with the FDA prior to performing the good laboratory practice (GLP)-compliant toxicology studies to be sure the plans are acceptable. In any case, the meeting questions should be clear, concise, and the relevant background for a meaningful discussion on the topic must be available in time for inclusion in the meeting package due at FDA at least 4 weeks prior to the meeting date for a type B pre-IND meeting (FDA Guidance for Industry, Formal Meetings Between the FDA and Sponsors or Applicants 2009). Therefore, working backwards from the time the relevant data or information needed for the meeting package is available will aid in determining the appropriate timing of the request and targeted meeting date(s).

According to US guidance, meeting questions should be categorized into topics related to nonclinical studies (pharmacology, toxicology, and pharmacokinetics), CMC, or clinical protocol design. Typical CMC topics include the appropriateness of specifications, test methods, stability data, etc. Meeting types, content of the request, and meeting package expectations are clearly outlined in the FDA guidance document.

It is also strongly recommended and encouraged by the FDA to plan for an end-of-phase 2 (EOP2)/pre-phase 3 meeting prior to initiating any pivotal trial(s) that will form the primary basis of an efficacy claim as well as a presubmission meeting to discuss the planned content of the marketing application with the FDA review division not less than 2 months prior to the planned submission. Such issues can be discussed at both EOP2 (planned) and pre-Biological License Application (BLA) meeting (when the package is already defined), supporting the concept of keeping FDA informed throughout development.

8.3 Chemistry, Manufacturing, and Controls

The particulars around CMC information for an ADC product could comprise a complete chapter in this book or even a completely separate major publication in itself; however, it is summarized in this chapter as many sponsors of various product types understand that CMC issues or roadblocks can very abruptly stop or delay drug development or approval in its tracks. By the same token, getting the CMC foundation right early in development will result in an overall faster development time to the market.

From a regulatory perspective, given that the mAb and DL are considered to be intermediates in the manufacture of the conjugated BDS, this calls for a high level

of CMC information for the intermediates although the CMC requirements for each are quite separate and distinct. Furthermore, when considering the key factors in the control of the drug substance and DP, it is important to keep in mind that the antibody is directed to a cell surface target and the linker systems are designed to be stable in the bloodstream to release the potent cell-killing agent once inside targeted cancer cells. For many ADCs currently in development, it is believed that the mAb intermediate has little direct cytotoxic effect, and thus, the highly potent cytotoxic drugs conjugated to the mAb is in a sense the primary active ingredient. With this in mind, the demonstration of manufacturing control and consistency of the DL must meet the expectations of the regulatory reviewer.

Based on the biologic and chemical intermediates described above, ADC manufacture often involves at least three separate and unique facilities, adding complexity in addressing regulatory compliance needs. This will include facilities and processes for the manufacture of the mAb, DL, the conjugated BDS, and the site and process for producing DP. Furthermore, the facilities for manufacturing DL, conjugation, and DP manufacture must be able to properly handle highly potent compounds requiring experienced operators, sophisticated systems and equipment with robust procedures, training, and risk-reduction strategies. For those companies not able to handle these highly toxic substances in-house, there are limited options for outsourcing the conjugation of ADC products at this time although more and more companies are investing in this area.

The following discussion of CMC issues focuses on early development of a new ADC product (pre-IND through phase 2) during which time the main objective of the regulatory review of CMC information is to assure the safety of patients. During phases 2 and 3 clinical trials, CMC review continues to evaluate not only the safety but also the bridge of the clinical test material to the intended product to be marketed. Manufacturing development of the product gradually advances to consistent quality through process control and product testing.

8.3.1 Monoclonal Antibody

Biologics have three major differences from chemical drugs which are reflected by both FDA and European Medicines Agency (EMA) in various regulatory guidance documents specific for biologic products: (1) the use of living source materials to produce the biologic, (2) increased complexity of the manufacturing processes, and (3) increased complexity of the products themselves (Geigert 2013). Fortunately for industry, the available guidance and regulations for biologics are specific and extensive, and the open access to guidance on the websites of the FDA, EMA, as well as ICH provides a great deal of information. Several key areas of focus in the regulatory review of the biologic portion of the ADC application are discussed below.

Given the use of living source materials to produce the mAb intermediate, it is important to demonstrate the biologic product is as free as possible of impurities, contaminants, and adventitious agents. All materials of human or animal origin

utilized in the cell line generation and manufacturing process or coming into contact with the mAb intermediate or subsequently conjugated drug substance or DP must be identified with information evaluating the risk of potential contamination (ICH Q5A(R1) 1999). For example, studies for viral clearance and removal of host cell protein impurities, and the DNA of animal-derived materials, should be supported by study reports and documents of certification and testing. Additional detailed guidance is provided in the FDA's Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (FDA 1997). In particular, scientifically sound viral clearance studies to provide a quantitative estimate of the level of virus reduction provided by the removal/inactivation procedures are of great importance. Also, demonstration of genetic stability is a key requirement in both the USA and EU (ICH Q5B 1996 and ICH Q5D 1998).

The mAb component of the ADC must be a well-characterized product. General guidance for biotechnology protein product specifications can be found in ICH Q6B "Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products" (1999). In addition, the sponsor should consider general aspects of mAb characterization with respect to glycosylation profile and other posttranslational modifications such as deamidation, lysine variability, etc.

The ADC products currently in clinical development include mAbs of the IgG1, IgG2, and IgG4 isotypes; however, the majority of current ADC products include an IgG1 antibody (Trail 2013). The isotype of the mAb will influence whether the ADC has the potential to kill cells via immune-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC). IgG1 mAbs have the potential to mediate CDC, via binding the complement component C1q, and ADCC, via binding to Fcγ receptors expressed on various effector cells, whereas IgG2 and IgG4 mAbs are inefficient in these effector functions (Jiang 2011). The mAb isotype may also influence conjugation, and whether the ADCC and/or CDC activity of a given IgG1 mAb is retained following conjugation of the DL is only a regulatory issue in that the sponsor should evaluate and understand the biologic activity of the ADC product as part of early nonclinical studies.

8.3.2 Drug-Linker

For regulatory purposes, the cytotoxic drug and linker (or DL) is considered as one component in this chapter although the linker has more recently been seen as a central part of the ADC construct as it ideally imparts stability in circulation, favorable pharmacokinetic properties, and efficient release of the toxic agent at the tumor site (Ducry and Stump 2010; see also Chap. 4). The added complexity of providing CMC information for a second intermediate is dependent on the DL selected. In the current ADC development environment, many products in clinical trials are employing a handful of DLs in-licensed from other companies such as the auristatins (Seattle Genetics' monomethyl auristatin E (MMAE) and MMAF) and

maytansinoids (Immunogen's DM1 and DM4; Trail 2013; Mullard 2013) which may mean that a DMF filed with certain regulatory authorities may limit the amount of information submitted by the sponsor in the IND or clinical trial authorization (CTA). A DMF is a submission made directly to the regulatory authority at the discretion of the DMF holder to provide confidential information about a facility, processes, or materials used in the manufacturing of one or more human drugs. The DMF is not approved or disapproved but may be cross-referenced by a sponsor in a product application and is reviewed only in connection with the review of the regulatory application.

However, for novel DLs that are not already described in a DMF, this charges the sponsor with conducting all of the necessary studies and control/stability testing required of a new chemical entity for inclusion in the regulatory application. Because of the importance placed on the linker for ADC product stability in circulation, favorable pharmacokinetic properties, and efficient release of cytotoxic agent at the tumor sites, some sponsors are evaluating small differences in linkers which may have regulatory impact calling for new studies and additional testing. Again, the phase-specific nature of CMC information required during development is emphasized; however, the minimum CMC information for a novel DL means an added layer to the already complex regulatory dossier for an ADC product.

With regard to testing and specifications, the DL is typically a less heterogeneous product in comparison to the mAb, and specific guidance for specifications can be found in ICH Q6A (1999) Product quality attributes for a new chemical entity focuses on impurities such as heavy metals, solvents, catalysts, and/or genotoxic compounds with chemistry and safety aspects and reporting criteria listed in ICH Q3A Impurities in New Drug Substances. (ICH Q3A(R2) 2006). The following considerations are noted for DL testing:

- Structural characterization
- Impurity profile
  - DL-related impurities
  - Process-related impurities
  - Stereochemistry diastereomers
- Phase 1 IND/CTA must consider the following:
  - Free drug-related impurities in clinical lot should be toxicologically qualified (no new impurities should be found in the material used for toxicology testing).
  - Comparable drug-to-antibody ratios should be maintained between the toxicology lot and the clinical lot.
- For phase 3 trial(s): Characterization of the impurity profile of DL intermediates, including structure determination of individual impurities at levels >0.1% is recommended prior to pivotal clinical trials.

Typically, the impurity profile (quality and quantity) for subsequent lots of DL should be equal to or better than the phase 1 material. The goal is to have no new

impurities and demonstration of process clearance of any impurities introduced into the manufacture of the ADC.

8.3.3 Conjugated Bulk Drug Substance and ADC Drug Product

Finally, the conjugated BDS and the DP must be described, characterized, and test results presented in the regulatory application. ADC products pose challenges from an analytical perspective during early development based on the need to develop multiple assays to support process and formulation development, release and stability testing, as well as having orthogonal methods of product characterization.

Due to multiple conjugation site options, lysines, and carbohydrates (via disulphide reduction, via cysteines, and site-specific conjugation), control and characterization can be challenging for the conjugated BDS. At a minimum, the following areas should be given special attention in BDS testing and characterization for preparing the necessary regulatory documentation:

- Impact of conjugation on antibody binding (affinity), stability of the antibody backbone, and aggregation
- Control and characterization of drug load (or drug-to-antibody ratio), including residual unconjugated antibody
- Non-proteinaceous impurities
- Residual conjugation solvent
- Cytotoxicity assays in BDS release and stability (including understanding of overall potency of BDS and DP as demonstrated by binding, cytotoxicity assay, drug-to-antibody ratio, etc.)

With regard to impurities, the conjugated BDS requires special attention to evaluate the multiple areas with potential to introduce harmful impurities, including:

- The antibody plus associated process and product-related impurities
- The small molecule plus associated process and product-related impurities
- Conjugation reaction-associated impurities

One often challenging area for sponsors developing a new product type can be the setting of early acceptance criteria for release and stability studies. For ADC products, in addition to the control parameters noted above, the sponsor must consider certain acceptance criteria for the mAb and BDS together since the subsequent conjugation step may require tighter specifications for the mAb intermediate (i.e., levels of aggregates and fragments may increase upon conjugation). The effect of the conjugation process must be understood to effectively control for specific parameters that may be impacted by conjugation. In addition, certain test parameters of the conjugated drug substance are interdependent and must be considered in concert when setting acceptance criteria for both release and stability studies.

One very specific regulatory requirement for ADC products intended for phase 1 dose-escalation study communicated by members of FDA at industry conferences

necessitates that the acceptance criterion for the required biologically relevant cytotoxicity assay should not be broader than the dose-escalation scheme; an alternative to this requirement is to complete the phase 1 dose-ranging study with a single lot of DP (Miksinski and Shapiro, FDA presentation 2012). In other words, the product potency acceptance criterion for release and stability testing of an ADC cannot be set too wide given the potential toxicities of ADC products. The regulatory review will evaluate the acceptance criterion to ensure that consistent dosing using subsequent lots of the ADC product is attainable especially during a first-in-man dose-escalation study. However, with limited manufacturing experience and stability data in early development, setting specifications in line with a 20 or 30% dose-escalation scheme with a biological assay having inherent assay variability is very challenging and involves the risk of rejecting batches or contributing to out-of-specification results during ongoing stability studies. If the acceptance criterion cannot be sufficiently tightened to be consistent with the dose-escalation scheme in the phase 1 study, the sponsor must provide a strong scientific justification for the acceptance criterion, including information on the state of the assay, additional quality attributes of the ADC that may contribute to the cytotoxic effect of the product, controls in place for such quality attributes, manufacturing capability, and the potential safety impact on patients. Fortunately for sponsors, the FDA is permitting the alternative approach of allowing the phase 1 dose-escalation safety study to proceed (given all other safety and quality review needs are met) if the sponsor can commit to completing the phase 1 study with a single batch. This commitment obviates the need for careful consideration as to when and how subsequent batches of DP are introduced into clinical studies and the acceptance criteria for cytotoxicity and related assays following the initial dose-escalation study.

Because first-generation ADCs use naturally occurring amino acids (cysteines, lysines) in the antibody as anchor points to attach a cytotoxic agent at non-saturating stoichiometries, this results in a heterogeneous mixture of conjugated drug substance, including some percentage of unconjugated mAb (no cytotoxic drugs attached). Determination of the average number of drugs that are conjugated to an antibody molecule (average drug-to-antibody molar ratio or DAR; also referred to as MR_D by some sponsors) is one of the most important quality attributes of an ADC. The DAR determines the amount of cytotoxic drug or "payload" that can be delivered to the tumor cell and can directly affect both safety and efficacy. A variety of methods have been used to measure this key quality attribute, depending on the properties of the drug and how it is linked to the protein (i.e., site of conjugation and structure of the linker; Wakankar et al. 2011). Different methods utilized to determine DAR may yield slightly different results, requiring careful consideration and additional studies to determine which assay will provide results closest to the true value.

The level of unconjugated antibody in the final ADC product is another key ADC control parameter noted in regulatory review. Depending on the isotype of the mAb intermediate and various parameters influencing conjugation, the amount of unconjugated mAb in the BDS may be at levels that, according to the regulatory authorities, do not demonstrate control of the process. The regulatory expecta-

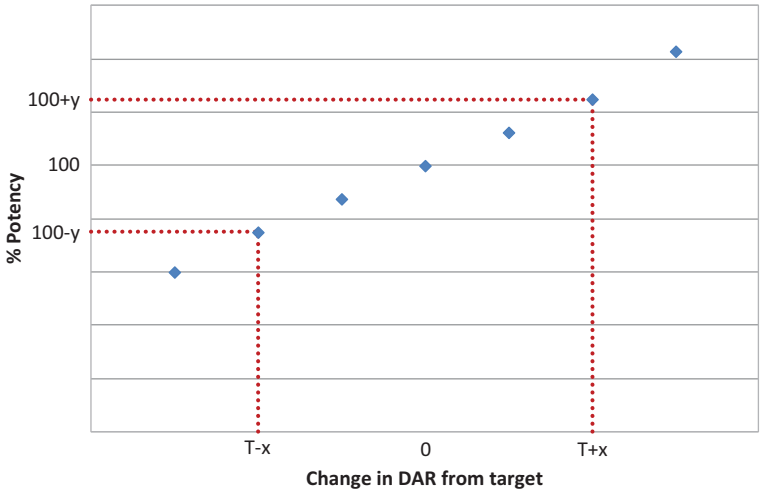

Fig. 8.4 Direct relationship of key ADC parameters—DAR and cytotoxicity

tion is for control of this "impurity" as the unconjugated mAb, although intended as a sophisticated delivery system for the antitumor cytotoxic agent, may have a pharmacologic affect. For instance, unconjugated antibodies can compete with the ADC for tumor-specific binding sites which may affect efficacy or pharmacokinetics (Hansen 2013). From a regulatory perspective, it is not the intended product described in the regulatory dossier (investigator brochure during development) and ultimately the product label upon approval. One of two post-marketing CMC-related sponsor commitments for brentuximab vedotin was to "reassess the acceptance limits for the bulk drug substance and drug product specifications for average drug load MR_D and percent unconjugated cAC10 and further tighten the currently proposed limits…" (FDA CDER Chemistry Review for brentuximab vedotin August 2011).

Moreover, the presence of high DAR species (conjugated antibody with 8, 10, 12, etc. drugs attached) is as much a concern from a safety and product consistency/control point of view. To this end, many firms are pursuing ways to more specifically target and control conjugation at predetermined sites to achieve a specific DAR value with next-generation ADC technologies to produce a more homogeneous ADC DP.

Finally, it has been presented and discussed informally at industry conferences that the DAR, the percent unconjugated antibody, and cytotoxicity results have been shown to be interrelated, calling for studies to understand the relationship between these attributes for a given ADC product. Moreover, the assays employed will have inherent variability, calling for careful consideration to determine even early specifications to assure that these interrelated parameters do not adversely impact one another. For example, Fig. 8.4 depicts the direct relationship observed between DAR and percent potency—two key ADC quality attributes.

8.3.4 Quality Aspects of Comparability

Contributing to the CMC challenges of ADC development, the multifaceted nature of an ADC further influences and adds complexity to the approach needed to demonstrate comparability, especially during the development stages when changes are common and likely at various intermediates and points involved in manufacturing the ADC product. Many types of changes may be made for improved manufacturability such as mAb cell line change, process changes to one or both intermediates, change in site, scale of conjugation, etc., and the sponsor must demonstrate the appropriate degree of similarity or comparability as a result of such changes. Careful consideration must be given to the comparability testing strategy and analytical methods depending on the specifics of the change and its potential impact to subsequent materials and ultimately the quality, safety, and efficacy of the final DP. A major point is that the ability to detect changes is directly related to the quality and capability of the analytical methods used, and as a result, ADC products may in fact require the most advanced analytics possible.

The goal of the comparability exercise is to collect the relevant technical information to serve as evidence that the change will not have an adverse impact on the quality, safety, and efficacy of the ADC DP. Harmonized guidance in this area from ICH (ICH Q5E 2004) greatly aids the sponsor in having a common scientific approach accepted in the ICH regions.

For instance, changes to the mAb intermediate may propagate downstream to the conjugated BDS and final DP, requiring studies be conducted on each material to demonstrate comparability and the lack of an adverse impact on the quality, safety, and efficacy of the ADC. A step-by-step data-driven exercise comparing the pre- and post-change products must be carried out consisting of analytical studies, including routine batch analysis, characterization, and stability studies. In addition, given the biological component of the ADC product, biological characterization is important to include in the testing. Depending on the differences observed in these sets of data that may adversely impact safety and efficacy, the sponsor may need to conduct additional nonclinical (animal pharmacokinetic/pharmacodynamic studies) and clinical bridging studies.

Sponsors should carefully plan the CMC development path to minimize late-stage changes which may significantly extend timelines.

8.4 Nonclinical Study Requirements

In comparison to the requirements of other indications, the entry of new anticancer agents into phase 1 clinical trials is typically based on a more modest preclinical package. However, due to the ADC construct having several intermediates, new ADC products may actually require a more extensive preclinical package in comparison to other types of anticancer agents (ICH S9 2009 and DeGeorge et al. 1998).

Specific chapters of this book are dedicated to the development issues related to pharmacokinetic/pharmacodynamic and toxicology studies for ADCs, but this section more generally outlines the regulatory requirements in early development.

In the USA, the original IND for any new DP should include data from nonclinical studies to demonstrate or determine the below listed items to support the clinical study proposed in the application.

- Proof of principle and pharmacological activity of the investigational drug
- Mechanism of action
- Dose–response relationship
- Toxicological effects predictive of the human response

Rationale for the following:

- Safe starting dose for clinical trials
- Clinical route of administration
- Administration schedule
- Duration of exposure
- Duration of the follow-up time to detect adverse reactions qualifying as "dose-limiting toxicities"
- Duration of the follow-up time to detect all drug-related adverse reactions
- The identity of target organs for toxicity

Some exceptions to the standard nonclinical study requirements do exist for ADC products. For instance, the Harmonized Tripartite Guideline, ICH S6 (R1), recommends that safety of biotherapeutics should be evaluated in two relevant species (2011) and a relevant species is defined in the guidance as one in which the drug candidate is pharmacologically active due to the expression of the receptor or epitope. However, when a second relevant species cannot be identified, the requirement for two species may be altered. The use of one species is also supported in the ICH S9 guideline for Nonclinical Evaluation of Anticancer Pharmaceuticals (2009). Other aspects of nonclinical studies specific to ADC products are listed below:

- GLP toxicology studies in two biologically relevant species, one of which is usually the cynomolgus monkey (unless a second species cannot be identified); toxicity of the drug alone.
- Measurement of concentrations of all ADC components in circulation from nonclinical and clinical studies to characterize the pharmacokinetics of these molecules in totality (conjugated antibody, total antibody, total drug, free drug).
- Immunogenicity assessments should include characterization to determine if anti-drug antibodies are directed towards the antibody or directed towards the DL.
- An additional short-term study or arm in a short-term study should be conducted in at least one species with the unconjugated toxin (ICH S6 Addendum 2011).
- Evaluation of effector function (of both the mAb intermediate and conjugated BDS) and other biological activity and whether or not this may contribute to the overall mechanism of action of the ADC DP.

As mentioned previously, based on information in the public domain, one can see that many of the ADCs currently in the clinic use a small handful of common DLs: the auristatins, maytansinoids, or pyrrolobenzodiazepines. New DL technology is needed to further explore the potential of ADCs; however, the development of novel payloads and compatible linkers is a challenging task and progress has been slow.

Next-generation ADC products with a novel DL will require objective testing of the DL and/or its main metabolite, obviating the need to identify the main metabolite(s) early in nonclinical studies. A standard battery of nonclinical safety testing such as that conducted for a small-molecule drug would not be out of scope (see Sect. 4.2 for CMC needs). Some of this information may be collected by including a separate arm in the pivotal GLP-compliant toxicology study, but others may have to be separate studies designed expressly to investigate the DL (ICH M3(R2) 2009). Sponsors entering into development of ADC products should be aware of the availability of regulatory review information of marketing applications on certain regulatory authority websites such as the FDA. For example, based on the experience with brentuximab vedotin, the current regulatory expectation is that data showing that pharmacokinetics (PK) was characterized, metabolism and drug–drug interaction potential evaluated, QT/QTc interval prolongation potential assessed, immunogenicity assessment was performed, and evaluation in specific populations (e.g., renal/hepatic impairment) will be included in marketing applications of ADCs (Thudium et al. 2012).

Finally, one question raised with regulatory reviewers by the ADC development community in open discussion has been the amount of information that can be borrowed from prior submissions with respect to use of the same DL since many ADCs currently in clinical trials employ maytansinoid or auristatin free drugs. Since regulatory agencies consider molecule-specific data and information with regard to efficacy, safety, and overall benefit to patients, the situations in which previously submitted information is allowed may be supplemental only and may be considered on a case-by-case basis.

8.5 Discussion and Conclusions

Since the withdrawal of gemtuzumab ozogamicin (Mylotarg) from the market in 2010, two new ADC products (brentuximab vedotin and ado-trastuzumab emtansine) have demonstrated clinical and regulatory validation and many more are in clinical trials with the challenges of development ahead. In the current setting, ADCs utilizing novel payload, linker, and conjugation technologies are one of the most active areas of biopharma research and investment.

The components making up the ADC product lead to development complexities, unique challenges, and can be more resource intensive for industry sponsors; therefore, it is important to consider the development stage-specific nature of CMC and nonclinical information required during the various phases. A proactive approach is to consider the information/data needed at each phase of clinical study to ensure

that CMC development, for example, is keeping pace with clinical development and to effectively utilize formal agency interaction to share development plans and de-risking strategies at the appropriate times. For example, the EOP2 meeting with the FDA may sometimes call for a discussion dedicated to CMC issues separate from clinical topics. Fortunately for this audience, regulatory agencies are considering ways to allow for more rapid access of promising new therapies to patients when a product is shown to have a favorable benefit-to-risk profile. However, an accelerated approval is only possible if the CMC expectations are also met and manufacturing is in compliance with current good manufacturing practice standards. As next-generation ADC products advance into clinical development, the CMC challenges are likely to change, requiring new insights into this technically and financially demanding area.

From a clinical perspective, one current challenge with the many ADCs employing the same DL moiety is that the collective safety data on a given DL being utilized by many ADC sponsors may not be known by the individual sponsors but known by regulatory agencies as the recipient of multiple applications and safety data containing the same DL.

As the research and development of ADC products matures, the lessons learned are leading sponsors to develop new technologies by employing synthetic cytotoxic agents and stable linker systems that attach the cytotoxic agents to the antibody at specific sites, eliminating the heterogeneous group of molecules in which the linkers can attach in a number of different places on the antibody (Hansen 2013). The promise of targeted therapy for cancer is attracting new players each year, and new sponsors to the area of ADC research and development are encouraged to maintain open communications with the FDA and other regulatory authorities.

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Part IV
Landscape, IP and Pharmacoeconomics

Chapter 9
Major ADC Companies, Current Clinical Trials, Recent Patents Issued and Patent Applications, and Cost Analysis of Drug Therapy

Jeffrey Wang and Jonathan Hirohiko Watanabe

9.1 Introduction

Prototype antibody-drug conjugates (ADCs) or immunoconjugates were explored as therapeutics in the 1960s and 1970s in experimental animals with promising results (Arnon and Sela 1982; Hurwitz et al. 1975 Rowland et al. 1975). The first generation of ADCs using monoclonal antibodies and conventional chemotherapeutic drugs such as methotrexate and doxorubicin were subsequently tested in animal models and clinical trials (Hurwitz et al., 1983). The clinical results were not satisfactory, mainly due to the inefficient internalization of the conjugate and intracellular release of the active drug species to the target site. To solve these problems, novel anticancer drugs with 100–1000 more potency have been used for the construction and testing of the second generation of ADCs. The three Food and Drug Administration (FDA)-approved ADCs (Mylotarg®, Adcetris®, and Kadcyla®) all belong to this category. Due to their excellent efficacy and desirable side effect profiles, the pharmaceutical industry and the medical field have accepted ADCs as a new class of anticancer medications. Many pharmaceutical and biotechnology companies have recognized the trend and dived into the discovery and development of ADC anticancer drugs. These efforts have enriched the research and the pipelines, which will benefit millions of cancer patients. This chapter tries to draw a sketch of the global ADC landscape and provides a brief cost and outcomes analysis of the available ADC therapeutics.

J. Wang (✉)
Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, Pomona, CA, USA
e-mail: jwang@westernu.edu

J. H. Watanabe
Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, CA, USA

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9.2 Major ADC Developing and Contract Manufacturing Companies

There are two major industry leaders in ADC drug discovery and clinical development, Seattle Genetics, Inc. and ImmunoGen, Inc. Each company has unique payloads and linker technologies, leading to FDA approval of Adcetris® of Seattle Genetics in 2011, and Kadcyla® developed by Roche/Genentech using a proprietary drug by ImmunoGen, Inc. in 2013. Pfizer has been involved in the ADC development process for a long time. Nearly every major pharma and many biotech companies have established collaborations with Seattle Genetics and/or ImmunoGen in developing ADCs. Here we describe some of these companies with active clinical ADC programs and a list of contract manufacturing organizations (CMOs).

9.2.1 Seattle Genetics, Inc.

Seattle Genetics (www.seattlegenetics.com) is based in Bothell, Washington. It was founded by Clay B. Siegall in 1998 and completed an initial public offering in March 2001. It is traded on the Nasdaq Stock Market under the symbol SGEN and its market capitalization is US$ 4.77 billion as of August 7, 2014. The company has approximately 600 employees and Clay B. Siegall continues to serve as its president, chief executive officer, and chairman of the board. Peter Senter leads research in ADC technologies, including payloads development, novel linker design, conjugation methodology, and studies on mechanism of action.

Seattle Genetics initially licensed SGN-15 (BMS-182248, BR96-DOX), a first-generation ADC using doxorubicin as the payload, from Bristol-Myers Squibb in 1999 and conducted clinical trials (Smith 2001). It terminated this ADC program due to limited efficacy (Ross et al. 2006). Seattle Genetics licensed, from Arizona State University in 2000, the right to use microtubule-disrupting monomethyl auristatin E (MMAE) discovered by George Pettit's research laboratory as the potent payload for their ADCs. They developed a cysteine-reactive novel protease-cleavable linker to attach monomethyl auristatins to the antibody. The resulting ADCs are stable in the bloodstream and effective at releasing the cell-killing agent once inside targeted cancer cells (Senter and Sievers 2012).

Seattle Genetics has been collaborating with Takeda Pharmaceutical Company in developing Adcetris® (brentuximab vedotin). Adcetris® is stable in the bloodstream, but it can release MMAE upon internalization into CD30-expressing tumor cells. Adcetris® was approved by the FDA in 2011 and now is commercially available in 40 countries for two indications: (1) The treatment of patients with Hodgkin lymphoma after failure of autologous stem cell transplant (ASCT) or after failure of at least two prior multi-agent chemotherapy regimens in patients who are not ASCT candidates. (2) The treatment of patients with systemic anaplastic large-cell lymphoma (ALCL) after failure of at least one prior multi-agent chemotherapy regimen. According to the co-development agreement, Seattle Genetics has

full commercialization rights to Adcetris® in the USA and Canada while Takeda has exclusive rights to commercialize the product in all other countries. Seattle Genetics is currently expanding the therapeutic potential of Adcetris® and conducting clinical investigations to evaluate the drug in earlier lines of its approved indications and other lymphomas and non-lymphomas.

In addition to its auristatin-based ADC technology, Seattle Genetics is also developing technology employing antibodies with site-specifically engineered cysteines and a more potent pyrrolobenzodiazepine dimer payload that kills cells by a different mechanism than auristatins. SGN-CD33A and SGN-CD70A are two such ADCs with loading of two drugs per antibody. There are >20 ADCs in clinical development using Seattle Genetics' proprietary technologies in both internal and collaborator programs. Its internal pipeline includes SGN-CD19A, SGN-CD33A, SGN-LIV1A, SGN-CD70A, as well as ASG-22M6E and ASG-15ME, ADCs being codeveloped by Agensys (Table 9.1).

Seattle Genetics has established multiple collaborations with leading pharmaceutical and biotechnology companies. There are currently 15 collaborator ADC programs in various stages of clinical development (Table 9.2). The collaborations with AbbVie, Agensys, Bayer, Celldex, Daiichi-Sankyo, Genentech, GlaxoSmithKline, Millennium, Pfizer, and Progenics are under license agreements. These companies

Table 9.1 ADCs in the internal pipeline of Seattle Genetics

ADC program	Therapeutic area	Development stage
SGN-35	Post-transplant HL relapse prevention	Phase 3
(Brentuximab vedotin)	Relapsed CD30-positive cutaneous T cell lymphoma	Phase 3
	Frontline HL (+ chemotherapy)	Phase 3
	Frontline CD30-positive mature T cell Lymphomas (+ chemotherapy)	Phase 3
	Relapsed/refractory CD30-positive non-Hodgkin lymphomas	Phase 2
	Frontline HL in patients 60+ (± dacarbazine)	Phase 2
	Frontline diffuse large B cell lymphoma (+ RCHOP)	Phase 2
	CD30-positive nonlymphoma malignancies	Phase 2
	Second-line HL (+ bendamustine)	Phase 1
SGN-CD19A	Acute lymphoblastic leukemia	Phase 1
	Non-Hodgkin lymphoma	Phase 1
SGN-CD33A	Acute myeloid leukemia	Phase 1
SGN-LIV1A	Breast cancer	Phase 1
ASG-15ME[a]	Bladder cancer	Phase 1
ASG-22ME[a]	Solid tumors	Phase 1
SGN-CD70A	Lymphoma, renal cell carcinoma	Preclinical

HL Hodgkin's lymphoma
[a] Co-developing with Agensys

Table 9.2 ADCs in the collaborators' pipeline using Seattle Genetics' ADC technology

Collaborator	ADC program	Therapeutic area	Development stage
Celldex	Anti-GPNMB ADC	Breast cancer	Phase 3
Genentech/ Roche	Anti-CD79b (RG7596, DCDS4501A)	Non-Hodgkin lymphoma	Phase 2
	Anti-CD22 (RG7593, DCDT2980S)	Non-Hodgkin lymphoma	Phase 2
	Anti-NaPi2b (RG7599, DNIB0600A)	Ovarian cancer	Phase 2
	Anti-STEAP1 (RG7450, DSTP3086S)	Prostate cancer	Phase 2
	Anti-MUC16 (RG7458, DMUC5754A)	Ovarian cancer	Phase 1
	Anti-ETBR (RG7636)	Melanoma	Phase 1
	RG7598		
	RG7600	Multiple myeloma	Phase 1
	Undisclosed ADCs	Pancreatic, ovarian cancer	Phase 1
			Preclinical
Progenics	Anti-PSMA ADC	Prostate cancer	Phase 2
Agensys	Anti-AGS-16 ADC	Renal cell carcinoma	Phase 1
	Anti-CD37 ADC Undisclosed ADCs	Solid tumor	Phase 1 Preclinical
Takeda/ Millennium	Anti-GCC ADC	Gastrointestinal malignancies	Phase 1
Pfizer	Anti-5T4 ADC	Solid tumors	Phase 1
AbbVie	Anti-EGFR ADC Undisclosed ADCs	Squamous cell tumors, Glioblastoma	Phase 1 Preclinical
Bayer	Undisclosed ADCs		Preclinical
Daiichi-Sankyo	Undisclosed ADCs	Solid tumors	Preclinical
GSK	Undisclosed ADCs		Preclinical
Genmab	Anti-Tf ADC	Solid tumors	Phase 1
Oxford BioTherapeutics	Undisclosed ADCs		Preclinical

are responsible for all development, manufacturing, and commercialization activities while Seattle Genetics receives up-front payments, milestones, and royalties on net sales of any resulting ADC products. Under the co-development agreements with Agensys and Genmab, Seattle Genetics can supplement its internal pipeline through opt-in rights to 50:50 co-development and profit-sharing for ADC product candidates. In addition, Seattle Genetics has formed a strategic collaboration with Oxford BioTherapeutics. Monoclonal antibodies against novel tumor-specific antigens generated by Oxford BioTherapeutics' Oxford Genome Anatomy Project database will be used for Seattle Genetics' ADC drug discovery and development.

9.2.2 ImmunoGen, Inc.

ImmunoGen (www.immunogen.com) is headquartered in Waltham, Massachusetts, with its manufacturing facility in Norwood, Massachusetts. It was founded in 1981 and currently has approximately 300 employees with Daniel Junius as its president and chief executive officer and John M. Lambert as its executive vice president of research and development and chief scientific officer. ImmunoGen is traded on the Nasdaq Stock Market under the symbol IMGN since 1990 and its market capitalization is US$ 950 million as of August 7, 2014. ImmunoGen's ADC technology comprises highly potent maytansinoids that can be attached to the surface lysine residues of antibodies via novel thioether linkers. The stable linkers keep the payload attached to the antibody in the bloodstream and then the drug is released inside a cancer cell after internalization (Lambert and Chari 2014).

Genentech/Roche Group licensed ImmunoGen's ADC technology to develop Kadcyla® (ado-trastuzumab emtansine, formerly referred to as T-DM1). The ADC consists of ImmunoGen's proprietary maytansinoid agent DM1 attached to trastuzumab, the HER2-binding antibody. It was approved by the US FDA for the treatment of patients with HER2-positive metastatic breast cancer who have received prior treatment with Herceptin® and a taxane chemotherapy. Kadcyla® has also been approved in the European Union (EU) and Japan. Genentech/Roche is conducting a number of clinical trials to assess Kadcyla for additional clinical uses (Table 9.3).

ImmunoGen has also out-licensed its ADC technology to Amgen, Eli Lilly, Novartis, Sanofi, Bayer, and Biotest for their anticancer ADC drug development (Table 9.3). Most ADCs are in preclinical or early-stage clinical investigation. However, Sanofi's SAR3419 is in phase 2 studies for diffuse large B cell lymphoma.

With the experience gained through the development of Kadcyla®, ImmunoGen is also developing ADCs in-house. It currently has three wholly owned ADCs in phase 1 clinical trials. The company is evaluating IMGN853 for folic acid alpha receptor positive solid tumors such as epithelial ovarian cancer and non-small cell lung cancer, IMGN529 for non-Hodgkin lymphoma (NHL), and IMGN289 for epidermal growth factor receptor (EGFR)-positive lung cancer, head and neck cancer, and other EGFR-positive solid tumors.

9.2.3 Genentech, Inc./Hoffmann-La Roche Ltd.

Genentech (www.gene.com), a member of the Switzerland-based Roche Group, is located in South San Francisco, California. It probably has the broadest and deepest experience with ADCs in the pharmaceutical and biotech industry. The company licensed ADC technologies from both Seattle Genetics and ImmunoGen. It also developed its own proprietary ADC techniques for site-specific conjugation (Panowksi et al. 2014; Junutula et al. 2008). Besides its successful commercialization of Kadcyla® (see Sect. 9.2), it has more than eight ADCs in various stages of clinical development (Table 9.2). Among them, anti-NaPi2b ADC (RG7599) comprises a

Table 9.3 ADCs in collaborators' pipeline using ImmunoGen' ADC technology

Collaborator	ADC program	Therapeutic area	Development stage
Genentech/ Roche	Kadcyla	$HER2^+$ metastatic breast cancer 1st line	Phase 3
	Kadcyla	$HER2^+$ breast cancer, others	Phase 3
	Kadcyla	$HER2^+$ gastric cancer	Phase 2
Sanofi	SAR3419	Diffuse large B cell lymphoma	Phase 2
	SAR566658	$CA6^+$ breast, ovarian, cervical, lung, and pancreatic tumors	Phase 1
	2 undisclosed ADCs		Preclinical
Biotest	BT-062	Multiple myeloma	Phase 1
		Breast, bladder cancer	Phase 1
Bayer	BAY 94-9343	$Mesothelin^+$ solid tumors	Phase 1
Amgen	AMG 595	Recurrent malignant glioma	Phase 1
	AMG 172	Clear cell renal cell carcinoma	Phase 1
	2 undisclosed ADCs		Preclinical
Eli Lilly	1 undisclosed ADC		Preclinical
Novartis	3 undisclosed ADCs		Preclinical

monoclonal antibody directed against NaPi2b, sodium-dependent phosphate transport protein 2b, linked to MMAE. This ADC is in a phase 2 clinical trial for platinum-resistant ovarian cancer. Another ADC in phase 2 trial, pinatuzumab vedotin (RG7593), is one composed of a monoclonal antibody directed against CD22, also linked to MMAE.

9.2.4 Pfizer, Inc.

Pfizer inherited its ADC technology from Wyeth. Mylotarg® (gemtuzumab ozogamicin) is an ADC comprising a monoclonal antibody to CD33 linked to a cytotoxic agent from the class of calicheamicins. It was approved in 2000 by the FDA through the accelerated-approval process to treat acute myelogenous leukemia. But it was voluntarily withdrawn from the market in 2010 because of safety and benefit concerns resulting from a randomized phase 3 comparative controlled trial (FDA 2010). Pfizer is continuing its clinical investigation, and recent results show positive results, which may lead to a resurrection of the product (Gamis et al 2014; Hills et al. 2014; Borthakur et al. 2014; Kharfan-Dabaja 2014; O'Hear and Rubnitz 2014). The company is also developing another ADC, inotuzumab ozogamicin, for slow-growing "indolent" NHL and acute lymphocytic leukemia. It is also being tested in combination with Roche's Rituxan® (rituximab) in a pivotal phase 3 study for patients with relapsed/refractory aggressive NHL.

9.2.5 Agensys, Inc.

Agensys, Inc. (www.agensys.com), an affiliate of Astellas Pharma Inc., is located in Santa Monica, California. The company has been in operation since 1997 and was acquired by Astellas in 2007. Agensys currently has over 200 employees. It specializes in developing fully human monoclonal antibodies as therapeutics to treat cancer. In addition, Agensys is co-developing with Seattle Genetics two ADCs, ASG-15ME for bladder cancer and ASG-22ME for solid tumors. It has also in-licensed Seattle Genetics' technology to develop three additional ADCs in-house for cancer (AGS-16M8F, AGS-16C3F, and AGS-67E). All are in phase 1 clinical trials (Astellas R&D Pipeline 2014).

9.2.6 Celldex Therapeutics

Celldex Therapeutics (www.celldex.com) is a spin-off of the New Jersey-based biopharmaceutical company Medarex, now a subsidiary of the Bristol-Myers Squibb Company. Celldex retains the rights to develop proprietary monoclonal antibodies utilizing the Medarex UltiMAb technology platform. Anthony S. Marucci serves as its founder, president, and chief executive officer. Celldex is traded on the Nasdaq Stock Market under the symbol CLDX since 1990, and as of August 7, 2014, its market capitalization is US$ 1.24 billion. In addition to developing therapeutic antibodies, immune system modulators, and vaccines, Celldex has in-licensed from Seattle Genetics' ADC technology to develop CDX-011 (glembatumumab vedotin), an ADC comprising MMAE conjugated with a fully human monoclonal antibody against glycoprotein NMB (GPNMB) using the valine–citrulline enzyme-cleavable linker. Glembatumumab vedotin is in phase 3 clinical trial for the treatment of locally advanced or metastatic breast cancer. This program is also in development for the treatment of stages 3 and 4 melanoma.

9.2.7 Progenics Pharmaceuticals, Inc.

Progenics (www.progenetics.com) with its location in Tarrytown, New York, focuses on developing diagnostic and therapeutic agents for prostate cancer. Mark R. Baker serves as chief executive officer and Hagop Youssoufian leads research and development. Its leading development compound, prostate-specific membrane antigen (PSMA) ADC, is licensed in from Seattle Genetics and is in phase 2 clinical trial for prostate cancer. Progenics acquired Molecular Insight in early 2013 to enrich and add its portfolio with radiopharmaceutical therapeutics and diagnostic imaging agents for cancer. Progenics is listed on the Nasdaq Stock Market with the symbol PGNX, and as of August 7, 2014, its market capitalization is US$ 321 million.

Table 9.4 Major ADC contract manufacturing organizations (CMOs)

Company	Website	Headquarter location	Main service specialty
Lonza Group Ltd.	www.lonza.com	Basel, Switzerland	Antibody, payload, linker, conjugation
Carbogen Amcis AG	www.carbogen-amcis.com	Bubendorf, Switzerland	Conjugation
Novasep	www.novasep.com/technologies/HPAIs.asp	Pompey, France	Antibody, payload, linker, conjugation
SAFC	www.sigmaaldrich.com/safc.html	St. Louis, MI, USA	Payload, linker, conjugation
ADC Biotechnology	www.adcbio.com	Denbighshire, Wales, UK	Conjugation
Piramal Healthcare	www.piramalpharmasolutions.com/products-services/antibody-drug-conjugation.html	Mumbai, Maharashtra, India	Conjugation
Fujifilm Diosynth	www.fujifilmdiosynth.com	Morrisville, NC, USA	Antibody
Catalent Pharma Solutions	www.catalent.com	Somerset, NJ, USA	Antibody

9.2.8 ADC CMOs

The construct of an ADC is simple in concept but complex in practice, involving careful selection and optimization of the payload, the antibody, the linker, and the conjugation method. Making an ADC is a multi-step endeavor and requires expertise in many scientific areas. This has provided business opportunities for many large and small companies. Table 9.4 summarizes some of the ADC contract manufacturing organizations (CMOs) around the globe (Thayer 2014).

9.3 Major ADC Clinical Trials

The combined pipeline of ADC therapeutics from various companies is rich. There are over 40 ADCs in various stages of clinical investigation (Table 14.1, Chap. 14) for a large spectrum of both blood and solid tumors. Companies such as Seattle Genetics, Progenics, Roche/Genentech, Agensys, ImmunoGen, and Immunomedics have more than one ADC under clinical investigation. Table 9.5 lists most of phase 2/phase 3 and some of the phase 1 clinical trials registered at www.clinicaltrials.gov. In addition to brentuximab vedotin (Adcetris®) and trastuzumab emtansine (Kadcyla®), two other ADCs, gemtuzumab ozogamicin (Mylotarg®) and inotuzumab ozogamicin are in phase 3 clinical trials. If the trials are successful, inotuzumab ozogamicin may soon become the fourth ADC approved to treat cancer patients.

Table 9.5 Major ADC clinical trial registered at www.ClinicalTrials.gov

ADC	ClinicalTrials.gov Identifier	Trial name	Clinical phase	Sponsor	Status	Estimated study completion date
Brentuximab vedotin	NCT01100502	A Phase 3 Study of BrentuximabVedotin (SGN-35) in Patients at High Risk of Residual Hodgkin Lymphoma Following Stem Cell Transplant (The AETHERA Trial)	3	Seattle Genetics Millennium	Ongoing, but not recruiting participants	April 2016
Brentuximab vedotin	NCT01777152	ECHELON-2: A Comparison of BrentuximabVedotin and CHP with Standard-of-care CHOP in the Treatment of Patients with CD30-positive Mature T Cell Lymphomas	3	Seattle Genetics Millennium	Recruiting participants	December 2019
CDX-011	NCT01997333	Study of GlembatumumabVedotin (CDX-011) in Patients with Metastatic, gpNMB Over-Expressing, Triple Negative Breast Cancer (METRIC)	2	Celldex	Recruiting participants	September 2016
PSMA ADC	NCT01695044	A Study of PSMA ADC in Subjects With Metastatic Castration-Resistant Prostate Cancer (mCRPC)	2	Progenics	Ongoing; not recruiting participants	July 2015
PSMA ADC	NCT02020135	An Open-Label Treatment Extension of PSMA ADC in Subjects with Metastatic Castration-Resistant Prostate Cancer (mCRPC)	2	Progenics	Enrolling participants by invitation only	July 2015
ASG-15ME	NCT01963052	Study of Escalating Doses of AGS15E Given as Monotherapy in Subjects with Metastatic Urothelial Cancer	1	Agensys Seattle Genetics	Recruiting participants	October 2015
AGS-22M6E	NCT01409135	A Study of the Safety and Pharmacokinetics of AGS-22M6E in Subjects with Malignant Solid Tumors That Express Nectin-4	1	Astellas Agensys Seattle Genetics	Ongoing; not recruiting participants	December 2014

Table 9.5 (continued)

ADC	ClinicalTrials.gov Identifier	Trial name	Clinical phase	Sponsor	Status	Estimated study completion date
Trastuzumab emtansine (Kadcyla®)	NCT02144012	A Study to Evaluate the Efficacy and Safety of TrastuzumabEmtansine Versus the Combination of Trastuzumab Plus Docetaxel in Patients with HER2-Positive Breast Cancer	3	Hoffmann-La Roche	Recruiting participants	July 2018
SAR3419	NCT01472887	SAR3419 as Single Agent in Relapsed-Refractory Diffuse Large B Cell Lymphoma (DLBCL) Patients (STARLYTE)	2	Sanofi	Ongoing; not recruiting participants	February 2015
ABT-414	NCT01741727	A Study of ABT-414 in Subjects with Solid Tumors	2	AbbVie	Recruiting participants	April 2016
DNIB0600A	NCT01991210	A Randomized Study of DNIB0600A in Comparison with Pegylated Liposomal Doxorubicin in Patients with Platinum-Resistant Ovarian Cancer	2	Genentech	Recruiting participants	July 2016
Gemtuzumab ozogamicin	NCT00091234	Gemtuzumab Ozogamicin in Treating Older Patients with Previously Untreated Acute Myeloid Leukemia (AML-19)	2/3	European Organization for Research and Treatment of Cancer	Recruiting participants	December 2013
IMMU-132	NCT01631552	Phase 1/2 Study of IMMU-132 in Patients with Epithelial Cancers	1/2	Immunomedics	Recruiting participants	June 2015
BT062	NCT01001442	Safety and Dose Determining Multidose Study of BT062 in Patients with Relapsed or Refractory Multiple Myeloma	1/2	Biotest	Active; not recruiting participants	December 2014
Inotuzumab ozogamicin	NCT01564784	A Study of InotuzumabOzogamicin Versus Investigator's Choice of Chemotherapy in Patients with Relapsed or Refractory Acute Lymphoblastic Leukemia	3	Pfizer	Recruiting participants	August 2017

Table 9.5 (continued)

ADC	ClinicalTrials.gov Identifier	Trial name	Clinical phase	Sponsor	Status	Estimated study completion date
IMMU-130	NCT01605318	Dose Finding Study of Once or Twice Weekly IMMU-130 in Metastatic Colorectal Cancer	1/2	Immunomedics	Recruiting participants	December 2015
IMGN901	NCT01237678	A Study to Test Safety and Efficacy of IMGN901 in Combination with Carboplatin/Etoposide in Patients with Advanced Solid Tumors and Extensive Stage Small-Cell Lung Cancer	1/2	ImmunoGen	Ongoing; not recruiting participants	July 2015
hLL1-DOX	NCT01101594	A Study of hLL1-DOX (Milatuzumab–Doxorubicin Antibody–Drug Conjugate) in Patients with Multiple Myeloma	1/2	Immunomedics	Completed	July 2013
DCDT2980S	NCT01691898	A Study of DCDT2980S in Combination with MabThera/Rituxan or DCDS4501A in Combination with MabThera/Rituxan in Patients with Non-Hodgkin's Lymphoma	2	Genentech	Ongoing; not recruiting participants	December 2015
DCDS4501A	NCT01691898	A Study of DCDT2980S in Combination with MabThera/Rituxan or DCDS4501A in Combination with MabThera/Rituxan in Patients with Non-Hodgkin's Lymphoma	2	Genentech	Ongoing; not recruiting participants	December 2015
SC16LD6.5	NCT01901653	SC16LD6.5 in Recurrent Small-Cell Lung Cancer	1/2	Stem CentRx	Recruiting participants	June 2015

9.4 Recent ADC Patents and Patent Applications

A search in the US patent database (http://patft.uspto.gov/netahtml/PTO/search-bool.html) using the search term "antibody-drug conjugates" or "immunoconjugates" reveals that for a long time Seattle Genetics and ImmunoGen own the largest number of US patents on ADCs. This group is followed by Immunomedics and Bristol-Myers Squibb. However, Genentech has recently been most active in filing and receiving US patents. Tables 9.6 and 9.7 list US patents issued in the first 7 months of 2014 and published US patent applications in the 3 months from May to July 2014. A detailed analysis of these patents would be beyond the scope of this chapter. However, it is obvious to note that many more companies (including those in Asia) and research organizations are building intellectual properties on ADC discovery and development. The future of ADC supply for targeted therapy looks extremely promising.

9.5 Cost and Outcomes Considerations

The price of cancer treatment for a single month with new targeted agents is roughly US$ 10,000. This represents a doubling of price in the past 10 years (Kantarjian et al. 2013). Fojo et al. reported in 2009 that more than 90 % of oncology therapeutics in the past 4 years cost in excess of US$ 20,000 per 12-week course of treatment (Fojo and Grady 2009). ADCs are not exceptions to the high price point. Indeed, the addition of "seek-and-destroy" capability comes at a premium in price beyond established biologic oncology therapeutics. The complexity of these molecules and the challenges of expression, production, and purification of protein-derived therapies require, in many cases, singular manufacturing infrastructure and expertise. These demands figure prominently into the pricing scheme of these agents. Moreover, these new targeted agents display the characteristics of other specialty biologic pharmaceuticals in necessitating additional effort to distribute, handle, educate, and mitigate risks (Sullivan 2008). Additional justifications of the pricing include inordinately high costs of bringing drugs to market in the current environment and funding necessary to support future research and development of new molecules. It is important to recall that the vast majority of investigational drugs do not make it to the market yet consume a large amount of research and development spending. The funds expended in the research program of these abandoned molecules by firms have been linked to pricing structure of agents that do see market life (DiMasi et al. 2003). The most persuasive justification in price may lie in the purported safety profile of these agents. ADCs are designed to locate and deliver anticellular payload solely to cancerous cells, deftly avoiding healthy tissue and the adverse effects concomitant with damaging noncancerous cells (Goldenberg 2013). Traditional oncology agents are virtually synonymous with quality of life depleting adverse events, including hair loss, muscle pain, weakness, and blood cell disorders.

Table 9.6 US patents on ADCs issued in January–July 2014

Patent number	Title	Inventor	Assignee	Issue date
8,790,649	EGFR-binding molecules and immunoconjugates thereof	Setiady J, Park PU, Rui L, ChittendenT, Payne G	ImmunoGen, Inc.	July 29, 2014
8,785,600	Anti-GCC antibody molecules and related compositions and methods	Nam SS, Greenfield EA, O'Keefe TL, Qin S, Babcook J	Millennium Pharmaceuticals, Inc. Amgen British Columbia	July 22, 2014
8,772,543	Antiproliferative compounds, conjugates thereof, methods therefor, and uses thereof	Cheng H, Gangwar S, Cong Q, Zhang Q	Medarex, LLC	July 8, 2014
8,772,542	Antiproliferative compounds, conjugates thereof, methods therefor, and uses thereof	Cheng H, Gangwar S, Cong Q	Medarex, LLC	July 8, 2014
8,772,457	BACE1 antibodies	Atwal J, Chen Y, Chiu CPC, Lazarus RA, Wang W, Watts RJ, Wu Y, Zhang Y	Genentech, Inc.	July 8, 2014
8,771,966	Immuno-PET imaging of antibodies and immunoconjugates and uses therefor	Dennis M, Marik J, Polakis P, Rubinfeld B, Williams S	Genentech, Inc.	July 8, 2014
8,771,685	Anti-BV8 antibodies and uses thereof	Wu X, Wu Y, Yu L, Ferrara N, Liang WC, Meng YJG, Tien J	F. Hoffmann-La Roche AG	July 8, 2014
8,765,917	CD37-binding molecules and immunoconjugates thereof	Deckert J, Park P, Tavares D, Rui L	ImmunoGen, Inc.	July 1, 2014
8,765,740	Cytotoxic benzodiazepine derivatives	Li W, Miller M, Fishkin N, Chari RVJ	ImmunoGen, Inc.	July 1, 2014
8,758,758	Post-relapse treatment of CD30 expressing lymphomas	Sievers E, Kennedy D	Seattle Genetics, Inc.	June 24, 2014
8,748,587	Molecules and methods for modulating TMEM16A activities	Gaither LA, Rothwell CJ	Novartis AG	June 10, 2014
8,747,857	Calicheamicin derivative-carrier conjugates	Kunz A, Moran JK, Rubino JT, Jain N, Vidunas EJ, Simpson JM, Merchant N, Dijoseph JF, Ruppen ME, Damle NK, Robbins PD, Popplewell AG	Wyeth Holdings LLC	June 10, 2014

Table 9.6 (continued)

Patent number	Title	Inventor	Assignee	Issue date
8,742,076	Nemorubicin metabolite and analog reagents, antibody-drug conjugates and methods	Cohen RL, Ha EHS, Reynolds ME	Genentech, Inc.	June 3, 2014
8,735,098	Methods and composition for secretion of heterologous polypeptides	Marrichi M, Reilly DE	Genentech, Inc.	May 27, 2014
8,729,024	Targeted delivery of pharmaceutical compounds	Stracher A, Kesner L, Shulman A	The Research Foundation of State University of New York	May 20, 2014
8,722,860	Anti-TNF-alpha antibodies and their uses	Harding FA, Akamatsu Y, Dubridge RB, Powers DB	AbbVie Biotherapeutics Inc.	May 13, 2014
8,722,857	Anti-CD79B antibodies and immunoconjugates and methods of use	Chen Y, Dennis M, Dornan D, Elkins K, Junutula JR, Polson A	Genentech, Inc.	May 13, 2014
8,710,189	Anti-FGFR3 antibodies and methods using same	Ashkenazi A, Qing J, Wiesmann C, Wu Y	Genentech, Inc.	April 29, 2014
8,709,431	Enediyne compounds, conjugates thereof, and uses and methods therefor	Chowdari NS, Gangwar S, Sufi B	Bristol-Myers Squibb Company	April 29, 2014
8,703,714	Monomethylvaline compounds capable of conjugation to ligands	Doronina SO, Senter PD, Toki BE, Kline TB	Seattle Genetics, Inc.	April 22, 2014
8,697,688	Pyrrolobenzodiazepines used to treat proliferative diseases	Howard PW, Masterson L, Tiberghien A, Jeffrey S, Burke P, Senter P	Seattle Genetics Inc. Spirogen Sarl	April 15, 2014
8,691,531	Anti-CD79B antibodies and immunoconjugates and methods of use	Chen Y, DennisM, Dornan D, Elkins K, Junutula JR, Polson A, Zheng B	Genentech, Inc.	April 8, 2014
8,691,222	NLRR-1 antagonists and uses thereof	Hazen M, Hongo JAS, Smith V, Stinson S	Genentech, Inc.	April 8, 2014
8,680,247	Monoclonal antibodies against glypican-3	Terrett JA, Lu LS, Huang H, Yao D, Pan C, Leblanc H, Sproul T, Yamanaka M	Medarex, LLC	March 25, 2014

Table 9.6 (continued)

Patent number	Title	Inventor	Assignee	Issue date
8,679,767	Multiple reaction monitoring LC-MS/MS method to detect therapeutic antibodies in animal samples using framework signature peptides	Kaur S, Saad O, Xu K	Genentech, Inc.	March 25, 2014
8,668,910	Antibodies that specifically bind to the EphA2 receptor	Bouchard H, Commercon A, Fromond C, Mikol V, Parker F, Sassoon I, Tavares D	Sanofi	March 11, 2014
8,664,407	Chemical linkers with single amino acids and conjugates thereof	Chen L, Gangwar S, Guerlavais V, Lonberg N, Zhang Q	Medarex, LLC	March 4, 2014
8,663,643	Combinations of an anti-HER2 antibody-drug conjugate and chemotherapeutic agents, and methods of use	Berry L, Phillips GL, Sliwkowski MX	Genentech, Inc.	March 4, 2014
8,663,642	Anti-CD70 antibody-drug conjugates and their use for the treatment and prevention of cancer and immune disorders	Law CL, McEarchern J, Drachman JG	Seattle Genetics, Inc.	March 4, 2014
8,658,774	Meditopes and related meditope-monoclonal antibody delivery systems, synthesis and therapeutic uses thereof	Williams JC, Horne DA, Ma Y, Chang HW	City of Hope	February 25, 2014
8,658,175	Anti-EGFR antibodies and their uses	Dubridge RB, Powers DB, Forsyth CM	AbbVieBiotherapeutics Inc.	February 25, 2014
8,652,479	Antibody-drug conjugates and methods	Ebens Jr. AJ, Jacobson FS, Polakis P, Schwall RH, Sliwkowski MX, Spencer SD	Genentech, Inc.	February 18, 2014
8,647,624	Treatment of immune disorders with anti-CD70 antibody	Law CL, McEarchern J, Wahl AF	Seattle Genetics, Inc.	February 11, 2014
8,642,742	Anti-CEA antibodies	Hofer TU, Hosse R, Moessner E, Umana P	Roche Glycart AG	February 4, 2014
8,637,642	Antibody drug conjugates (ADC) that bind to 191P4D12 proteins	Satpayev D, Morrison RK, Morrison KJM, Gudas J, Jakobovits A, Torgov M, An Z	Seattle Genetics, Inc. Agensys, Inc.	January 28, 2014
8,637,641	Antibodies with modified isoelectric points	Dahiyat BI, Lazar GA, Bernett MJ	Xencor, Inc.	January 28, 2014

Table 9.7 US patent applications on ADCs published in May–July 2014

Patent application number	Title	Inventor	Assignee	Publication date
20140194601	Antibody–drug conjugates (ADC) that bind to 161p2f10b proteins	Torgov M, Morrison RK, Jakobovits A, Gudas J, An Z	Agensys, Inc.	July 10, 2014
20140193438	Enediyne compounds, conjugates thereof, and uses and methods therefor	Chowdari NS, Gangwar S; Sufi B	Bristol-Myers Squibb Company	July 10, 2014
20140193437	Hydrophilic self-immolative linkers and conjugates thereof	Lin RH, Lin SY, Hsieh YC, Huang CC	BioAlliance CV AbGenomics International Inc.	July 10, 2014
20140193436	Extracellular targeted drug conjugates	Prudent JR	Centrose, LLC	July 10, 2014
20140187756	Methods of preparing antibody-active agent conjugates	Kim Y, Park T, Woo S, Lee H, Kim S, Cho J, Jung D, Kim Y, Kwon H, Oh K, Chung Y, Park YH	LegoChem Biosciences, Inc.	July 3, 2014
20140187754	Anti-fgfr3 antibodies and methods using same	Ashkenazi A, Qing J, Wiesmann C, Wu Y	Genentech, Inc.	July 3, 2014
20140187500	Compounds, conjugates and compositions of epipolythiodiketopiperazines and polythiodiketopiperazines	Movassaghi M, Kim J, Hergenrother PJ	The Board of Trustees of the University of Illinois Massachusetts Institute of Technology	July 3, 2014
20140179905	Methods and compositions for targeting polyubiquitin	Gordon NC, Kelley RF, Pham A	Genentech, Inc.	June 26, 2014
20140178416	Cross-linkers and their uses	Chari RVJ, Zhao RY, Kovtun Y, Singh R, Widdison WC	Immunogen, Inc.	June 26, 2014
20140178415 20140178414	Maytansinoid derivatives	Li S, Deng X, Tan S, Tang W, Qin C	Bio-Thera Solutions, Ltd., Co.	June 26, 2014
20140178413	Compounds and methods for the treatment of erb b2/neu positive diseases	Tang W, Li S, Yu JC, Deng X	Bio-Thera Solutions, Ltd., Co.	June 26, 2014

Table 9.7 (continued)

Patent application number	Title	Inventor	Assignee	Publication date
20140178412	Compounds and methods for the treatment of EGFR positive diseases	Qin, C, Li S	Bio-Thera Solutions, Ltd., Co.	June 26, 2014
20140170063	Dosages of immunoconjugates of antibodies and SN-38 for improved efficacy and decreased toxicity	Govindan SV, Goldenberg DM	Immunomedics, Inc.	June 19, 2014
20140161877	Pederin and psymberin agents	Floreancig PE, Day BW, Wan S, Wu F	University of Pittsburgh	June 12, 2014
20140161829	Antibody-active agent conjugates and methods of use	Kim Y, Park T, Woo S, Lee H, Kim S, Cho J, Jung D, Kim Y, Kwon H, Oh K, Chung Y, Park YH	LegoChem Biosciences, Inc.	June 12, 2014
20140161827	Compositions and methods for treating cancer	Santen RJ, Aiyar SE	University of Virginia Patent Foundation	June 12, 2014
20140155861	Extrusion methods and devices for drug delivery	Gallagher E, Moreno-Gonzelez A, Lund Sr. W, Lund Jr. W, Grenley M	Presage Biosciences, Inc.	June 5, 2014
20140155590	Conjugates of pyrrolo[1,4]benzodiazepine dimers as anticancer agents	Commercon A, Gauzy-Lazo L	Sanofi	June 5, 2014
20140155584	Conjugated anti-CD38 antibodies	Elias KA, Landes G, Singh S, Korver W, Drake AW, Haak-Frendscho M, Snell GP, Bhaskar V	Takeda Pharmaceutical Co., Ltd.	June 5, 2014
20140140993	Combinations of an anti-her2 antibody-drug conjugate and chemotherapeutic agents, and methods of use	Ross LB, Phillips G L, Sliwkowski MX	Genentech, Inc.	May 22, 2014

Table 9.7 (continued)

Patent application number	Title	Inventor	Assignee	Publication date
20140134193	Spliceostatin analogs and methods for their preparation	Subramanyam C, Koehn FE, Dirico KJ, Eustaquio AS, Green ME, He H, He M, O'Donnell CJ, Puthenveetil S, Ratnayake A, Teske JA, Yang HY	Pfizer, Inc.	May 15, 2014
20140128580	Antibody-drug conjugates and methods	Ebens Jr. AJ, Jacobson FS, Polakis P, Schwall RH, Sliwkowski MX, Spencer SD	Genentech, Inc.	May 8, 2014
20140127241	Antibody-drug conjugates and methods of use	Leuschner C, Alila H	Esperance Pharmaceuticals, Inc.	May 8, 2014
20140127240	Novel binder–drug conjugates (ADCs) and use of same	Lerchen HG, Linden L, Sheikh SE, WilludaJ, Kopitz CC, Schuhmacher J, Greven Simone, Mahlert C, Stelte-Ludwig B, Golfier S, Beier R. Heisler I, Harrenga A, Thierauch K, Bruder S, Petrul H, Jorien H, Brokowski S	Bayer Intellectual Property GmbH	May 8, 2014
20140127239	Pyrrolobenzodiazepines and conjugates thereof	Howard PW	Spirogen Sarl	May 8, 2014
20140127211	Anti-notch3 antibodies and antibody-drug conjugates	Geles KG, Gao Y, Sapra P, Tchistiakova LG, Zhou BBS	Pfizer, Inc.	May 8, 2014
20140127197	Anti-cd22 antibodies and immunoconjugates and methods of use	Ebens AJ, Gray AM, Liang WC, Wu Y, Yu SF	Genentech, Inc.	May 8, 2014
20140120118	Pyrrolobenzodiazepines and conjugates thereof	Howard PW	Spirogen Sarl	May 1, 2014

Specifically, the adverse side effects of diarrhea, fatigue, nausea, neuropathy, and vomiting have been statistically tied to reductions in quality of life metrics in prior cancer trials (Boyd et al. 2011). The notion of potential willingness to pay for quality of life enhancement in oncology treatment has been previously described and figured into pharmacoeconomics (Tonia et al. 2012). The simplified premise being that 1 year of feeling perfectly healthy is equal to 2 years of feeling half of perfectly healthy. If drug A and drug B equally extend a person's life by 1 year, but drug A results in better quality of life for that additional year, the value of drug A is considered greater than drug B. For years, validated means of measuring quality of life have been broadly used in health care via rating scale surveys that evaluate patient preferences for different states of health (Drummond et al. 1997). These special considerations contribute to the premium in costs versus traditional small-molecule oncology agents. These justifications have fallen under greater scrutiny as a significant portion of the often cited estimate of US$1 billion to bring a drug to market includes nonresearch spending as well as evidence that a portion of the preliminary research is taxpayer or government funded (Goozner 2004).

The two FDA-approved agents currently on the market are Adcetris® (brentuximab vedotin), indicated for third-line therapy for HL and second-line for systemic ALCL, and Kadcyla® (ado-trastuzumab emtansine), approved for second-line monotherapy for HER2-positive metastatic breast cancer. FDA approved in 2011, Adcetris® costs approximately US$ 13,500 a cycle with trial patients receiving an average of seven to nine doses totaling US$ 94,500 to US$ 121,500 for the annual course (Newland et al. 2013). FDA approved in 2013, a 1-month cycle of Kadcyla® is listed at US$ 9800 with the 9-month regimen estimated at US$ 94,000 (Goldenberg 2013). Outcomes analysis in the oncology space is complicated by the application of several different endpoints commonly used. The gold standard measurement is the straightforward outcome of overall survival (OS) and is defined as the time from randomization to death. The true virtue of OS is the absence of clinician interpretation in rating the outcome. Application of the other commonly used endpoints in cancer studies can be frustrated by additional challenges in interpretation of the endpoints. Tumor size reduction equal to or greater than a pre-specified amount can also serve as an endpoint in cancer studies. The percentage of patients who experience a tumor size reduction of a minimum magnitude for a pre-specified minimum amount of time is called the objective response rate (ORR). The time from first clinical response until documented tumor progression is deemed the response duration. The endpoint of progression free survival (PFS) is the time from randomization to objective tumor progression or death. Comparative effectiveness analyses from observational studies or registry data are crucial in revealing differences in outcomes for patients who are taking these agents versus standard of care. This matter is further complicated as Adcetris® was granted market approval based on the results of phase 2 trial without a second treatment arm for comparison. Defining the methods to determine an appropriate clinical comparator for these new agents will be an important next step. Systematic considerations of clinical, humanistic, and cost outcomes will all likely be needed to determine an ideal, real-world comparator. At the time of this writing, neither approved agent has been available on

the market long enough for robust comparative effectiveness and cost-effectiveness analyses to be performed. However, the available data does allow us to make some conjectures about drug cost implications for this important new category in the cancer treatment armamentarium.

Adcetris® HL is no longer a lethal diagnosis as 75% of patients are now cured worldwide with less than 1,500 patients per year dying from HL in the USA. Unfortunately, toxicities associated with the standard treatments for HL themselves carry long-term health consequences. Adcetris® presents a potential additional step forward in a relatively successful battle to eradicate HL by lowering mortality and morbidity complications of treating HL. Adcetris® was lauded for positive findings in the phase 2 open-label, single-arm pivotal trials that provided the evidence deemed sufficient for market approval (Younes et al. 2012). The primary endpoint was the overall ORR determined by an independent radiology review facility. The ORR was 75% with complete remission (CR) in 34% of patients. The median progression-free survival time for all patients was 5.6 months, and the median duration of response for those in CR was 20.5 months. After a median observation time of more than 1.5 years, 31 patients were alive and free of documented progressive disease. Importantly, this study did not proceed using the gold standard of efficacy assessment: the randomized clinical trial. Thus, the absence of masked randomization of similar study patients to a clinically congruent comparator presents several limitations in gauging effectiveness and even efficacy. The ongoing phase 3 randomized trial will shed additional light on efficacy, but given the comparison in the phase 3 trial is to placebo rather than active comparator; effectiveness will be left to speculation. It is important to note that despite the positive findings related to the primary outcomes of PFS and ORR, fewer than half of the 102 patients were alive at the end of 2 years (Younes et al. 2012). Other oncologists have described the importance of outcomes to justify the high price tag of Adcetris® given the availability in inexpensive, generic form of the gold standard regimen of doxorubicin, bleomycin, vinblastine, and dacarbazine termed ABVD therapy (Canellos 2012). Drug cost per cycle of ABVD therapy due to generic availability is approximately US$ 100 per cycle (Center for Medicare and Medicaid Services 2013). This contrasts markedly with US$13,500 per cycle for Adcetris®. The value proposition of Adcetris® will rely heavily on demonstrating from the payer and societal perspective that cost savings in terms of adverse event avoidance and improved quality of life offset the large increase in drug costs.

Kadcyla® Kadcyla® was compared to Herceptin® (trastuzumab) plus the generic taxane medication docetaxel in phase 2 randomized clinical trials. In terms of the main study endpoint of the time from randomization to objective tumor progression or death referred to as PFS (Pazdur 2008), Kadcyla® was associated with an additional 5 months of PFS compared to Herceptin® plus docetaxel (14.2 months PFS vs. 9.2 months PFS, respectively; Hurvitz et al. 2013). At an estimated cost of US$ 5,000 per cycle for Herceptin® (Kantarjian et al. 2013), Kadcyla® represents a near doubling in price per cycle for this additional 5 months in PFS. Using results from the phase 2 clinical trial, it is possible to gain additional perspective on the cost implications. The

median number of cycles of Herceptin® used per patient was 12 (range 2–43 cycles) plus a median number of cycles of docetaxel of 8 (range 1–31 cycles) compared to 16 cycles for Kadcyla® (range of 1–41 cycles; Hurvitz et al 2013). Generic docetaxel is relatively inexpensive with an average drug cost per cycle of US$ 600 (Center for Medicare and Medicaid Services 2013; Sacco et al. 2010). Based on the trial results, assuming the listed US$ 9,800 per cycle for Kadcyla®, this yields a non-adjusted drug cost per course of US$ 65,000 and US$ 156,800 for Herceptin® plus docetaxel and Kadcyla®, respectively. This represents an approximate additional treatment course of US$ 91,800 for Kadcyla® for the 5 months in additional PFS. Applying an incremental lens, this equates to US$ 18,360 per month for each of the additional 5 month of PFS. This translates to US$ 220,320 for an additional year of PFS. Pharmacoeconomic studies commonly use a threshold in which a health technology is deemed cost-effective if it falls in the range of US$ 75,000–US$ 150,000 per year of "'perfect health" with US$ 100,000 per year of perfect health commonly applied as the cost-effectiveness threshold (Cutler 2004). It is important to observe based on the wide range in number of cycles per treatment arm, that the uncertainty in Kadcyla® costs for patients undergoing chemotherapy is expensive, making oncology cost forecasting particularly frustrating. Similar to Adcetris®, robust data will be needed to demonstrate value based on improved quality of life and cost offsets from the payer and societal perspective.

9.6 Additional ADC Resources

Published materials on ADC discovery and development stemmed from the 1960s. Conjugation chemistry and preclinical research results are usually published in biomedical and pharmaceutical journals such as *Journal of Medicinal Chemistry* and *Bioconjugate Chemistry*. Clinical findings usually appear in clinical journal such as *New England Journal of Medicine*. Timely findings are presented in annual meetings of pharmaceutical and medical associations (American Association of Pharmaceutical Association, American Association for Cancer Research, American Society of Clinical Oncology, etc.). Several ADC workshops have been organized by the American Association of Pharmaceutical Association. A specialized conference, World ADC Summit (www.adc-summit.com), is held annually with its 2015 meeting site in San Diego, California. Humana Press has published two books on ADCs. The book entitled *Antibody-Drug Conjugates and Immunotoxins* was edited by Gail Lewis Philips of Genentech. It focuses on the general concept of ADCs with many development examples. The other more recent one, *Antibody-Drug Conjugates* edited by Lonza's Laurent Ducry, is a book in the series of *Methods in Molecular Biology* and provides experimental protocols in ADC preparation and analysis. Roots Analysis (www.rootsanalysis.com) is a UK-based business research and consulting firm providing ADC marketing analysis and projection.

9.7 Conclusions

With the recent approvals of Adcetris® and Kadcyla®, the concept of ADC has been accepted by the pharmaceutical and medical fields. These twenty-first-century "magic bullets" are redefining the practice of cancer therapy and greatly benefiting many cancer patients. With the increasing number of pharmaceutical and biotech companies joining the trend, clinical candidates in the pipeline and clinical trials, issued patents, and patent applications, it is foreseeable that in the next 5–10 years, many new ADCs will be added to the approved list and cancer therapy outcomes will be vastly improved.

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Part V
Case Studies

Chapter 10
Mylotarg: Revisiting Its Clinical Potential Post-Withdrawal

Jennica L. Zaro

10.1 Introduction

Under the newly introduced accelerated approval process by the US Food and Drug Administration (FDA), gemtuzumab ozogamicin (GO; Mylotarg®) was approved in May 2000 for the treatment of acute myeloid leukemia (AML). However, due to concerns about the product's safety, and its lack of improving clinical benefit in post-approval clinical trials, it was voluntarily withdrawn by the market by Pfizer in June 2010. The antibody–drug conjugate (ADC) consists of an antibody targeting the CD33 antigen expressed on most myeloid and monocytic leukemia cells linked to an antitumor antibiotic, calicheamicin, via a pH-sensitive hydrazone linker (Fig. 10.1). Following binding to CD33 antigens, GO is internalized and routed to the acidic lysosome, where the hydrazone linker is cleaved, freeing the conjugated drug. The drug is then able to be internalized into the nucleus, where it induces breaks in DNA. The activity of GO depends on several factors, including the expression and saturation levels of CD33, binding of GO at the cell surface, release of calicheamicin and induction of DNA breaks in the nucleus, and the efflux of calicheamicin via multidrug resistance (MDR)-related proteins (Fig. 10.2). Over the past decade, numerous review articles have been published discussing the development and approval of Mylotarg® for AML treatment (Cowan et al. 2013; Sievers 2003; Tanimoto et al. 2013). Therefore, the details of the ADC, its approval process, and clinical trials will only be briefly introduced. The main focus of this case study will be on the revival of Mylotarg® in the clinics through recent clinical trials.

J. L. Zaro (✉)
Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, California, USA
e-mail: zaro@usc.edu

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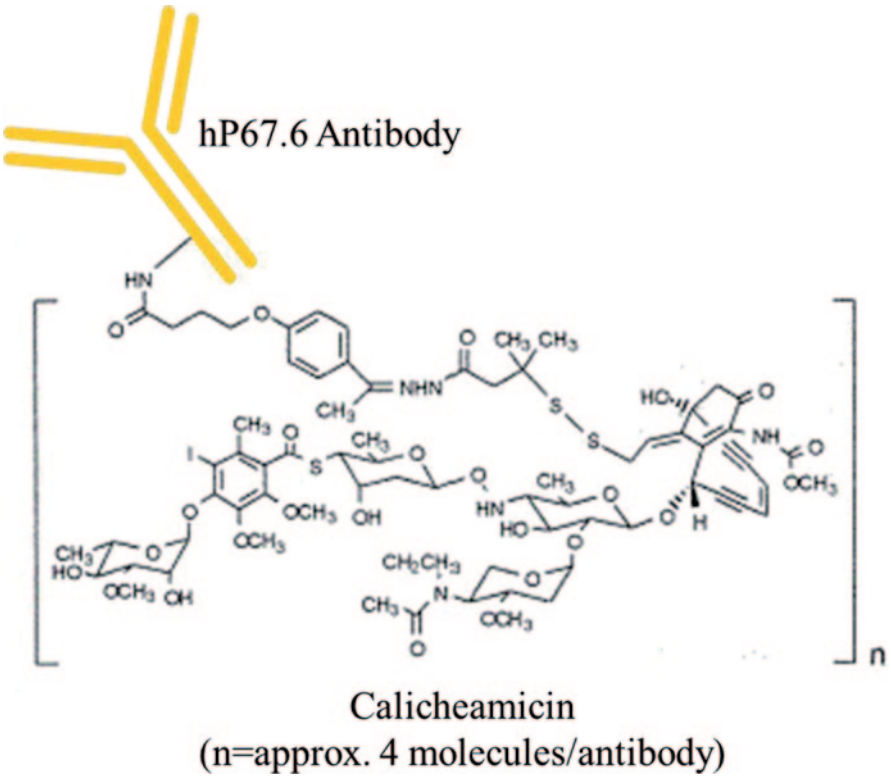

Fig. 10.1 Structure of GO. GO consists of the drug, calicheamicin, linked to the anti-CD33 hP67.6 antibody with an average drug–antibody ratio (DAR) of 3–4. The drug is linked to the lysine residues of the antibody via a pH-sensitive hydrazone linker. *GO* gemtuzumab ozogamicin

10.1.1 The Antibody: Gemtuzumab

Mylotarg® employs a monoclonal antibody, gemtuzumab (hP67.6), which is targeted against the CD33 antigen. Gemtuzumab is a humanized antibody derived from the complementarity-determining regions of the murine antibody p67.6. (Hamann et al. 2002). Its IgG4 isotype influences the effector function of the antibody, where the effector functions correlate with the Fc-receptor binding affinity (IgG1 > IgG3 > IgG4 > IgG2; Janeway et al. 2001). Therefore, consistent with its isotype, Mylotarg® does not exhibit any effector functions. Although the engagement of secondary immune functions is considered beneficial for antitumor activity, the lack of binding to effector cells could conceivably lead to higher tumor localization. Another advantage of IgG4 is that it has the longest circulating half-life compared to the other isotypes.

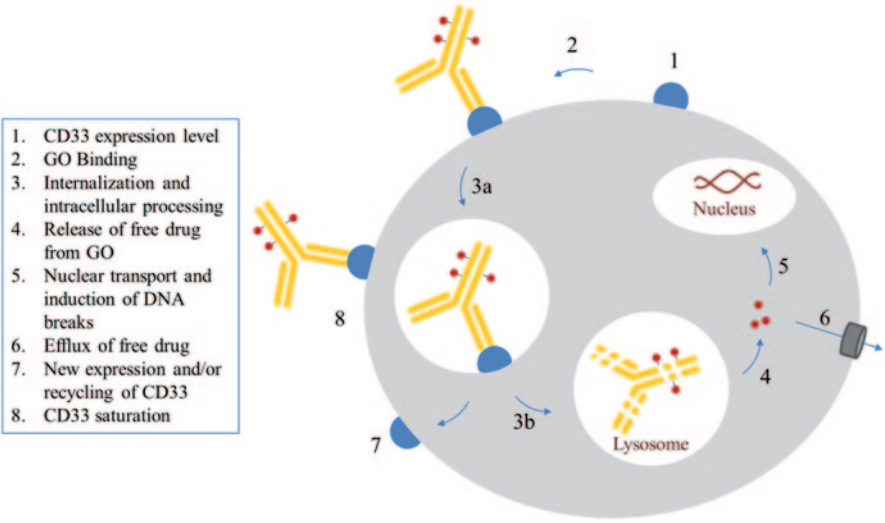

Fig. 10.2 Important criteria for the activity of GO. The activity of GO at the cellular level depends on several factors including the expression level of the antigen at the cell surface of target cells (*1*), the binding (*2*), internalization (*3a*), and intracellular processing (*3b*) of GO to the acidic lysosomal environment, where the hydrazone linkage is cleaved. Once freed from the antibody (*4*), the drug can enter the nucleus (*5*), where it then induces DNA breaks. The drug is also susceptible to efflux via MDR proteins (*6*). The CD33 antigens are continuously renewed at the cell surface (*7*). Following infusion with GO, the CD33 antigens are rapidly saturated (*8*), which also affects activity. *GO* gemtuzumab ozogamicin, *MDR* multidrug resistance

10.1.2 The Target Antigen: CD33

The antigen target, CD33, is a 67-kDa transmembrane protein belonging to the sialic acid-binding immunoglobulin-like lectin (Siglec) family. As part of the hematopoietic cascade, the CD33-negative pluripotent stem cells (PSCs) differentiate into CD33-positive myeloid cells. The antigen is found on normal multipotent myeloid precursors, unipotent colony-forming cells, maturing granulocytes, and monocytes, and is not expressed on cells outside the hematopoietic system or on pluripotent hematopoietic stem cells (Andrews et al. 1989; Robertson et al. 1992). There are several advantages in targeting the population of cells expressing CD33. AML has been shown to derive predominantly from malignant CD33-positive myeloid cells, and therefore a large majority of patients (>80%) express CD33 antigen on leukemic cells (Dinndorf et al. 1986; Griffin et al. 1984; Peiper et al. 1988). Another advantage of the CD33-target is that any normal CD33-positive cells that are destroyed by the treatment can be regenerated by differentiation of the CD33-negative PSCs. CD33 antigens are also continuously renewed on the cell surface of myeloid cells (van Der Velden et al. 2001), thereby replenishing the population of available binding sites for GO. Finally, studies have suggested that CD33 stimulation is inhibitory. Binding and phosphorylation of the transmembrane protein leads to suppression of signals generated by receptor systems containing the immunoreceptor tyrosine-

based activation motif (Paul et al. 2000). Therefore, gemtuzumab binding may also exert antileukemia effects on its own, however, this has not been confirmed.

Disadvantages of the CD33 antigen target include its relatively low expression levels and slow internalization following antibody binding. Quantitative flow cytometry studies have estimated that leukemic cells express a relatively low number of CD33 molecules (approximately 10^4 CD33 molecules/cell; Jilani et al. 2002; Scheinberg et al. 1989). Consequently, the CD33 antigenic sites are saturated after treatment with GO. As shown by van Der Velden et al. (2001), the CD33 sites were saturated within 3 h after the start of a 2-h infusion of 9 mg/m^2 of Mylotarg®. Following binding to bivalent antibodies, the CD33-antibody complex internalizes, but the rate is relatively slow compared to other antigens including the transferrin receptor (van der Jagt et al. 1992; Audran et al. 1995). Following internalization in myeloid blast cells and monocytes, results have shown that the complex is routed to the lysosomes where it is proteolytically degraded (van der Jagt et al. 1992; Press et al. 1996; Caron et al. 1992; van Der Velden et al. 2001). Taken together, the low receptor number, combined with the slow internalization, limits the amount of drug that can be delivered by the ADC. Therefore, it is crucial that a highly potent drug is utilized.

10.1.3 The Drug: Calicheamicin

Calicheamicin is an antitumor antibiotic that binds to the minor groove of DNA and causes cleavage in a sequence-dependent manner. The DNA damage causes cell cycle arrest followed by cell death via apoptosis predominantly via the mitochondrial pathway involving the release of cytochrome c and involvement of Bcl-2 proteins and caspase activation (Walter et al. 2012; Linenberger 2005). Studies have shown that treatment of CD33-expressing cells with GO results in induction of apoptosis (Naito et al. 2000; van Der Velden et al. 2001) within 72–96 h of culturing cells. Due to its high potency and delayed toxicity in animal models, this drug has not been utilized therapeutically on its own as a single agent therapy.

10.1.4 The Conjugation Chemistry and Hydrazone Linker

In GO, a semisynthetic derivative of calicheamicin, *N*-acetyl-γ-calicheamicin 1,2-dimethyl hydrazine, is utilized. For conjugation, the lysine residues of the antibody are first activated to the amine-reactive *N*-hydroxy succinimide (NHS)-ester. The cytotoxic drug is then conjugated to the NHS-antibody by covalent linkage of a bifunctional linker, 4-(4-acetylphenoxy)butanoic acid (AcBut linker), resulting in an acid-labile hydrazone linkage. The average drug–antibody ratio (DAR) of Mylotarg® is ~4 mol/mol, and approximately 50% of the antibody is unconjugated. Following binding to CD33 and receptor-mediated endocytosis, the ADC is presumably exposed to an acid lysosomal compartment, where the calicheamicin derivative is hydrolytically released intracellularly (Ricart 2011).

10.1.5 Approval Process, Post-marketing Analysis, and Withdrawal

As part of the FDA-accelerated approval process, Mylotarg® was the first ADC to be approved in the USA in 2000. The approval of Mylotarg® was based on three open-label, multicenter single-arm phase II clinical trials that enrolled 142 patients at >60 years old, where the clinical data showed an overall response rate of 26% (complete remission, CR, of 13% and CR with incomplete platelet count recovery of 13%; Sievers et al. 2001; Bross et al. 2001). The patients typically received two 9 mg/m^2 doses of GO over 14 days apart, which was shown in earlier phase 1 studies to saturate CD33 binding sites without dose-limiting non-hematologic toxicity (Sievers et al. 1999). It is important to point out that half of the CRs were in complete response with incomplete platelet recovery (13%), supporting the activity of the drug, but questioning the effect on survival rate. A subsequent confirmatory Phase III trial (S0106) by the Southwest Oncology Group (SWOG) failed to demonstrate a clinical benefit compared to standard chemotherapy, and also showed an increased mortality in the group of patients who received the ADC (Petersdorf et al. 2013).

This SWOG0106 study has received several critiques regarding the results obtained and the design of the experimental groups. First, the results showed a significantly worse mortality in GO (6 mg/m^2) than control (5.8 versus 0.8%). However, the induction death rate obtained with GO treatment is within the normal range (5–7%) usually found in the younger patients age group represented in the trial. Also, the SWOG trial compared a daily dose of 60-45 mg/m^2 daunorubicin + GO. Studies have shown that, in younger patients, a high-dose daunorubicin (90 mg/m^2) improves overall survival and complete remission compared to a low dose (45 mg/m^2). Therefore, the data could be interpreted as a compensation by GO for the reduction of the daunorubicin dose in the GO treatment group. Nevertheless, based on this study, Mylotarg® was voluntarily withdrawn from the US market in June 2010. Since the SWOG study, there have been several randomized trial reports that suggest Mylotarg® may improve clinical outcomes, indicating that its clinical use should be revisited.

10.2 Recent Clinical Studies

Following withdrawal of Mylotarg® in 2010, several clinical studies that have been reported or are currently underway (Table 10.1). The main difference in the newer studies is the lower dose of GO administered to the patients. As previously mentioned, new CD33 binding sites are continuously renewed at the cell surface, with CD33 levels returning to pretreatment levels within 72 h after GO administration (van Der Velden et al. 2001; Caron et al. 1994). Therefore, these data indicate that the intracellular drug accumulation may be enhanced by the administration of lower doses of GO every 3 days. The more recent clinical trials, summarized below, indicate that a lower dose with more frequent administration may improve the efficacy and toxicity profile of GO.

Table 10.1 Summary of clinical trial results for GO. (Source: Petersdorf et al. 2013; Kirby 2012; Aplenc et al. 2013; Fernandez et al. 2011; Lowenberg et al. 2010; Amadori et al. 2013; Burnett et al. 2012)

Study	Number of Patients	Age (years)	Description	GO dose (mg/m^2)	Improved CR with GO	Improved RFS, EFS, DFS or OS with GO	Increased mortality rate	Increased hepatic toxicity
Completed								
NCT00085709 (SWOG 0106)	637	18–60	DA/C+GO versus DA/C	6	No	No	No	No
NCT00927498 (ALFA 0701)	280	50–70	DA/C+GO versus DA/C	3		Yes (in favorable/intermediate group)	No	No
NCT00372593 (AAML0531)	1022	<30	DA/C+GO versus DA/C	3	No	Yes: improved EFS, DFS, RFS	n/a	n/a
NCT00049517 (E1900)	657	17–60	DA/C+GO versus DA/C	6	No	No	n/a	n/a
NCT00121303 (HOVON-43)	814	>60	DA/C+GO versus DA/C	6	No	No	No	Yes
NCT00052299 (EORTC-06012, AML17)	472	61–75	GO+MICE for remission induction followed by GO+mini-ICE for consolidation	6	No	No	Yes	No (slightly higher)
NCT00454480 (AML16)	1115	51–84	GO+Chemo	3	No	Yes (in favorable/intermediate group)	No	No

Table 10.1 Summary of clinical trial results for GO. (Source: Petersdorf et al. 2013; Kirby 2012; Aplenc et al. 2013; Fernandez et al. 2011; Lowenberg et al. 2010; Amadori et al. 2013; Burnett et al. 2012)

Study	Number of Patients	Age (years)	Description	GO dose (mg/m^2)	Improved CR with GO	Improved RFS, EFS, DFS or OS with GO	Increased mortality rate	Increased hepatic toxicity
NCT00962767 (0903X-101128)	168	18–61	GO versus ATRA plus 6-MP and MTX	2 × 6	n/a (completed Dec 2007)			
Ongoing								
NCT00860639	327	18–60	DA/C + GO versus DA/C	6	n/a (PCD Oct 2016)			
NCT00893399 (AMLSG 09-09)	588 (est)	≥18	IEC/ATRA + GO versus IEC/ATRA	3	n/a (PCD Jan 2020)			

CR complete remission, *DA/C*: daunorubicin/cytarabine, *DFS* disease-free survival, *EFS* event-free survival, *IEC/ATRA* idarubicin, etoposide, cytarabine/all-trans retinoic acid, *MICE* mitoxantrone; idarubicin; cytarabine; etoposide, *MTX* methotrexate, *n/a* not available, *OS* overall survival, *PCD* primary completion date, *RFS* relapse-free survival, *6-MP*-6-mercaptopurine

10.2.1 AML15

The phase II AML15 trial included 1113 patients who were predominantly <60 years old. These patients received two induction courses with and without a single dose of GO (3 mg/m^2). The results showed that in a predefined subgroup of patients with favorable cytogenetics, there was an improved OS at 5 years (79 versus 70%), but no benefit for patients with unfavorable cytogenetics (Burnett et al. 2011).

10.2.2 AML16 Trial

The AML16 phase III trial from the UK Medical Research Council, AML patients (51–84 years, $n=1115$) were randomized to receive different chemotherapy regiments, with and without a 3-mg/m^2 dose of GO on day one. The lower dose was established in a pilot study in younger patients (Burnett et al. 2006) as well as the AML15 trial described above (Burnett et al. 2011). Although patients receiving GO induction showed no overall difference in rate of relapse, OS or RFS, the 3-year cumulative incidence of relapse was significantly lower with GO (68 vs. 76%, $p=0.007$), and the 3-year survival was significantly better (25 vs. 20%, $p=0.05$). Additionally, patients with favorable cytogenetics whose leukemia was CD33$^+$ showed an overall survival benefit (OS 79 vs. 51%). There was no difference in 30- or 6-day mortalities and no major increase in toxicity with GO treatment (Burnett et al. 2012).

10.2.3 ALFA0701 Trial

In the Acute Leukemia French Association trial, ALFA 0701, fractionated doses of GO (3 mg/m^2 on days 1, 4, and 7) were administered to older AML patients (age 50–70 years, $n=280$). In this study, both EFS (17.1 versus 40.8%, $p=0.0003$) and OS (53.2 versus 41.9%, $p=0.0368$) were improved at 2 years in the GO group. Subgroup analysis showed that the improvements were predominantly in those patients with cytogenetically favorable or intermediate-risk disease (Renneville et al. 2014). Hematological toxicity was found to be significantly higher in the GO treated versus the control group (16 versus 3%, $p<0.0001$); however, there was no increase in the risk of death from toxicity (Kirby 2012).

10.2.4 AML 2006 Trial

In the study by the *Groupe Ouest Est d'Etude des Leucémies et Autres Maladies du Sange*, AML 2006 IR, patients with an intermediate-risk karyotype (age 18–60 years) were randomized to receive 6 mg/m^2 with both induction and consolidation chemotherapy. The patients in the GO showed an improved 3-year EFS (53.7 vs. 27%, $p=0.0308$), but there was no improvement in OS (Casasnovas et al. 2011).

10.2.5 AML17 Trial

In order to investigate the feasibility of a sequential induction strategy, a phase II trial was first carried out by the European Organization for Research and Treatment of Cancer (EORTC) and Gruppo Italiano Malittie Ematologiche dell'Adulto (GIMEMA) consortium. In this trial, 9 mg/m^2 of GO (days 1 and 15) was administered in older patients with a previously untreated AML, followed by standard chemotherapy. The trial resulted in approximately 70 % of patients able to complete the induction sequence and an overall response rate of 54 %. However, the induction mortality was high (14 % of patients dying as a result of toxicity), and therefore a lower dose was considered for the subsequent trial.

In the follow-up phase III trial, patients received a course of standard chemotherapy with or without a preceding course of a lower 6 mg/m^2 dose of GO (days 1 and 15). The results of the trial ($n = 472$ patients age 61–75) showed that the overall response rate was comparable between the two arms (with GO, 45 %; without GO, 49 %). However, the induction and 60-day mortality rates, as well as hematologic and liver toxicities, were higher in the GO-treated patients. Therefore, the trial failed to demonstrate an advantage of GO before standard chemotherapy, and also showed a higher risk of early mortality in the older age subgroups > 70 years (Amadori et al. 2013).

10.3 Summary and Prospective Future for GO

Overall, the response to the voluntary withdrawal of GO has been mixed. When comparing the five randomized trials, they all found that newly diagnosed AML patients with a favorable cytogenetic risk profile were likely to benefit from GO treatment. Furthermore, those with an intermediate-risk AML had the same benefit in four out of the five trials. Cytogenetic analysis has allowed for the identification of several gene mutations that have a prognostic significance in comparison to cytogenetically normal (CN) AML. Identification of these mutations has allowed a prognostic classification of CN-AML and improved the risk stratification in this subset of patients. Furthermore, results have shown a great benefit of GO in treatment of acute promyelocytic leukemia (APL), likely due to the higher surface expression of CD33. GO has been shown to result in durable molecular remissions in patients with relapsed APL (Lo-Coco et al. 2004) and can effectively replace anthracyclines in the management of APL (Breccia et al. 2007; Ravandi et al. 2009). Therefore, in line with moving toward more personalized medicine, it is clear that there are certain well-defined molecular subgroups that could benefit from GO treatment.

The optimal timing and dose of GO administration is another area that needs further consideration. The AML17 and ALFA0701 trials showed that the coadministration of low doses of GO with induction therapy may improve the outcome and be a safer alternative to sequential administration of higher doses. Therefore, it may be better to use lower, concomitant doses rather than the higher sequential doses used in several of the trials.

Taken together, the recent data supports the efficacy of GO when used at a dose range of 3–6 mg/m^2 in APL, and in newly diagnosed AML with favorable cytogenetics. Unfortunately, these data were not available when GO was withdrawn from the market. Since AML is a highly heterogeneous disease, basing the potential clinical benefit on average patient data may not fully reflect the true therapeutic potential of the drug. Therefore, GO may be an active anti-AML with an acceptable toxicity profile when using a more optimal dose in a subpatient population with favorable to intermediate cytogenetic risk profiles.

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Chapter 11
ADCETRIS: A Regulatory Case Study of a New Generation Antibody-Drug Conjugate

Bruce W. Hart

11.1 ADCETRIS Description

The ADCETRIS antibody–drug conjugate (ADC) consists of the antibody chimeric monoclonal antibody AC10 (cAC10, brentuximab) and the small-molecule microtubule inhibitor monomethyl auristatin E (MMAE) attached to the antibody via a protease cleavable linker (Fig. 11.1).

The cAC10 antibody is targeted specifically to CD30, an antigen with limited expression on normal human tissues but preferential expression on select hematologic malignancies including Hodgkin lymphoma (HL), anaplastic large cell lymphoma (ALCL), and some other non-Hodgkin lymphomas. Nonclinical data suggest that the anticancer activity of brentuximab vedotin is due to the binding of the ADC to CD30-expressing cells, followed by internalization of the ADC–CD30 complex, and the release of MMAE via proteolytic cleavage. Binding of MMAE to tubulin disrupts the microtubule network within the cell, subsequently inducing cell cycle arrest and apoptotic death of the cells. Brentuximab vedotin embodies several important ADC design principles including an internalizing antigen that is preferentially expressed on tumor cells rather than on normal tissues, a potent cytotoxic component that is rapidly released inside the tumor cell, and a stable linker that limits systemic release of the cytotoxic.

11.2 SGN-30

Prior to the clinical development of ADCETRIS, the naked antibody cAC10, also known as SGN-30, entered clinical development in 2003 and was studied in two phase 1 trials and two phase 2 trials. SGN-30, administered as weekly doses as

B. W. Hart (✉)
Regulatory Affairs, Seattle Genetics, Bothell, WA, USA
e-mail: bhart@seagen.com

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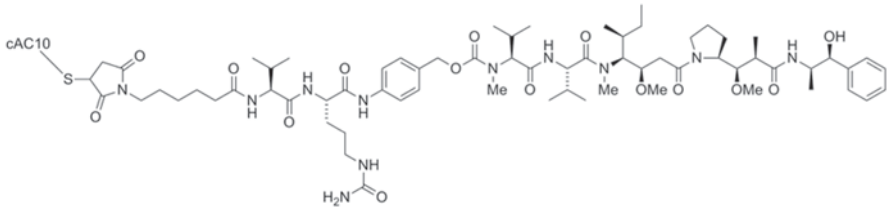

Fig. 11.1 Structure of brentuximab vedotin

high as 12 mg/kg, exhibited antitumor activity in systemic ALCL (sALCL) and CD30+cutaneous T cell lymphoma (CTCL). In phase 2 trials, the overall response rate (ORR) in ALCL patients was 17% and in CTCL patients was 70% (Forero-Torres et al. 2009; Duvic et al. 2009). However, the activity of SGN-30 in HL (0% ORR) did not warrant further development and therefore a corporate decision was made to discontinue the program.

11.3 ADCETRIS Regulatory Timeline

The major regulatory interactions during the development of ADCETRIS listed in chronological order are listed in Table 11.1. All of the regulatory information contained in this chapter is publically available on the Food and Drug Administration (FDA) website at drugs@fda.com and http://www.fda.gov/AdvisoryCommittees/default.htm.

11.4 Early Days: Phase 1 and the Food and Drug Administration

SGN-35 began its US regulatory life cycle in June 2006 with the submission of an investigational new drug (IND) application to the Division of Drug Oncology Products in the Office of Oncology Drug Products. The first phase 1 trial of SGN-35, as it was designated then, was designed to test the safety and antitumor activity of a dose of drug administered once every 3 weeks (q3w). Enrollment began in November 2006 and the last patient completed the study in July 2009. Forty-five patients were enrolled and received study drug. The ORR in this trial was 36% and was dose-level dependent. SGN-35 was also generally well tolerated (Younes et al. 2010).

A second phase 1 trial was submitted to the IND in September 2007. This trial was designed to explore the safety and antitumor activity of a once weekly dose schedule of SGN-35 on a 28-day cycle (3 weeks on, 1 week off). Enrollment began

Table 11.1 Major regulatory interactions

IND submission	June 2006
Orphan drug designation for HL	January 2007
End-of-phase-1 meeting with FDA	July 2008
Orphan drug designation for ALCL	October 2008
Special protocol assessment granted for HP pivotal trial	January 2009
Fast-track designation for HL	March 2009
Nonclinical/clinical pharmacology meeting with FDA	March 2009
End of phase 2 CMC meeting with FDA	January 2010
Clinical pre-BLA meeting with FDA	November 2010
CMC pre-BLA meeting with FDA	December 2010
BLA submission	February 2011
Oncologic Drugs Advisory Committee meeting	July 2011
Confirmatory trial meeting with FDA	July 2011
BLA approval by FDA	August 2011

IND investigational new drug, *HL* Hodgkin lymphoma, *FDA* Food and Drug Administration, *ALCL* anaplastic large cell lymphoma, *CMC* chemistry, manufacturing, and controls, *BLA* Biologics License Application

in March 2008 and the last patient completed the study in February 2010. Forty-four patients were enrolled and received the study drug. The ORR in this trial was 53 % and SGN–35 was generally well tolerated (Fanale et al. 2012).

Based on the compelling early tumor response data, Seattle Genetics requested a Type B end-of-phase-1 (EOP1) meeting with the FDA. This meeting occurred in July 2008 and during the meeting, the design of two single-arm pivotal trials was discussed. For HL, the FDA agreed that patients who had progressive or relapsed HL following autologous stem cell transplant (ASCT) were an unmet need. When asked if a single-arm trial with ORR as the primary endpoint would be acceptable for accelerated approval in this patient population, the FDA responded that a controlled randomized trial is preferred but a single-arm trial may be acceptable provided there is sufficient evidence of efficacy, i.e., high response rate with prolonged duration and with acceptable safety. For sALCL, FDA agreed that patients who had progressive or relapsed sALCL following at least one prior therapy were an unmet need. When asked if a single-arm trial with ORR as the primary endpoint would be acceptable for accelerated approval in this patient population, the FDA responded that the rarity of a disease does not necessarily preclude one from performing a randomized trial but a single-arm phase 2 study may support accelerated approval depending on the response rate, including proportion of complete responses (CRs), duration of response (DOR), and the risk–benefit ratio. A sufficiently large CR rate and duration might support full approval. Seattle Genetics also asked for FDA feedback on a proposed phase 3 trial in patients at high risk of progression following ASCT that was

to act as a confirmatory trial in the case of an accelerated approval. The FDA had several questions regarding the patient population and the endpoints and no agreements were gained at the meeting. The FDA did agree that 1.8 mg/kg of SGN-35 every 3 weeks was appropriate for future single-agent development and that a safety database of at least 175 patients would be sufficient for registrational purposes in the intended indications. The FDA reminded Seattle Genetics of pre-IND comments regarding the clinical pharmacology plan and specifically pointed out that the potential for QT/QTc interval prolongation would need to be addressed and requested the submission of an electrocardiography (ECG) evaluation plan for review.

11.5 Pivotal Trial Regulatory Agreements

During the EOP1 meeting, the FDA requested that the proposed pivotal trials be submitted for special protocol assessment (SPA). Seattle Genetics submitted a request for SPA for the single-arm phase 2 pivotal trial for relapsed/refractory HL. In January 2009, the FDA granted a SPA for the HL protocol, agreeing to trial design aspects including the patient population (patients who had relapsed after or were refractory to an ASCT), the number of patients (100), and the primary endpoint (ORR).

HL and ALCL are both rare diseases and orphan drug designation was granted for HL in January 2007 and for ALCL in October 2008. Based on the potential for ADCETRIS to treat a serious condition and to fill an unmet medical need, fast-track status was granted for HL in March 2009.

11.6 Nonclinical and Clinical Pharmacology Development

The nonclinical and clinical pharmacology development plans were discussed with the FDA in a teleconference held in March 2009. The FDA agreed with the proposed nonclinical plan including completed rat and monkey toxicology and safety pharmacology studies and in vitro metabolism and transport studies and an as yet-to-be-conducted panel of genotoxicity studies of MMAE, a single chronic toxicology study of the ADC and MMAE in cynomolgus monkeys, and a single reproductive toxicology study of MMAE in rats. The FDA confirmed that no carcinogenicity study was required.

The FDA disagreed with Seattle Genetics proposal to not conduct several human clinical pharmacology studies. The FDA did not agree to a proposal to conduct a mass balance study of SGN-35 in cynomolgus monkeys and to extrapolate the results to cancer patients. Seattle Genetics then proposed to collect MMAE and metabolites in urine and feces of healthy subjects. The FDA responded that the plan may be acceptable provided the agency concurred with the dose and the results of genetic toxicology and safety pharmacology studies.

The FDA stated that drug–drug interaction (DDI) studies of SGN-35 with a strong cytochrome P450 3A4 (CYP3A4) inhibitor, a strong CYP3A4 inducer, and a sensitive CYP3A4 substrate should be conducted during drug development and be included in the new drug application (NDA) to provide important safety information. Seattle Genetics queried whether a study in healthy subjects can be conducted. FDA responded that results of the genetic toxicology and safety pharmacology studies need to be submitted for review and FDA concurrence prior to initiating the studies.

The FDA stated that special population (renal or hepatic impairment) studies should be conducted during drug development and be included in the NDA when it is submitted to provide important safety information on the use of your drug. If a population pharmacokinetics (PK) approach was used, the FDA recommended a sufficient number of patients with a wide range of hepatic and renal function be enrolled in the studies and enough samples collected to characterize the PK.

Seattle Genetics proposed to perform intensive ECG monitoring in a subset of patients treated with SGN-35 as a single agent to address the potential for QT/QTc prolongation. The FDA responded that the proposed ECG sub-study was adequate to characterize large effects on the QT interval due to SGN-35 but noted there would be significant confounding due to comorbidities and prior therapies. The FDA agreed that if there were no QTc signal in this assessment, no further QT studies would be required. Seattle Genetics stated that to enroll 24 subjects from the pivotal trial will be challenging as this study would enroll only 100 patients in total and enrollment had already commenced. There were three potential alternatives proposed to FDA in order to obtain the necessary number of patients for the QT study (1) enroll patients from other single-agent studies where SGN-35 is given at the same dose and schedule as on the pivotal study, (2) include patients from a retreatment protocol, and (3) include patients who have already received one or more doses of SGN-35. All three scenarios were considered potentially acceptable to the FDA.

Prior to submission of the marketing application, a change in designation occurred that changed the submission to a Biologics Licensing Application (BLA) (see 11.10). For the BLA submission, Seattle Genetics conducted two dedicated clinical pharmacology studies and submitted the data in support of prescribing information language. SGN35-007 was designed to characterize the potential for ADCETRIS to prolong the QT interval. Administration of brentuximab vedotin to 46 evaluable patients did not prolong the mean QTc interval > 10 ms from baseline. SGN35-008 was designed to characterized MMAE excretion, DDI, and the effects of renal or hepatic impairment (special populations). Coadministration of ADCETRIS with a potent CYP3A4 inhibitor increased exposure to MMAE by approximately 34 % and coadministration of ADCETRIS with a potent CYP3A4 inducer reduced exposure to MMAE by approximately 46 %. The final US prescribing information (USPI) included language advising that patients who are receiving strong CYP3A4 inhibitors concomitantly with ADCETRIS should be closely monitored for adverse reactions. Coadministration of ADCETRIS did not affect exposure to a CYP3A4 substrate and ADCETRIS is not expected to alter the exposure to drugs that are metabolized by CYP3A4 enzymes.

11.7 Phase 2 Registrational Trials and AETHERA

The phase 2 HL trial (SGN35-003) enrolled its first patient in February 2009. All patients were required to have relapsed or refractory disease following an ASCT and the target enrollment was 100 patients. The phase 2 sALCL trial (SGN35-004) enrolled its first patient in June 2009. All patients were required to have relapsed or refractory sALCL following at least one prior therapy and the target enrollment was 50 patients.

A request for SPA was submitted for the AETHERA phase 3 confirmatory trial in April 2009. After review, the FDA did not agree to the SPA. Seattle Genetics requested a Type A meeting to discuss the areas of disagreement with the FDA. This meeting occurred in October 2009. At the center of the disagreement were the two issues identified at the EOP1 meeting, namely the patient population to be enrolled and the appropriate primary endpoint. The FDA did not agree that even with proposed modifications to the protocol that progression-free survival (PFS) was an appropriate primary endpoint for the trial. They noted that the clinical study as defined appeared to be enrolling a heterogeneous population with up to 40 % of patients being in partial response (PR)/CRu (CR unconfirmed) and 60 % in CR. The FDA recommended that the trial focus on a single population. PFS could not be recommended as an endpoint in a trial with patients who are already in CR because the study would be a maintenance trial in patients who may not need additional therapy and overall survival (OS) was considered to be the appropriate endpoint for those patients. Additionally, the FDA questioned whether achieving stable disease for those patients who are in PR/CRu would represent a clinical benefit. The agency recommended that the sponsor carefully write the exclusion/inclusion criteria to avoid enrollment of patients who are less than a PR and if so, the proposal to use PFS as the primary endpoint and OS as a key secondary endpoint might be acceptable.

11.8 Phase 2 Clinical Data

The phase 2 HL trial last patient visit was in August 2010. One hundred two patients were enrolled and received the study drug. The median age was 31 years with a range of 15–77. Forty-seven percent of patients were male. Patients had received a median of 3.5 prior therapies (range 1–13). The results of the trial described significant antitumor activity and a generally well-tolerated safety profile. Brentuximab vedotin demonstrated an ORR of 73 % and a CR rate of 32 %. The median DOR was 6.7 months. The most common adverse reactions ($\geq$20 %), regardless of causality, were neutropenia, peripheral sensory neuropathy, fatigue, upper respiratory tract infection, nausea, diarrhea, anemia, pyrexia, thrombocytopenia, rash, abdominal pain, cough, and vomiting (Younes et al. 2012).

The phase 2 sALCL trial last patient visit was in June 2011. Fifty-eight patients were enrolled and received the study drug. The median age was 52 years with a range of 14–76. Fifty-seven percent of patients were male. Seventy-two percent of patients had anaplastic lymphoma kinase (ALK)-negative disease. Patients had received a median of two prior therapies (range 1–6) and 26% had received a prior ASCT. The results of the trial described significant antitumor activity and a generally well-tolerated safety profile. Brentuximab vedotin demonstrated an ORR of 86% and a CR rate of 57%. The median DOR was 12.6 months. The most common adverse reactions (≥20%), regardless of causality, were neutropenia, anemia, peripheral sensory neuropathy, fatigue, nausea, pyrexia, rash, diarrhea, and pain (Pro et al. 2012).

11.9 Chemistry, Manufacturing, and Controls Plan

Seattle Genetics had two major discussions with the FDA regarding the chemistry, manufacturing, and controls (CMC) for a brentuximab vedotin marketing authorization application. The first was an EOP2 meeting that occurred in January 2010. At that time, the FDA agreed in general with the proposed control strategy for the GMP intermediate SGD-1006, including raw materials, in-process controls, and release testing was acceptable for commercial manufacture. However, they did not agree with the designation of only three starting materials and proposed that there were actually six potential starting materials. These included three custom-synthesized starting materials and three commercially available starting materials. The FDA stated that these three additional compounds significantly contribute to the structure of the drug substance (DS) and might be considered as more appropriate starting materials. For the commercially available starting materials, the FDA requested at least two methods of identification, assay, impurity profile, chiral purity, diastereomer content (if applicable), acceptance criteria, test methods, and certificates of analysis from the supplier and the applicant. For the custom-synthesized starting materials, the FDA requested a brief description of synthesis, and literature reference or reference to a DMF with purging studies to show the removal of carryover impurities in the downstream process, plus all of the attributes mentioned for the commercially available starting materials. Seattle Genetics stated that they would consider this proposal. The FDA agreed that the proposed control strategy for cAC10, brentuximab bulk DS and drug product (DP), including raw materials, in-process controls, release, and stability testing were acceptable for commercial manufacture and the characterization plans for cAC10, bulk DS, and DP were adequate to support filing of a NDA for brentuximab vedotin.

The FDA agreed with the proposed structure of module 3 for the planned NDA submission, which included cAC10 and SGD-1006 being described in separate DS sections as intermediates in the manufacture of brentuximab vedotin BDS. They added that the section describing cAC10 should essentially be a stand-alone unit, containing all of the information that would be expected for a BLA for a monoclonal antibody.

That meeting was followed nearly a year later by a pre-BLA meeting in December 2010. The key topics of discussion for that meeting were the establishment of release specifications and the adequacy of the justification of the specifications. The FDA stated that the proposed plan appeared reasonable and added that using tolerance intervals (TI) to justify specifications is acceptable. However, as the number of lots manufactured increases, the TI should decrease and the sponsor should continue to analyze lots and reevaluate specifications as additional lots of cACl0 and SGN-35 DS and DP are manufactured. Seattle Genetics stated that they planned to reevaluate specifications as additional lots of cAC10 and SGN-35 DS and DP were manufactured. The FDA also agreed that proposed designations and controls for the three previously identified additional starting materials appear to be acceptable to support the registration. The FDA agreed that submission of a simple stability update for DP within the planned timeframe would not extend the Prescription Drug User Fee Act (PDUFA) review date of the application.

11.10 After Phase 2 and Onward to the Biologics License Application

A clinical pre-NDA meeting was held with the FDA in November 2010. A key discussion topic was whether the submission would qualify for regular or accelerated approval. The FDA responded that response rate in a single-arm trial is generally not adequate for regular approval and whether a high response rate with an acceptable duration will be considered a surrogate for clinical benefit (and acceptable for accelerated approval) will be a review issue. The FDA continued by stating that regular approval of an oncologic new molecular entity (NME) usually requires two adequate and well-controlled clinical trials establishing that the NME provides clinical benefit and has an acceptable benefit to risk ratio. Accelerated approval, covered under Subpart E 21 Code of Federal Regulations (CFR) 601.40–46, may be applicable to brentuximab vedotin and would take into account magnitude and durability of response, and require a confirmatory trial for regular approval. The FDA stated that the confirmatory study for conversion to regular approval does not necessarily have to be in the exact indication that holds accelerated approval. FDA reiterated that PFS is not an acceptable efficacy endpoint in a single-arm trial and whether the PFS analysis is utilized for regulatory action will be a review issue. During the meeting, the FDA indicated that the application would likely go to the Oncologic Drugs Advisory Committee (ODAC) which would be scheduled after the submission. Specific for the sALCL submission, the FDA agreed that a single update of response duration could be submitted early in the BLA review without triggering an action date extension.

Subsequent to the pre-NDA meeting, Seattle Genetics was informed that the marketing application would be reviewed as a BLA rather than an NDA. An important outcome of this change in designation is the term of data exclusivity which is 12 years for BLAs and 7 years for NDAs.

11.11 A Changing Landscape

On February 8, 2011, the FDA held a meeting of ODAC to discuss requirements and expectations for the accelerated approval regulatory pathway. Overall, members agreed that randomized controlled trials should be the standard and that single-arm trials should be the exception except for rare diseases and agents with a high level of activity or pronounced treatment effect. Overall, members also agreed that at least two controlled trials should be needed for accelerated approval commitments, with the caveat that in rare diseases and pediatrics this may not be feasible. Overall, members felt that a well-designed development plan is needed prior to the application being filed. Most also preferred that the sponsor have confirmatory studies already ongoing at the time of application.

11.12 The Biologics Licensing Application

The ADCETRIS BLA was submitted on February 28, 2011. The application requested priority review based on the potential for ADCETRIS to be a significant improvement in the safety and effectiveness of the treatment of HL and sALCL. The application also requested a waiver from the PDUFA fee and pediatric data requirements based on the designation of ADCETRIS as an orphan drug for both indications. The BLA was administratively split into two by the FDA to allow for separate regulatory actions if needed. Reponses to requests for information were submitted to both BLAs during the review. The two BLA applications were officially filed on April 29 and priority review was granted with an action date (PDUFA date) of August 30, 2011.

11.13 Meeting with the Committee

ADCETRIS was the subject of two sessions at ODAC on July 14. The morning session involved the HL indication and followed a standard advisory committee agenda with the sponsor presenting first and the FDA presenting after. This was followed by a question and answer period, public hearing, discussion, and finally the vote. The vote for accelerated approval for HL was 10 for and 0 against. The vote was followed by a discussion about the AETHERA trial as a confirmatory study to convert the accelerated approval to regular approval. There was general discomfort by the advisory committee members with the design of the AETHERA trial as a confirmatory trial. The afternoon session involved the sALCL indication and again followed the standard agenda. The vote for accelerated approval for sALCL was 10 for and 0 against. The vote was followed again by a discussion about confirmatory trials required to convert the accelerated approval to regular approval.

11.14 Confirmatory Trials

On July 21, a face-to-face meeting was held between Seattle Genetics and the FDA to discuss the design of potential confirmatory trials. In addition to the previously discussed AETHERA trial, two other designs were discussed including a phase 3 trial of ADCETRIS in combination with Adriamycin, vinblastine, and dacarbazine (AVD) in newly diagnosed HL patients and a phase 3 trial of ADCETRIS in combination with cyclophosphamide, doxorubicin, and prednisone (CHP) in patients with newly diagnosed mature T cell lymphomas (MTCL). The FDA considered both of these designs to be acceptable as confirmatory trials.

11.15 Chemistry, Manufacturing and Controls Issues during the Review

During the BLA review, several teleconferences were held to discuss CMC issues that the FDA had identified. These included (1) deficiencies and harmonization of the drug master file for the drug linker, (2) the introduction of new products into contract manufacturing facilities, (3) the potential for glass lamella formation, (4) specification reassessment, and (5) criteria for determination of product quality criticality assessment. All issues were resolved or commitments made prior to the BLA approval.

11.16 Biologics Licensing Application Approval

The BLA was granted accelerated approval on August 19, 2011, for (1) treatment of patients with HL after failure of ASCT or after failure of at least two prior multi-agent chemotherapy regimens in patients who are not ASCT candidates and (2) treatment of patients with sALCL after failure of at least one prior multi-agent chemotherapy regimen. While the pivotal HL trial did not include patients who were not candidates for ASCT, the BLA package included data from 21 patients who were enrolled on other trials. The final indication allows for these patients to receive ADCETRIS.

11.17 After Biologics Licensing Application Approval

As a condition of the accelerated approval, Seattle Genetics is required to meet a number of post-market requirements for additional clinical trials (confirmatory trials). The two phase 3 trials in newly diagnosed HL and MTCL patients discussed with the FDA in July were listed as post-market requirements in the approval letter for confirmation of the accelerated approval. The success of either trial will be

sufficient to convert the BLA to regular approval for both of the initial approval indications. Both of these trials are currently enrolling patients and study reports are expected in 2019. In addition, the approval letter listed several other post-approval commitments including the completion of the AETHERA clinical trial and submission of the clinical study report characterizing the severity, duration, and reversibility of treatment emergent neuropathy. Orphan drug status was conferred for both indications at the time of approval, granting Seattle Genetics 7 years of market exclusivity (superseded by the BLA exclusivity) and tax credits up to 50% for qualified clinical research costs.

Subsequent to the approval of the initial BLA, the USPI for ADCETRIS has been revised three times. In January 2012, a boxed warning for progressive multifocal leukoencephalopathy was added to the PI. In August 2013, the PI was revised to remove the 16-cycle limit from the dosing and administration section. Dosing guidance for patients with renal and hepatic impairment and revisions to warnings and precautions were added in September 2013.

11.18 Conclusion

ADCETRIS, when it was approved in August 2011, became the first ADC approved since the Mylotarg approval in 2000. The ADCETRIS development pathway involved frequent interactions with the FDA and utilization of many regulatory incentive programs. As the first of a new generation of ADCS, the ADCETRIS case will be informative for others considering the ADC development. In February 2013, Kadcyla (ado-trastuzumab emtansine) became the third approved ADC when the FDA approved it for patients with human epidermal growth factor receptor 2 (HER-2)-positive late-stage breast cancer. ADCs will continue to grow as a group of new entities within the larger class of pharmaceuticals.

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Chapter 12
Ado-Trastuzumab Emtansine

Yun Luo, Jérôme J. Lacroix and Sunil Prabhu

12.1 Introduction

Ado-trastuzumab emtansine (T-DM1) is one of only two antibody-drug conjugates (ADC) currently on the market. It was specifically developed by Roche (trade name Kadcyla) as anti-HER2 ADC for metastatic breast cancer (MBC) patients and also shows promising application against gastric cancers (Barok et al. 2011).

HER2 is a cell-surface receptor member of the epidermal growth factor receptor (EGFR) family which contains four members (HER1–4) and is normally expressed at low levels in epithelial tissues where it plays an important role by promoting normal cell proliferation and differentiation. However, amplification of the *HER2* gene and/or overexpression of the HER2 receptor are linked to the development of many human cancers in breast, ovarian, and gastrointestinal tissues and correlates with poor prognosis. This is due to the fact that, unlike HER1–3, the presence of HER2 at the cell surface is required to enhance the strength of the EGFR signaling. Hence, an estimated 15~30 % of human breast cancers are linked to HER2 amplification/overexpression (Mitri et al. 2012).

The accessibility of the extracellular portion of the receptor has made HER2 a potential target for antibody therapy. Binding of the anti-HER2 humanized monoclonal antibody trastuzumab, or Herceptin, to the HER2 receptor was demonstrated to significantly prevent growth of cancer cells by preventing dimerization of the receptor and by activating antibody-mediated immunity against the targeted cell. Hence, trastuzumab was approved by the Food and Drug Administration (FDA) in 1998 for promising use for the treatment of HER2-dependent MBC. However,

Y. Luo (✉) · S. Prabhu
Department of Pharmaceutical Sciences, Western University of Health Sciences, Pomona, CA, USA
e-mail: luoy@westernu.edu

J. J. Lacroix
Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA, USA

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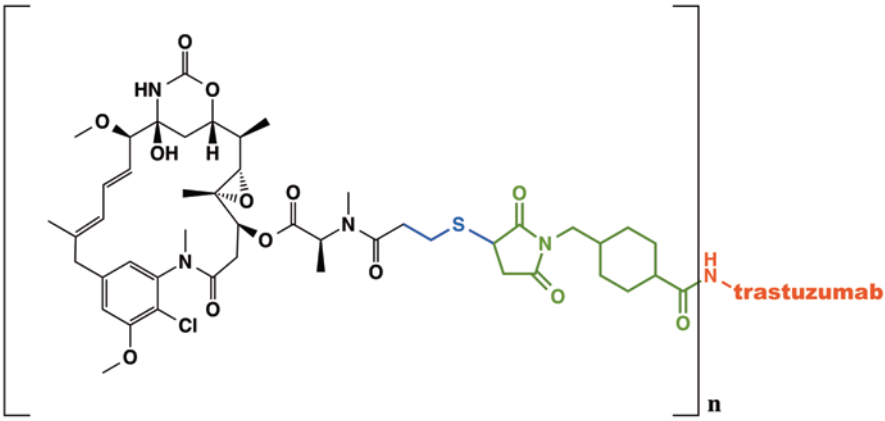

Fig. 12.1 Schematic representation of trastuzumab emtansine. The maytansine skeleton is shown on the left (*black*). The thioether group is shown in *blue,* and the linker group is shown in *green*. The linker group is bound to the amino group of a lysine residue in the trastuzumab molecule shown in *red*

despite increased survival rates, a significant proportion of patients treated with trastuzumab either did not respond initially or relapsed after a period of clinical response (Slamon et al. 2001; Vogel et al. 2001).

HER2 overexpression does not recess when cancer cells become refractory to HER2-directed therapies (Nahta et al. 2005, Ritter et al. 2007). Thus, to improve the naked antibody therapy, trastuzumab was later engineered as a vehicle to deliver antimitotic drugs to HER2-overexpressing cells. A drug of choice was mertansine (DM1), a potent antimitotic drug. DM1 is a derivative of an old cytotoxic agent maytansine that inhibits microtubule dynamics by binding to tubulins. This targeted strategy helps to minimize the systemic side effects exhibited by maytansine-based chemotherapy. Limited side effects are also diminished by the relatively high stability of the nonreducible thioether linkers between trastuzumab and DM1, which prevents nonspecific systemic release of the drug. In the EMILIA phase 3 clinical trial of women with advanced HER2-positive breast cancer who were already resistant to trastuzumab alone, it improved progression-free survival (PFS) alone with overall survival (OS) and safety compared to the combination of the chemotherapeutic agents lapatinib (EGFR inhibitor) and capecitabine (DNA synthesis inhibitor; Verma et al. 2012). Based on that trial, the US FDA approved marketing on February 22, 2013.

12.2 Chemistry

Similarly, to other ADCs, T-DM1 is composed of several DM1 molecules covalently connected to the antigen-specific humanized antibody trastuzumab via a chemical linker.

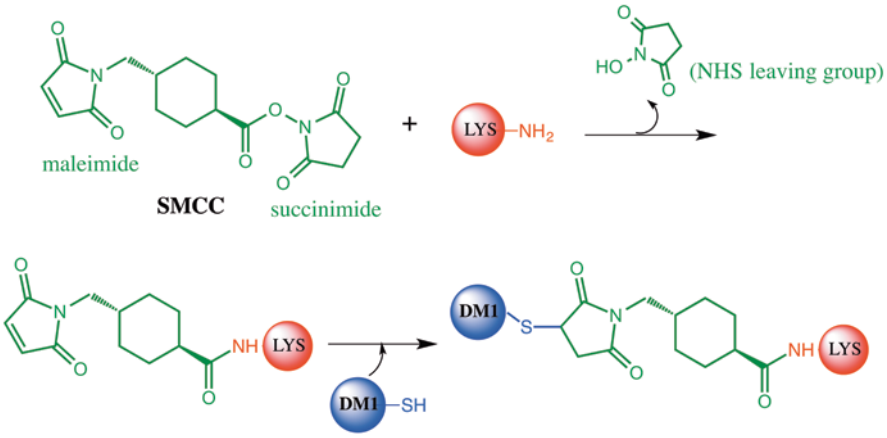

Fig. 12.2 Linking mechanism of DM1 molecules to Trastuzumab. The cross-linking reagent trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate, also known as SMCC is shown in *green*. The succinimide ester group reacts with lysine residues in the trastuzumab molecule (shown in *red*) to form an amide bond and N-hydroxysuccinimide (NHS) leaving group. The maleimide moiety links to the free sulfhydryl group of DM1 (shown in *blue*), forming a thioether bond between the linker and DM1. DM1 mertansine

DM1 is a derivative of cytotoxic agent maytansine. Maytansine (shown in black in Fig. 12.1) is not only an ansamycin family antibiotic initially isolated from the Ethiopian shrub *Maytenus serrate* but also found in other plants from the genus *Maytenus,* which grow in a wide diversity of climates around the world. The structure represents the benzoansamacrolide family that comprises an aromatic moiety bridged by a long-branched unsaturated aliphatic chain. Maytansine inhibits the microtubule assembly by binding to the same site on tubulin as rhizoxin, a bacterial antimitotic agent (Partida-Martinez and Hertweck 2005).

Maytansine exhibits cytotoxicity against many tumor cell lines at subnanomolar concentrations. However, it failed as an anticancer agent in human clinical trials because of the lack of tumor specificity and high systemic toxicity (Iwasaki 1993; Liu et al. 2005; Lopus et al. 2010; Sawada et al. 1993; Tassone et al. 2004; Widdison et al. 2006). Mertansine is a derivative of maytansine containing a sulfhydryl (-SH) group. DM1 has an in vitro cytotoxicity three- to tenfold greater than maytansine (Junttila et al. 2011; Kovtun et al. 2006), but still presents nonselective systemic toxicity. Interestingly, the sulfhydryl group allows the creation of DM1 conjugates with cross-linking reagent through the thioether group (shown in blue in Fig. 12.1).

The cross-linking reagent used in T-DM1 is succinimidyl *trans*-4-(maleimidylmethyl)cyclohexane-1-carboxylate, also known as SMCC (Fig. 12.2). SMCC has two functional groups—a succinimide ester and a maleimide. The succinimide ester group reacts with lysine residue in the trastuzumab molecule to form an amide bond and N-hydroxysuccinimide (NHS) leaving group. The maleimide moiety links to the free sulfhydryl group of DM1, forming a thioether bond between the linker and DM1. Each trastuzumab molecule is linked to 3.5 DM1 molecules on average with a maximum of eight payloads (Krop et al. 2010). The conjugation

has been shown to preserve the activity and binding specificity of the antibody component (Junttila et al. 2011). The DM1 moiety together with the linker make emtansine, hence the generic name trastuzumab emtansine for the final ADC.

Choosing a suitable cross-linking reagent is one of the most critical points in the design of an ADC. The linker should stabilize the ADC in circulation and once the compound enters the cell, it liberates the cytotoxic agent. Antibody–DM1 conjugates were originally designed with a disulfide-based linker. Indeed, after internalization of the ADC, the endosomal reduction of the disulfide bond separates the antibody from the DM1, and releases the latter into the cytoplasm. Several disulfide-linked trastuzumab ADCs were tested such as trastuzumab–SPDP–DM1, trastuzumab–SPP–DM1, trastuzumab–SSNPP–DM3, and trastuzumab–SSNPP–DM4 (Lewis Phillips et al. 2008). It was found that introducing steric hindrance near the disulfide bond would slow down the reduction rate of disulfide bond. However, even with the most hindered disulfide linker, once the DM1 molecules are liberated from the antibody, it is cleared rapidly. Thus, with the disulfide-linked ADC, the concentration of DM1 inside the tumor cell may drop too quickly to ensure prolonged antitumor effects. The SMCC linker, in contrast, forms a thioether bond instead of a disulfide bond, and thus is nonreductible (cannot undergo enzymatic reduction of disulfide bond within the cell). Among the thioether-linked ADCs, the superior thioether stability of T-DM1 compared to the cysteine-linked ADCs has been reported (Erickson and Lambert 2012). It is possible that for the thio-maleimide exchange reaction, the Michael donor reactivity of the sulfhydryl group of DM1 is higher than the sulfhydryl group of cysteine side chains (Baldwin and Kiick 2011). The SMCC-linked ADC is internalized upon binding to HER2 and is postulated to undergo intracellular proteolytic degradation to release active Lys–MCC–DM1 (MCC is the linker moiety) molecules into the cytoplasm of the targeted tumor cell. This postulation is supported by the blocked activity of T-DM1 in the presence of protease inhibitors in cell culture experiments (Lewis Phillips et al. 2008). The fact that Lys–MCC–DM1 molecules were detected as the major metabolites in rats and mice plasma, as well as plasma samples of patients in a phase 2 clinical study (Burris et al. 2011; Krop et al. 2010; Lu et al. 2011; Shen et al. 2012) also supports this mechanism. The slow release of DM1 by proteolytic degradation inside a tumor cell guarantees the prolonged antitumor efficacy and reduces systemic toxicity. More detailed review on the effect of T-DM1 linker can be found in Lambert and Chari (2014).

12.3 HER2 and the EGF Receptors Family

In humans, the EGFR family consists of four transmembrane receptors: HER1, first identified and initially called “the EGF receptor”; HER2; HER3; and HER4. HER2 was identified as a homolog of the avian erythroblastosis oncogene B, or ErbB, and hence was named ErbB2 by homology. In rodents, it was independently identified in a glioblastoma cell line, a type of neuronal cancer, and was hence named Neu. Genome sequencing later confirmed that HER2, ErbB2, and Neu are in fact

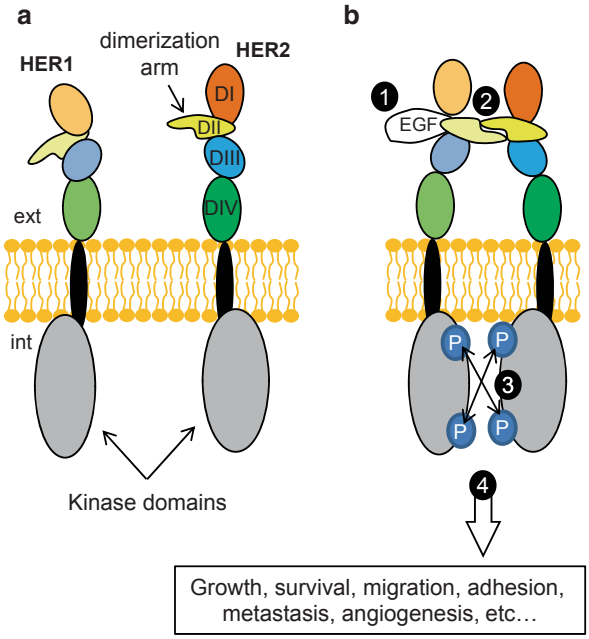

Fig. 12.3 Mechanism of cell proliferation mediated by EGFR receptors. **a** In absence of ligand, EGFR members are mainly present as monomers in the epithelial cell surface (here only HER1 and HER2 are shown as examples). EGFR monomers consist on a large extracellular N-terminal region composed of four structural domains named DI to DIV (indicated with different colors), a unique transmembrane segment, and a cytoplasmic C-terminal catalytic domain. Ligand-dependent EGFR activation requires the interaction between two receptors via their activation arms formed by DII. In the orphan HER2 receptor, the activation arm is in an open conformation readily available for dimerization, while in HER1, HER3, and HER4 it remains in a tethered conformation in absence of ligand. **b** Binding of the EGF ligand to HER1 (1) opens its dimerization arm and mediates the formation of a stable heterodimer with HER2 (2). The physical proximity of the catalytic domains induce several transphosphorylations of hydroxylated side chains in the catalytic domains (3) which activate several proliferative signaling pathways (4) promoting the survival and proliferation of the cell. EGFR epidermal growth factor receptor, HER human epidermal growth factor receptor

ortholog proteins, suggesting that they play similar functions in different vertebrate organisms (Coussens et al. 1985).

EGFR proteins share a homologous structure of a large N-terminal extracellular domain followed by a single transmembrane-spanning segment and a C-terminal intracellular kinase domain (Bessman and Lemmon 2012) (Fig. 12.3). The extracellular domain of HER1 binds at least seven ligands: EGF, transforming growth factor α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), betacellulin, epiregulin, amphiregulin, and the recently identified epigen (Linggi and Carpenter 2006; Schneider and Yarden 2014). HER3 binds neuregulin 1 and neuregulin 2, while HER4 recognizes neuregulins 1–4, epiregulin, betacellulin, and HB-EGF.

Binding of specific ligands to HER1, HER2, or HER3 causes these receptors to dimerize, either with a receptor of the same type (homodimerization) or with another family member (heterodimerization). In contrast, HER2 does not interact with any known ligands and is thus considered an orphan receptor. HER2 cannot homodimerize and is exclusively found in heterodimers with ligand-bound HER1, HER3, or HER4 (Rubin and Yarden 2001). Hence, HER2 is thought to enhance EGFR-mediated signals by stabilizing heterodimers containing a ligand-bound EGFR. Recent molecular dynamic simulations showed that, although being asymmetric, heterodimers harboring ligand-free HER2 monomers form quite stable complex as long as one ligand molecule remains bound to the other receptor (Arkhipov et al. 2013).

The EGFR extracellular region is subdivided into four domains DI–DIV (Fig. 12.3a). Dimerization is catalyzed by the opening of a dimerization arm formed by domain II in each monomer (Ogiso et al. 2002). In HER1, HER3, and HER4, the dimerization arm swings between an inactive "tethered" conformation to an active "open" conformation upon ligand binding (Fig. 12.3b). In contrast, the dimerization arm of HER2 remains in a constitutively open conformation. Although HER2 can dimerize with any EGFR members, the HER2/HER3 pair is one of the most efficient and often observed cancer studies (Way and Lin 2005). Receptor dimerization triggers transphosphorylation of hydroxylated residues in the intracellular domain. This event activates several proliferative intracellular signaling pathways (RAS/ERK and Pi3 K/AKT). During the tumorization process, naturally selected mutations of HER2 may promote cancer growth by inducing constitutive autophosphorylation of tyrosines in the cytoplasmic kinase domain (Wang et al. 2006) or by inducing constitutive dimerization (Muller et al. 1998).

An estimated 15–30 % of breast cancers are directly due to an increase of HER2 receptors at the cell surface (Mitri et al. 2012). Overexpression of HER2 also occurs in ovarian, stomach, and uterine cancer, and causes gallbladder adenocarcinoma in mouse (Kiguchi et al. 2001). In HER2-positive cancers, the amount of HER2 proteins may be up to two orders of magnitude higher compared to normal epithelial cells (Venter et al. 1987). More than 90 % of HER2-positive breast cancers are caused by allelic amplification of the *HER2* gene (Pauletti et al. 1996). Genic amplification is also the main mechanism for HER2 overexpression in the development of ovarian and gastric tumors (Hynes and Stern 1994). Interestingly, amplification of the *HER2* gene does not seem to occur in HER2-positive cancers located in specific organs such as lungs, bladder, mesenchyme, and esophagus. In those cases, overexpression of HER2 is thought to result from alterations of transcriptional/translational and degradation/recycling processes (Slamon et al. 1989).

12.4 Trastuzumab–HER2 Interaction

To investigate the molecular determinants of the antibody–antigen interaction between trastuzumab and HER2, Cho et al. (Cho et al. 2003) co-crystallized the extracellular domain of the human HER2 receptor with Fab fragments obtained digesting

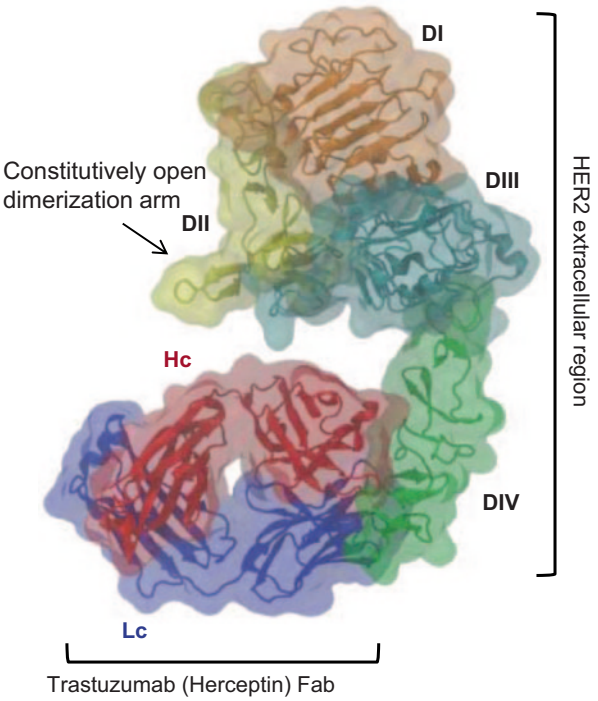

Fig. 12.4 HER2–trastuzumab interaction. Crystal structure of a trastuzumab (Herceptin) antigen-binding fragment (Fab, Hc: heavy chain; Lc: light chain) complexed to the extracellular domain of HER2 showing that the antibody binds specifically to DIV (PubMed accession code: 1N8Z; Cho et al. 2003). Note that the dimerization arm is located in DII (see Figure 12.3) and remains in a constitutively "open conformation" ready to engage dimerization with ligand-bound EGFR members. The figure was generated using the free-access software visual molecular dynamics (VMD, http://www.ks.uiuc.edu/Research/vmd). The protein backbones are shown in new cartoon representation. The large arrows indicate the presence of beta sheets. The transparent surface delineates the external surface of the protein side chains. EGFR epidermal growth factor receptor, HER human epidermal growth factor receptor

Herceptin antibodies with papain. The X-ray structure of the complex showed that trastuzumab binds the bottom part of DIV (Fig. 12.4). A closer look at the structure shows that the trastuzumab epitope in the human HER2 is formed by three adjacent loops (Fig. 12.5a) stretching from residues 557–561, 570–573, and 593–602, respectively. A sequence alignment of the three-epitope loops in HER2 homologs indicates poor residue conservation between rodents and hominids (Fig. 12.5b). This may explain why the trastuzumab antibody recognizes the primate HER2 receptor but not rodent Neu homologs (Poon et al. 2013).

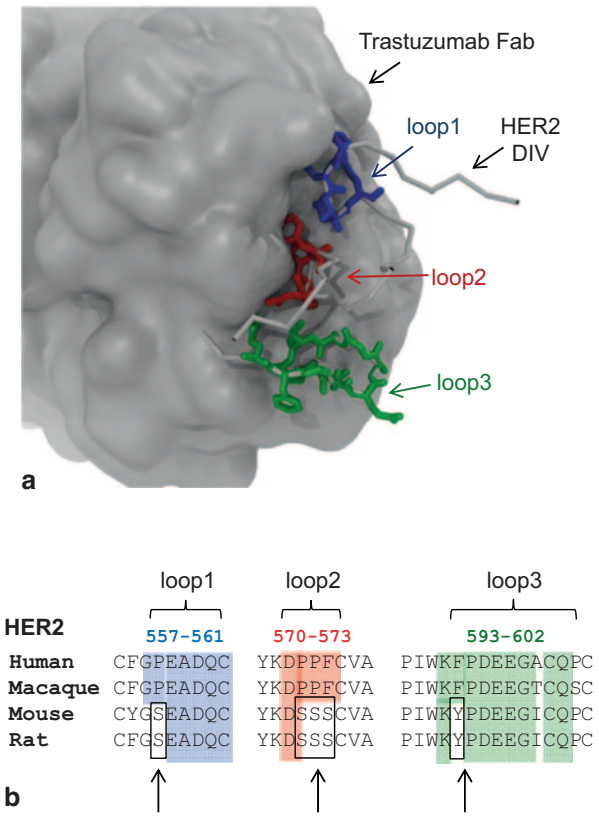

Fig. 12.5 Molecular determinants of the HER2 epitope. **a** Zoomed view of the structure shown in Fig. 12.4 showing the side chains from HER2 domain IV (licorice representation) making contact with the Herceptin Fab (represented as a white surface). The epitope is essentially formed by three contiguous loops (loop1 in *blue*, loop2 in *red,* and loop3 in *green*). **b** Sequence alignment of the three-loop regions in rodents and primate HER2 homologs (human GI: 119533, macaque GI: 544499689, mouse GI: 76363513 and rat GI: 3915663). Conserved residues in the three loops are indicated by colored boxes and the residue numbers are from the human HER2 amino acid sequence. Note the presence of residue divergence for some positions in each loop between primate and rodent HER2 proteins (open boxes indicated by *vertical arrows*). HER human epidermal growth factor receptor

12.5 Mechanisms of Action

T-DM1 helps fight HER2-positive cancer cells through several mechanisms. First, the binding of the antibody interferes with the transduction of HER2-mediated growth signals. Binding of trastuzumab to HER2-amplified cancer cell was shown to disrupt the ligand-independent interaction between HER2 and HER3, providing to the antibody an antiproliferative effect (Junttila et al. 2009; Fig. 12.6a). Second, cancer cells decorated with humanized antibodies exposing Fc regions can poten-

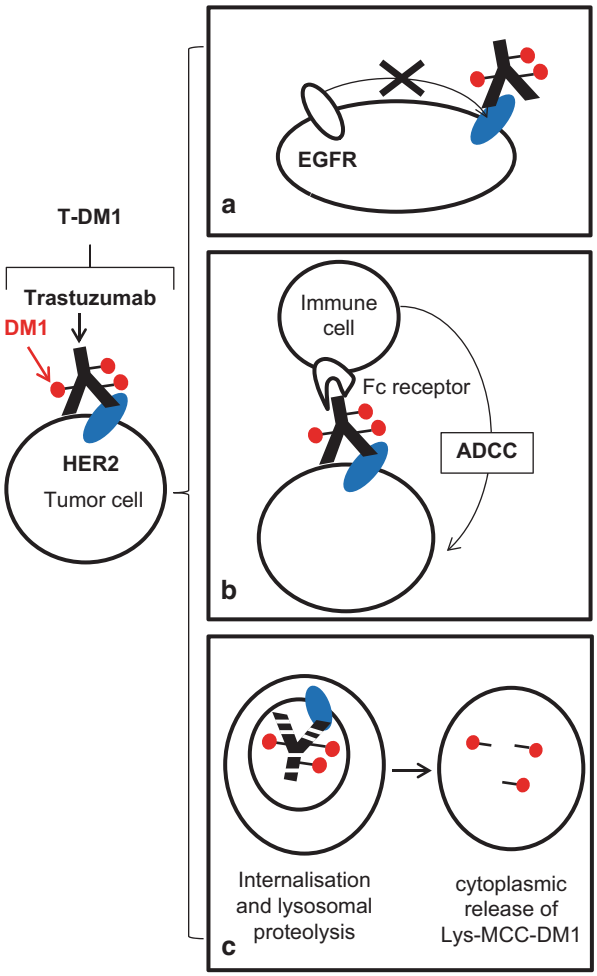

Fig. 12.6 T-DM1-mediated antimitotic mechanisms. **a** Binding of T-DM1 to a HER2-overexpressing tumor cell (left) prevents the formation of a stable heterodimer between HER2 and other ligand-bound members of the EGFR family and thus interferes with HER2-dependent proliferative signaling pathway. **b** Recognition of the Fc region of the trastuzumab antibody by Fc receptors present on macrophages or natural killer cells patrolling the surrounding tissue triggers antibody-dependent cell-mediated cytotoxicity (ADCC) directed toward the HER2-expressing tumor cell. **c** Binding of T-DM1 facilitates internalization of the ADC–HER2 complex. The following lysosomal proteolysis of the antibody moiety releases Lys–MCC–DM1 molecules in the cytoplasm of the tumor cell. The activated compounds bind tubulin molecules and inhibit mitotic division. T-DM1 Ado-trastuzumab emtansine, DM1 mertansine, ADC antibody–drug conjugate, EGFR epidermal growth factor receptor, HER human epidermal growth factor receptor

tially be recognized by immune cells such as macrophages or natural killer cells and targeted for elimination through a process called antibody-dependent cell-mediated cytotoxicity (ADCC; Fig. 12.6b). Both trastuzumab and its ADC (T-DM1) trigger

ADCC in clinical studies, showing the effective contribution of ADCC to fight tumor progression in vivo (Junttila et al. 2011). In principle, the Fc regions may also trigger complement-dependent cytotoxicity (CDC). Third, binding of trastuzumab favors internalization of the ADC–receptor complex leading to subsequent lysosomal proteolytic degradation of the antibody moiety. This process slowly releases Lys–MCC–DM1 molecules into the cytoplasm of the targeted HER2-amplified tumor cell (Fig. 12.6c) where they bind tubulin and inhibit microtubule polymerization.

12.6 Metabolism and Pharmacokinetics

Several absorption, distribution, metabolism, and excretion (ADME) studies of T-DM1 have been reported using mice and rats. The antibody component of T-DM1 does not cross-react with rodent antigens. Thus, there is no antigen-mediated contribution to the pharmacokinetics (PK). The major metabolites found in rats and mice plasma were lysine–MCC–DM1 (around 70%; Fig. 12.7 top), [*N*-maleimidylmethyl] cyclohexane-1-carboxylate-DM1 (MCC-DM1; Fig. 12.7 bottom) (Burris et al. 2011; Krop et al. 2010; Lu et al. 2011; Shen et al. 2012). These metabolites indicate that lyosomal degradation is the main metabolism pathway for the ADC, and the thioether linker is highly resistant against chemical cleavage during clearance. The major two metabolites identified in rats and mice were also detected in plasma samples from patients with HER2-positive MBC in a phase 2 clinical study (Shen et al. 2012). DM1 was detected at low level in human plasma. The primary active metabolite, lysine–MCC–DM1, does not readily cross the plasma membranes of neighboring cells and, therefore, does not induce a bystander effect (Lewis Phillips et al. 2008). In vitro studies indicate that DM1 is metabolized mainly by CYP3A4 isoenzyme (Lu et al. 2011).

The clearance profiles of several DM1 conjugates are found to correlate with the in vitro stability of their chemical linkers. The clearance rate in mice was found that the regular disulfide-linked conjugate is cleared faster than the more sterically hindered disulfide-linked conjugate. The latter is cleared faster than the uncleavable thioether-linked T-DM1 (Erickson et al. 2012; Kellogg et al. 2011). In nude mice, T-DM1 showed the serum concentrations of T-DM1 measured for 1 week were not different from total serum trastuzumab concentration (Lewis Phillips et al. 2008). Study on female Sprague-Dawley rats shows that following administration of radiolabeled T-DM1, the major circulating species in plasma was T-DM1, while DM1 concentrations were low (Shen et al. 2012).

T-DM1 shows no evidence of tissue accumulation in rats or metabolite accumulation in human plasma following multiple dosing. The average 3.5 DM1 molecules per antibody conjugating rate shows no detectable impact on the PK properties of the trastuzumab (Erickson and Lambert 2012; Mayo et al. 2005). The major pathway of DM1-containing metabolite elimination in rats was the fecal/biliary route, with the majority of T-DM1 eliminated in the feces (80%) and a small fraction in the urine. Those preclinical T-DM1 observations appear to translate to the clinic, although trastuzumab does not bind the rat or mouse HER2-like receptor.

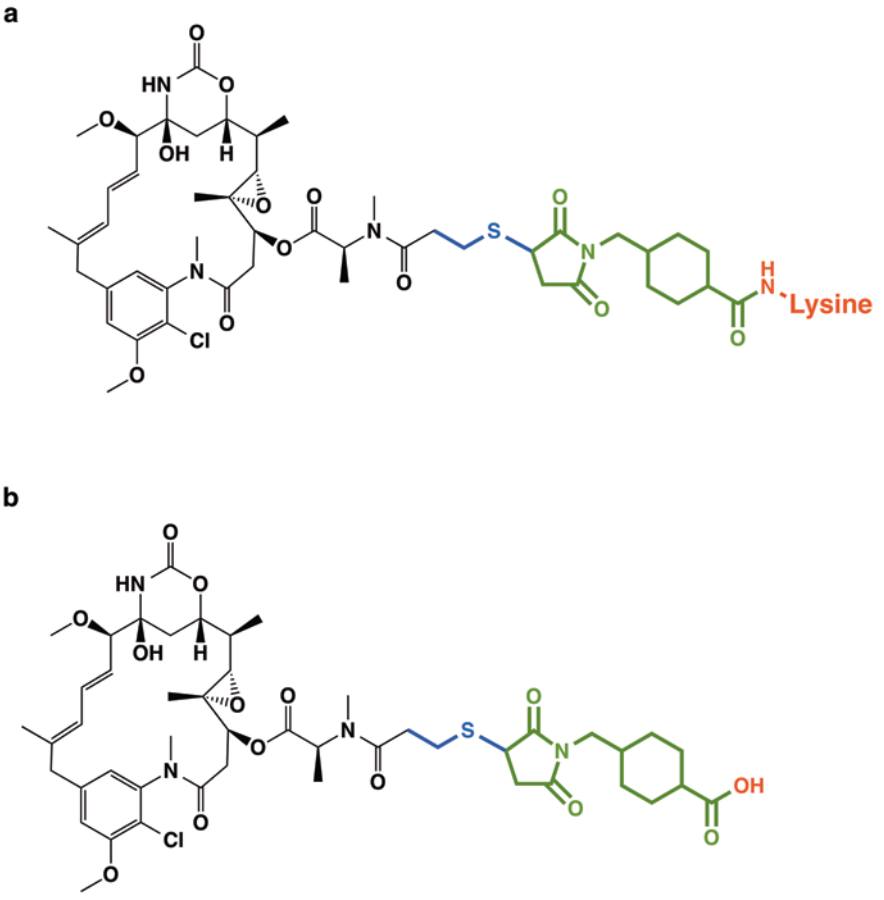

Fig. 12.7 The major metabolites found in rat plasma. **a** Lysine–MCC–DM1, **b** [N-maleimidyl-methyl] cyclohexane-1- carboxylate-DM1 (MCC-DM1)

PK data of T-DM1 is available from one phase 1 trial, four phase 2 trials and one phase 3 trial. The proposed dose characterized by noncompartmental methods is 3.6 mg/kg as an intravenous (IV) infusion over 30 min once every 3 weeks (q3w). The ranges of PK parameters following single and multiple doses of T-DM1 3.6 mg/kg q3w are summarized in Table 12.1. Clinical studies show that T-DM1 has predictable PK properties at clinical doses. Six clinical studies reported using the accepted maximum tolerated dose (MTD) of 3.6 mg/kg q3w have shown that the clearance rate in patients ranged from 6–13 mL/day/kg for T-DM1 and the terminal half-life is 3–4.5 days for T-DM1 and 9–11 days for total trastuzumab (Burris et al. 2011; Girish et al. 2012; Krop et al. 2010). T-DM1 accumulation was not observed following multiple dosing.

Table 12.1 Summary of T-DM1 PK parameters following single and multiple doses of T-DM1 3.6 mg/kg q3w, reproduced from (Qi and Nitin 2012)

Clinical trial	Cycle	*N*	C_{max} (μg/mL)	AUC (μg*d/mL)	$T_{½}$ (d)	CL (mL/d/kg)	V_d (mL/kg)
Phase 1	1	15	76.2	300	3.1	12.7	58.4
Phase 2	1	51–105	75.6–84.2	431–495	3.5–4.0	8.0–9.2	28.4–41.2
	3–5	39–82	68.9–80.7	456–475	4.2–4.5	6.7–8.4	33.6–45.2
Phase 3	1	292	83.4	489	3.7	7.8	29.5

T-DM1 Ado-trastuzumab emtansine, *PK* pharmacokinetics

12.7 Preclinical Efficacy and Toxicity Studies

Preclinical experiments have been conducted in cells and animal models to assess the tolerability of T-DM1 and the side effects observed upon administration of the drug. Taking advantage of the fact that trastuzumab does not cross-react with the rodent HER2 homolog Neu, Poon et al. determined the antigen-dependent and antigen-independent toxicity of T-DM1 by comparing the toxicity of T-DM1 administered intravenously to cynomolgus monkeys (*Macaca fascicularis,* also known as crab-eating macaques) and rats (Poon et al. 2013). Tolerability of T-DM1 was found relatively similar for both types of animals. Toxic effects start to develop above 40 mg/kg in rats (~4.4 mg DM1/body surface area in m^2) and 30 mg/kg (~6 mg DM1/m^2) in monkeys, indicating that T-DM1 toxicity mainly arises from an antigen-independent mechanism. Furthermore, the toxic effects were relatively proportional to the dose received, either acutely or chronically and corresponded to the expected effects of the emtansine molecules. In rats, toxic effects were decreased platelet, reticulocyte, and lymphocyte counts, increase in neutrophil counts, depletion, or necrosis of lymphoid organs, such as lymph nodes, spleen and thymus, and various degrees of hepatocellular degenerations associated with elevated concentrations of serum alanine and glutamyl aminotransferases, alkaline phosphatase, and bilirubin, the down-product of heme catabolism. Similar toxic effects on red and white blood cells counts, hepatic functions, and lymphoid system were observed for monkeys. In both animals, and particularly in rats, a large number of cells in tissues beyond the liver were found in mitotic arrest, strongly suggesting the occurrence of antigen-independent T-DM1 uptake with subsequent activation of antimitotic DM1 molecules. It is hypothesized by the authors that T-DM1 may trigger endocytosis via its Fc region or that circulating DM1 molecules may be released through catabolism. In monkeys, irreversible axonal degeneration was observed after four doses of 10–30 mg/kg or eight doses of 1–10 mg/kg, although no obvious effects were noted in neurologic tests. In comparison to T-DM1, when DM1 was administered in rats, the tolerability of the dose went down to about 1.6 mg DM1/m^2, which corresponds to about half the tolerable dose of T-DM1. Hence, this study demonstrates the ability of T-DM1 to increase tolerability of the chemotherapeutic drug in animal models.

Potential benefits from T-DM1 treatments in other HER2-positive epithelial cancers have been assessed by studying the effects of T-DM1 on four HER2-positive human gastric cancer cell lines (Barok et al. 2011). In vitro assays further indicated that T-DM1 was globally more effective than trastuzumab on N-87 and OE-19 cells, while it showed moderate and limited efficacy on MKN-7 and SNU-216 cells, respectively. The authors also noted that N-87 and OE-19 cells exhibited slightly higher *HER2* gene copies and about threefold higher HER2 surface expression than MKN-7 and SNU-216 cells. Hence, N-87 and OE-19 gastric cancer cells may be more susceptible to HER2-directed ADC therapy. To address T-DM1 efficacy in vivo, the authors further xenotransplanted N-87 and OE-19 cells into severely immunodeficient (SCID) mice and conducted various T-DM1 protocols. In all OE-19 and half of N-87 xenografts, T-DM1 induced a complete reversal of tumor growth leading to nearly complete remission, while trastuzumab alone did not prevent tumor progression. Interestingly, these effects remained potent against large established tumors and against trastuzumab-resistant tumors. Moreover, no delay was necessary for switching between trastuzumab and T-DM1 treatments to observe the full benefits of T-DM1, suggesting a rapid turnover of HER2 proteins in these gastric cancer cells. This discovery may help to reduce delays to switch ADC treatments in HER2-positive cancer patients. Since July 2013, a phase 3 clinical trial testing T-DM1 treatment for gastric cancer patients is ongoing (Fig. 12.8).

12.8 Clinical Studies

Trastuzumab emtansine (Kadcyla ™) was granted approval by the FDA in the USA in February 2013 and subsequently in Japan (September 2013) and the EU (November 2013), thus receiving global approval of this ADC. Clinical studies demonstrated increased median OS of MBC patients with decreased adverse effects, thus leading to an accelerated approval of this ADC (Ballantyne and Dhillon 2013).

Phase 1 studies evaluated the effects of trastuzumab emtansine under two different administration schedules for patients with locally advanced or metastatic HER2-positive breast cancer: (1) Once every 3 weeks ranging from 0.3–4.8 mg/kg ($n=24$) and (2) once weekly dosing ranging from 1.2–2.9 mg/kg ($n=28$). This was designed as an open-label dose escalation study. An MTD was determined as 3.6 mg/kg in the first cohort dosed once every 3 weeks. The clinical benefit rate was found to be 73% ($n=15$; 3.6 mg/kg; treatment = 238 days) whereas the objective response rate (ORR) was 44% ($n=9$; Krop et al. 2010).

The second cohort on the once-weekly dosing schedule demonstrated an MTD lower than the first cohort (2.4 mg/kg). The partial tumor responses were reported in 46% of the patients (13 of 28) up to ~18 months of therapy. Sixteen patients received 2.4 mg/kg once weekly dosage of which 15 patients had measurable disease at baseline. Of this, 40% had an objective partial response with a median duration of response (MDR) of 5.6 months (Beeram et al. 2012).

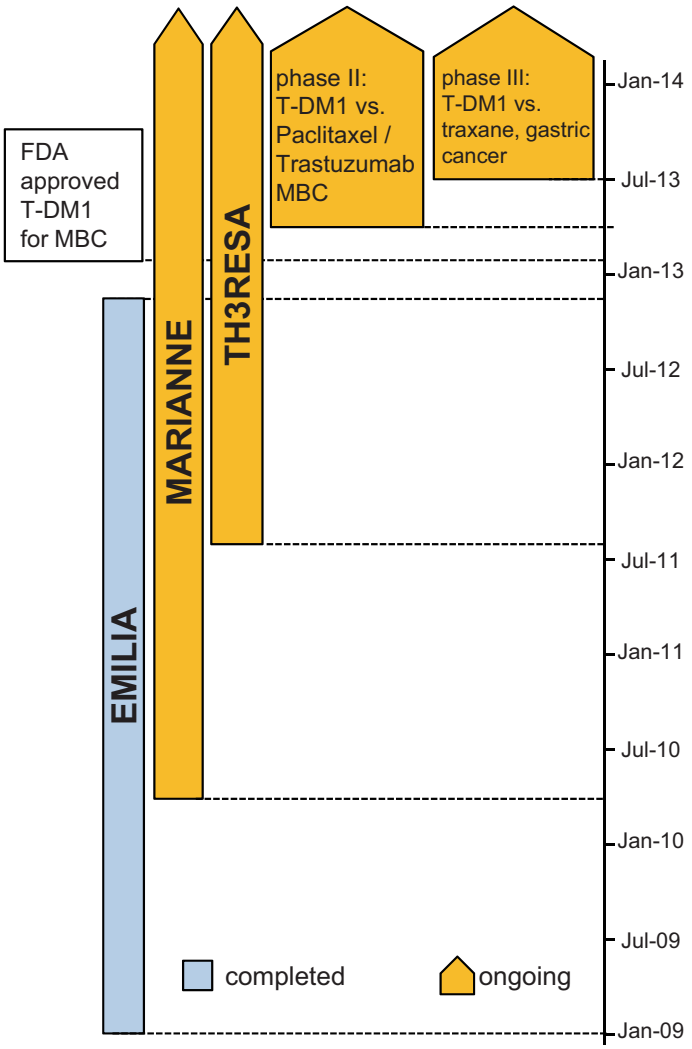

Fig. 12.8 Chronology of T-DM1 clinical trials. Four phases 2 or 3 clinical trials are presently ongoing using various protocols to test T-DM1 efficacy against MBC and gastric cancers. Only the EMILIA clinical phase 3 trials (see Sect. 12.8) have been completed in November 2012. The MARIANE trials started in April 2010 and consist of three arms: T-DM1/pertuzumab versus T-DM1 versus trastuzumab/taxane in HER2-positive MBC patients. The TH3RESA trials started in August 2011 and consist of two arms: T-DM1 versus the physician's choice of therapy in HER2-positive MBC patients. T-DM1 was approved by the FDA in February 2013 for HER2-positive MBC patients based on the EMILIA study. T-DM1 Ado-trastuzumab emtansine, MBC metastatic breast cancer, HER human epidermal growth factor receptor, FDA Food and Drug Administration

A similar phase 1 study in Japan used a single-arm, dose-escalation (Aogi et al. 2011) study enrolling patients with HER2-positive MBC who had received prior therapies including trastuzumab. This study also found the MTD in Japanese patients ($n = 10$) to be 3.6 mg/kg administered once every 3 weeks.

Phase 2 Studies: These were open-label studies in patients with HER2-positive MBC. Two treatment protocols followed were trastuzumab emtansine and tratuzumab with docetaxel. Patients received trastuzumab emtansine monotherapy ($n=67$) or trastuzumab (8 mg/kg loading dose followed by 6 mg/kg every 3 weeks) plus docetaxel (75 or 100 mg/m^2; $n=70$). Results from this first-line treatment with trastuzumab emtansine significantly prolonged median PFS (primary endpoint) to ~14 months compared to 9.2 months treatment with trastuzumab plus docetaxel, thus increasing PFS by 5 months (14.2 vs. 9.2 months; HR 0.59; 95% CI 0.36–0.97; Hurvitz et al. 2013). The ORR with trastuzumab emtansine was higher than the combination dose of trastuzumab plus docetaxel (64 vs. 58%). OS rates showed no significant between-group differences in a preliminary analysis conducted at a median follow-up of ~23 months (HR 1.06; 95% CI 0.477–2.352).

In an independent assessment, trastuzumab emtansine monotherapy was associated with an ORR of 25.9% (primary endpoint) and a median PFS of 4.6 months after a median follow-up of>12 months in a single-arm phase 2 study in patients ($n=112$) with HER2-positive breast cancer who were previously treated with trastuzumab (Burris et al. 2011).

In another single-arm phase 2 study in patients with HER2-positive MBC refractory to current therapies ($n=110$), patients were required to have received prior treatment with trastuzumab, lapatinib, a taxane, an anthracycline, and capecitabine in the neoadjuvant, adjuvant, locally advanced, or metastatic setting (Krop et al. 2012). In addition, patients were required to have had at least two HER2-directed therapy regimens in the metastatic or locally advanced setting and progression on the most recent regimen. Independent assessments demonstrated an ORR of 34.5% (primary endpoint) and a clinical benefit rate of 48.2%, at a median follow-up of 17.4 months of trastuzumab emtansine monotherapy. The median PFS in these patients was 6.9 months and the MDR was 7.2 months.

A single-arm phase 1b/2 study in patients with HER2-positive locally advanced or MBC evaluated the efficacy of combination therapy with trastuzumab emtansine plus pertuzumab (Dieras et al. 2010). The phase 1b dose-escalation phase of the study established the dosage of trastuzumab emtansine (3.6 mg/kg every 3 weeks) and pertuzumab (840-mg loading dose in cycle 1, followed by 420-mg maintenance dose every 3 weeks thereafter). Following combination treatment, 16 of 46 patients in the relapsed setting (confirmed ORR of 34.8%) and 12 of 21 patients in the first-line setting (confirmed ORR of 57.1%;primary endpoint) showed partial or complete responses. Another 22 patients in the relapsed setting and 5 patients in the first-line setting had stable disease with clinical response rates of 21 and 13%, respectively. Prior treatment for recurrent locally advanced or metastatic disease (relapsed setting) or newly diagnosed or previously untreated MBC (first-line setting) was received by the enrolled patients.

Phase 3 Study: The EMILIA study was a randomized, open-label, international trial involving 991 patients from 24 countries with HER2-positive, unresectable, and locally advanced or MBC who had previously been treated with trastuzumab and a taxane, to T-DM1 or lapatinib plus capecitabine. Patients received trastuzumab emtansine ($n=495$) or oral lapatinib (1250 mg/day on days 1–21 of a 3-weekly

Table 12.2 Safety profile (Kadcyla®) Phase 3 EMILIA study

Cancer drug treatment	Adverse events		Common adverse symptoms			
	Overall (%)	Grade 3 or 4 (%)	Nausea (%)	Fatigue (%)	Thrombo-cytopenia (%)	Elevated AST (%)
Trastuzumab Emtansine (Kadcyla®; 3.6 mg/kg)	95.9	40.8	39.2	35.1	28	4.3
Lapatinib + Capecitabine (1250 mg/day + 1000 mg/m^2)	97.7	57.0	44.7	27.9	2.5	0.8

AST aspartate aminotransferase

cycle) plus capecitabine (1000 mg/m^2 twice daily on days 1–14 of a 3-weekly cycle; $n=496$). The median duration of follow-up was ~13 months, with the second interim analysis of OS conducted at a median follow-up of ~19 months (Verma et al. 2012). PFS as assessed by independent review, OS, and safety were considered as the primary end points. Secondary end points included PFS (investigator-assessed), ORR, and the time to symptom progression (Figure 9). Two interim analyses of OS were conducted. The study showed that trastuzumab emtansine therapy significantly ($p<0.001$) prolonged PFS (co-primary endpoint) by 3.2 months relative to treatment with lapatinib plus capecitabine (9.6 vs. 6.4 months, as per independent review; hazard ratio; HR 0.65; 95% CI 0.55–0.77) in patients with HER2-positive advanced breast cancer previously treated with trastuzumab and a taxane. An increase of median OS by 5.8 months was seen as significant ($p<0.001$; 30.9 vs. 25.1 months at the second interim analysis; HR 0.68; 95% CI 0.55–0.85) relative to treatment with lapatinib plus capecitabine (co-primary endpoint). The survival rate was 85.2% (estimated 1-year) in trastuzumab emtansine recipients compared with 78.4% in lapatinib plus capecitabine recipients and the 2-year survival rates were 64.7 versus 51.8%, respectively. In addition, trastuzumab emtansine recipients had significantly ($p<0.012$) longer time to symptom progression (7.1 vs. 4.6 months) and higher ORR of 43.6 versus 30.8% than lapatinib plus capecitabine recipients; the MDR was 12.6 and 6.5 months in the respective groups. T-DM1 significantly prolonged PFS and OS with less toxicity than lapatinib plus capecitabine in patients with HER2-positive advanced breast cancer previously treated with trastuzumab and a taxane.

Safety Profile Trastuzumab emtansine was well tolerated in patients with HER2-positive advanced breast cancer previously treated with trastuzumab and a taxane. In general, most adverse events were of low grade as seen from the Phase 3 EMILIA study (Verma et al. 2012). As shown in Table 12.2, after a median follow-up of ~13 months of treatment, trastuzumab emtansine recipients ($n=490$) showed lower incidence of adverse events of any grade compared with lapatinib plus capecitabine recipients ($n=488$; Verma et al. 2012); Both treatment groups exhibited adverse events of grade 3 or 4; however, the incidence was again lower in the trastuzumab emtansine group. The most common (incidence > 25%) treatment-

emergent adverse events of any severity in the trastuzumab emtansine group were nausea, fatigue, and thrombocytopenia. The most common (incidence > 3 %) grade 3 or 4 adverse events in trastuzumab emtansine recipients were thrombocytopenia (12.9 vs. 0.2 %) and elevated aspartate aminotransferase (AST; 4.3 vs. 0.8 %). A total of 15.5 % of patients in the trastuzumab emtansine group reported serious adverse events compared with 18.0 % of patients in the lapatinib plus capecitabine group.

12.9 Perspectives

The approval of trastuzumab emtansine (Kadcyla ™) for MBC represents the first ADC to be approved by the FDA for HER2/neu-positive patients who have progressed on prior therapy with trastuzumab (Abraham 2013). This novel approach whereby combining the toxic drug (emtansine) with a targeted agent (trastuzumab) via a linker molecule provides a tremendous impetus on future drug development. Additionally, and perhaps more importantly, for patients with MBC, this ADC option improves PFS and OS rates at safety profiles far better than its predecessors. The addition of this ADC to the arsenal of treatments have helped redefine the natural progression of HER2-positive MBC allowing women to live with the disease for many years and maintain an excellent quality of life.

ADCs have generated tremendous interest for decades (Perez et al. 2014). More recently, FDA approvals of Adcetris ® and Kadcyla ® and a robust pipeline of additional ADCs have contributed to the promise of this therapeutic class of drugs. With nearly 30 additional ADCs currently in clinical development, it is anticipated that the potential of this new therapeutic class might finally be coming to fruition (Mullard 2013). While the complexities in the design of ADCs are enormous, evolving clinical data from approved products will continue to drive technological advancements in the field. Current methods for preclinical lead selection typically rely on systematic in vitro evaluation of a matrix of various mAbs, linkers, and cytotoxic payloads. However, early in vivo studies would be crucial until it is determined that in vitro models are sufficient to predict response of the ADCs. Progress in site-specific conjugation modalities, optimization of linkers with balanced stability, and identification of novel and potent cytotoxic agents should pave the way for greater insight into the contribution of these various factors to ADC efficacy, PKs, and safety. Challenges in target tumor selection will be addressed as the roles of antigen expression, heterogeneity, and internalization rate are further elucidated. Guiding principles for the selection of an ideal antibody Fc format are currently lacking and prompt validation of assumptions regarding antibody-dependent properties, such as specificity and immune effector functions, is needed. Ongoing efforts to address these issues will continue to broaden the impact of ADCs as targeted therapeutics for the treatment of cancer and potentially other diseases (Perez et al. 2014).

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Chapter 13
The Antibody-Drug Conjugate Glembatumumab Vedotin (CDX-011) and Its Use in Treatment of Breast Cancer

Linda T. Vahdat and Nancy Chan

13.1 Introduction

Breast cancer is the most common malignancy in women worldwide and despite advances in screening, diagnosis, and treatment, it remains one of the most significant causes of cancer-related deaths in women (Bray et al. 2013). Of the different subtypes of breast cancers, triple-negative breast cancer (TNBC) poses a special challenge due to its aggressive clinical course, predilection for metastases, and the lack of targeted therapy (Shuen and Foulkes 2012). Molecularly targeted therapies are becoming increasingly prominent as part of the armamentarium of treatments for breast cancer (Muller et al. 2009). Glycoprotein nonmetastatic B (GPNMB) was identified as an overexpressed gene in many malignancies, including TNBC, and is associated with lower disease-free and overall survival. The antibody-drug conjugate (ADC) glembatumumab vedotin (CDX-011) appears to selectively target GPNMB, and is being investigated in clinical trials for efficacy in patients with metastatic breast cancer and other GPNMB-expressing tumors. This chapter reviews the mechanisms of action, preclinical, and phase I/II results of glembatumumab vedotin, and ongoing studies of its role in the treatment of advanced breast cancer.

13.2 GPNMB as a Therapeutic Target

Glycoprotein non-metastatic melanoma protein B is a type I transmembrane protein, which is also known as osteoactivin (OA) (Saitoh et al. 1992). It was first described in 1995 by Weterman et al., as a highly expressed protein in melanoma cell line with low metastatic potential (Weterman et al. 1995). Subsequently, GPNMB

L. T. Vahdat (✉) · N. Chan
Department of Medicine, Weill Cornell Medical College, New York, NY, USA
e-mail: ltv2001@med.cornell.edu

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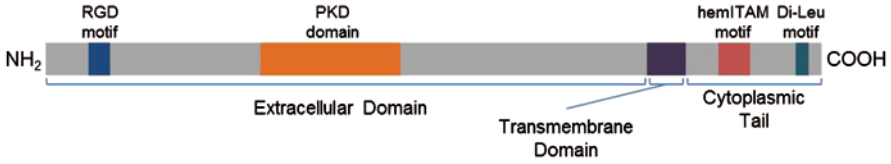

Fig. 13.1 Schematic representation of glycoprotein nonmetastatic B (GPNMB). hemITAM immunoreceptor tyrosine-based activation motif, Leu leucine, PKD polycystic kidney disease, RGD arginine—glycine—aspartic acid

was discovered to have high expression in a number of malignancies including glioblastoma multiforme (Kuan et al. 2006), cancers of the breast (Rose et al. 2007), prostate (Fiorentini et al. 2014), liver (Haralanova-Ilieva et al. 2005), and colon (Eldai et al. 2013). GPNMB has a complex role in tumor biology. Despite its originally perceived low invasive phenotype, it is associated with increased metastatic tendencies in malignancies such as breast cancer.[7]

The GPNMB is located on the small arm of chromosome 7 (7q15), and belongs to the Pmel17/NMB family. Pmel17 is the major structural component of melanosomes, and is essential in melanocyte pigment production (Yamaguchi and Hearing 2009). Also important is that GPNMB shares homology to the lysosomal-associated membrane protein (LAMP) glycoproteins that are implicated in cell adhesion and metastasis (Saitoh et al. 1992).

Under normal conditions, GPNMB is situated in intracellular compartments in macrophages and melanocytes (Tomihari et al. 2009; Ripoll et al. 2007). In tumor cells, however, GPNMB expression is enriched on the cell surface (Fig. 13.1; Maric et al. 2013).

Rose et al. found that when GPNMB is overexpressed in parental 4T1 mouse mammary carcinoma cells, they acquire a more invasive phenotype, leading to increased bone metastasis (Rose et al. 2007). A high level of expression correlated with negative estrogen receptor status and higher tumor grade. Moreover, tumors that express GPNMB have high endothelial cell density compared to those without its expression, suggesting GPNMB recruit endothelial cells to promote tumor growth and enhance the metastatic process. The quantification of apoptotic cells revealed fewer cells in GPNMB-expressing tumors was undergoing apoptosis assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which further elucidates its functional role in tumor progression (Rose et al. 2010a).

The interaction between the tumor and the microenvironment is emerging as an important component in metastasis, and GPNMB has also been implicated in this relationship. Ogawa et al. found that GPNMB can activate fibroblasts by inducing upregulation of pro-invasive metalloproteases such as matrix metalloproteinase-3 (MMP-3) and myelofibrosis with myeloid metaplasia (MMM-9) in mouse models (Ogawa et al. 2005).

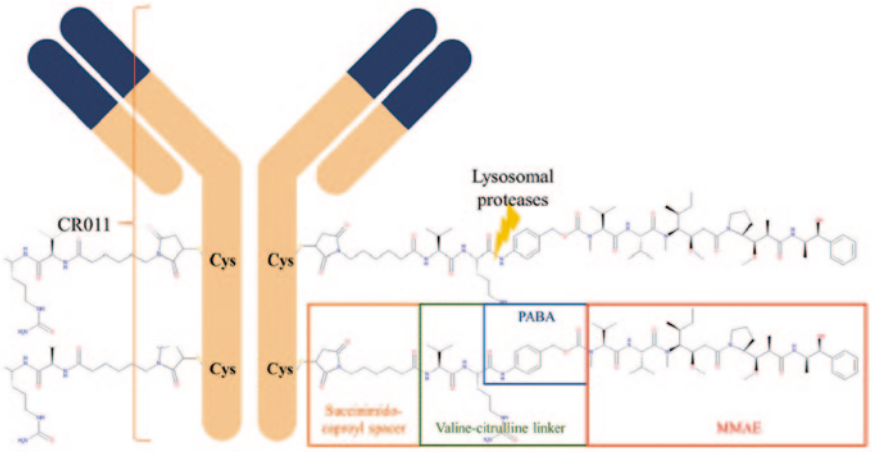

Fig. 13.2 Structure of glembatumumab vedotin. MMAE, monomethylauristatin E, PABA *p*-aminobenzoic acid spacer. (Vaklavas and Forero 2014)

13.3 Glembatumumab Vedotin

GPNMB has the ideal components required for targeted therapy. The selectively extracellular domain in tumor cells (not normal cells) enables the availability to antibody targets. Glembatumumab vedotin (or CDX-011, CR011-vcMMAE) is a fully humanized monoclonal ADC. The first component of the ADC is the tubulin destabilizing cytotoxin monomethylauristatin E (MMAE). It was originally derived from peptides found in the marine shell-less mollusk *Dolabella auricularia* known as dolastatins (Bai et al. 1990). This antimitotic agent has potency of up to 200 times compared to that of vinblastine and it was found to block the growth of GPNMB-expressing melanoma cells with half-maximal inhibitory concentration (IC_{50}) of 216–300 ng/mL (Vaklavas and Forero 2014). Therefore, the toxicity profile prevents its antineoplastic usage alone, but the high potency proves to be advantageous when MMAE is linked to an antibody, at which point it is referred to as vedotin. Vedotin is also the cytotoxic agent used with brentuximab targeting CD30 in Hodgkin's lymphoma and anaplastic large-cell lymphoma (Francisco et al. 2003).

Vedotin is conjugated through a cathepsin B-sensitive valine–citrulline linker to the antibody glembatumumab, which is directed against the extracellular domain of GPNMB (Fig. 13.2). On average, four or five MMAE molecules are bound to a single antibody (Vaklavas and Forero 2014). The ADC is highly stable in serum, and is released and internalized upon binding to GPNMB. After endocytosis, the synthetic dolastin analogue MMAE is released through enzymatic cleavage into the tumor cell cytoplasm where it binds to tubulin and inhibits tubulin polymerization. This result in G2/M phase arrest and apoptosis of the tumor cells (Tse et al. 2006).[1]

[1] http://www.cancer.gov/drugdictionary/?CdrID=599456.

13.4 Preclinical Models

Glembatumumab vedotin was first demonstrated to be efficacious in metastatic melanoma cells in culture and in xenograft assays. Pollack et al. used the human SK-MEL-2 and SK-MEL-5 melanoma xenografts in athymic mice to assess the antitumor efficacy of glembatumumab vedotin (Pollack et al. 2007). They found that it induced complete tumor regression in 100% of GPNMB-expressing xenografted melanoma cells. This was achieved at concentrations as low as 2.5 mg/kg, administered intravenously every 4 days for four treatments. Interestingly, tumors did not regrow during the nontreatment observation period of 200 days. In the breast cancer cell line MDA-MB-468, a single dose of 20 mg/kg glembatumumab vedotin was able to induce sustained tumor regression in vivo (Rose et al. 2010a).

13.5 GPNMB and Triple-Negative Breast Cancer

Rose et al. first investigated GPNMB as a potential therapeutic target in breast cancer by analyzing its expression in several breast cancer gene expression data sets and in primary human breast tumors. They found that GPNMB may serve as an important target, especially for TNBC patients whose clinical courses tend to be more aggressive with increased potential for metastasis. Since TNBC patients have limited therapeutic options, there is a tremendous need for the identification and development of precision therapy for these patients. In the Rose et al. study, the investigators first showed that GPNMB messenger RNA (mRNA) expression in 295 human breast tumors is associated with reduced metastasis-free and overall survival. This was accomplished through comparing GPNMB mRNA levels with clinical outcomes in three published data sets. GPNMB protein expression in tumor epithelium was also found to have significantly inferior outcome in human breast cancer evaluated by immunohistochemical (IHC) staining for GPNMB using breast tissue microarray data. Intriguingly, GPNMB expression correlated with recurrence within the TNBC patients. An independent cohort of breast cancer tissue microarrays enriched for TNBC samples showed that 29% of triple negative tumors are GPNMB-epithelial positive, compared with only 3.6% in luminal and 11.6% in HER2 tumors. The multivariate Cox regression survival analysis revealed that GPNMB was an independent prognostic indicator of distant metastasis in TN tumors ($p = 0.0024$). Therefore, GPNMB-epithelial expression is more common in TNBC, and within this subtype, its expression is associated with an increased risk of recurrence (Rose et al. 2010).

13.6 Clinical Trials of Glembatumumab Vedotin

Glembatumumab vedotin was first investigated in two multicenter phase I/II clinical trials for patients with unresectable melanoma and advanced stage or metastatic breast cancer (Hamid et al. 2010; Saleh et al. 2010; Burris et al. 2009). In the

melanoma study, tumor shrinkage was achieved in up to 56% of patients when glembatumumab vedotin was given intravenously at 1.88 mg/kg in the phase II maximum tolerated dose (MTD) expansion at dose frequencies ranging from weekly to q3 weeks. The median progression-free survival (PFS) was highest at 3.9 months in patients treated at the q3 week dose. Importantly, the subset of patients with strong GPNMB expression, defined as 3+by IHC with 90% staining, had higher tumor shrinkage at 86% and longer median PFS of 4.9 months. Of note, 57% of all patients developed varying degrees of skin rash, which also correlated with greater median PFS of 4.8 versus 1.2 months in those without rash. This may be related to the presence of GPNMB in skin, and act as a predictor of drug efficacy (Hamid et al. 2010).

Similarly, glembatumumab vedotin was used in heavily pretreated advanced breast cancer patients in phase I standard dose escalation followed by a phase II expansion at the MTD to assess a 12-week PFS. Eligible patients for this study had at least two prior chemotherapy regimens that included taxanes, anthracycline, and capecitabine. A total of 42 patients were enrolled, and 71% of the tumor specimens were positive for GPNMB defined as>5% of malignant epithelial or stromal cells with GPNMB expression. IHC with goat polyclonal antibody to GPNMB was performed on 25% of the patient biopsies that were available for the evaluation (done as a post hoc analysis). This study demonstrated median PFS of 9.1 weeks in all patients enrolled. In GPNMB-expressing patients at the MTD/phase II dose, PFS was doubled at 18.3 weeks. The objective response rate was also the highest amongst this subgroup at 29% (Burris et al. 2009). This suggestion that efficacy might be enhanced in tumors with higher GPNMB expression led to the design of future studies of glembatumumab vedotin in tumors that had expression of the target.

13.6.1 Toxicity Profile

Glembatumumab vedotin was generally well tolerated in the phase I/II study in advanced breast cancer patients. The most common adverse reactions (AEs) were rash (61%), fatigue (50%), and alopecia (50%). The most common grade 3 or 4 AEs are neutropenia (17%) and neuropathy (11%). Patients with baseline neuropathy worse than grade 1 were excluded after first two patients at 1.34 mg/kg dose experienced worsening of neuropathy (Burris et al. 2009).

13.6.2 Clinical Trials on the Horizon

The phase I/II studies demonstrated both efficacy and manageable safety profile of glembatumumab vedotin. The encouraging results led to the initiation of the electronic medical records and genomics (EMERGE) study, a phase II, open-label, randomized study designed to evaluate the anticancer activity of glembatumumab vedotin in advanced GPNMB-expressing breast cancer. Patients were stratified by GPNMB expression pattern (any tumor, low stromal, or high stromal) and were

Table 13.1 Clinical trials of glembatumumab vedotin

Trial	Primary endpoints	Randomization	No. of patients
Phase I/II in advanced melanoma	ORR = 15–33 %	No	117
Phase I/II in advanced breast cancer	12-week PFS = 33–100 % † in GPNMB-expressing patients	No	42
EMERGE study: Phase II in advanced breast cancer	ORR (trial completed, results pending)	Yes[a]	124
METRIC study: Phase II in metastatic GPNMB-expressing TNBC	ORR and PFS (currently enrolling)	Yes[b]	300

ORR overall response rate, *PFS* progression-free survival
[a] Randomization of glembatumumab vedotin versus investigator's choice chemotherapy
[b] Randomization to receive glembatumumab vedotin or capecitabine
† = in GPNMB-expressing patients

randomized in 2:1 fashion to glembatumumab vedotin (1.88 mg/kg IV every 3 weeks) or investigator's choice (IC) single-agent chemotherapy. Patients were treated until progression of disease (PD) or intolerance to therapy. In this study, patients on the IC arm were allowed to crossover at PD. The primary endpoint is overall response rate (ORR) and secondary endpoints are PFS, toxicity profile, and pharmacokinetics. Preliminary analysis of the study was presented at San Antonio breast symposium in 2012, and the results were promising in the enhanced activity in TNBC and high GPNMB-expressing breast cancer tumors. The study has been completed and the final results are expected to be published at the end of 2014 (Yardley et al. 2012).

In order to understand the activity of the drug in TNBC patients, the randomized phase II METRIC study has also been initiated to assess the efficacy in women with metastatic GPNNB-overexpressing TNBC. Patients with GPNMB-overexpressing TNBC (expression > 25 %) will be randomized to receive glembatumumab vedotin or capecitabine in a 2:1 ratio. The primary endpoints are ORR and PFS. This study is currently enrolling patients from multiple centers.[2]

Studies of glembatumumab vedotin in other GPNMB positive cancers such as osteosarcoma, squamous lung cancer, and melanomas are also under consideration (Table 13.1).

[2] Clinicaltrials.gov. Study of glembatumumab vedotin (CDX-011) in patients with metastatic, gpNMB overexpressing, triple-negative breast cancer (METRIC); 2014. http://clinicaltrials.gov/ct2/show/NCT01997333.

13.7 Conclusion

GPNMB is a relevant target in breast cancer, and the ADC glembatumumab vedotin has been demonstrated in clinical trials to be efficacious in patients with advanced and metastatic disease. Triple-negative breast cancers may have higher GPNMB expression, which provides the rare opportunity of targeted therapy for this subtype. Further confirmatory clinical trials are underway and we eagerly await the results of the randomized phase II EMERGE and METRIC trials. Glembatumumab vedotin will continue to be explored in other GPNMB-expressing malignancies.

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Part VI
From Today to Tomorrow

Chapter 14
Summary and Future Directions of ADCs

Jennica L. Zaro, Jeffery Wang and Wei-Chiang Shen

14.1 Summary on the Current Status of ADC Design

Antibody-drug conjugates (ADCs) have become a powerful class of therapeutics in oncology. With two recently approved drugs on the market, Kadcyla® and Adcetris®, and more than 40 in clinical trials (Table 14.1), ADCs represent a growing class as the next generation for cancer treatment. By 2018, the sales of ADCs are expected to exceed US$ 5 billion. Over 100 ADCs are currently being developed in the preclinical stage in various companies (Thayer 2014).

In the design of an ADC, there are five important considerations, including (1) the molecular target; (2) the antibody; (3) the details of the chemical conjugation; (4) the linker; and (5) the cytotoxic payload (Fig. 14.1).

14.1.1 Molecular Targets

There are currently a vast range of target antigens or receptors utilized in ADCs. The majority of the targets represent molecules that are internalized via receptor-mediated endocytosis (RME), while there are a few targets that remain at the cell surface or in the tumor vasculature. For targets that internalize, the optimal conditions in ADC design are that they should show abundant and uniform expression on tumor cells, have no or low expression in healthy tissues and organs, exhibit rapid internalization following ADC binding, and traffic to the lysosomes (Carter and Senter 2008;

J. L. Zaro (✉) · W.-C. Shen
Department of Pharmacology and Pharmaceutical Sciences,
University of Southern California, Los Angeles, CA, USA
e-mail: zaro@usc.edu

J. Wang
Department of Pharmaceutical Sciences, Western University of Health Sciences,
Pomona, CA, USA

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Table 14.1 ADCs in the clinic

ADC	Target	Drug	Linker	Lead sponsor(s)	Phase	Indication
ABBV-399	Undisclosed			AbbVie	I	Advanced solid tumors
ABT-414	EGFR	MMAF	Noncleavable (*maleimidocaproyl*)	AbbVie	I II	Glioblastoma multiforme Squamous cell tumors
AGS-16C3F/ AGS-16M8F	ENPP3	MMAF	Noncleavable (*maleimido-caproyl*)	Astellas Pharma; Agensys	I	RCC
AMG 172	CD27L	DM1	Noncleavable (*thioether, SMCC*)	Amgen	I	RCC
AMG 595	EGFRvIII	DM1	Noncleavable (*thioether, SMCC*)	Amgen	I	Glioma
Anti-5T4 (PF-06263507)	5T4	MMAF	Noncleavable (*maleimido-caproyl*)	Pfizer; Seattle Genetics	I	Solid tumors
Anti-MUC16 (DMUC5754A, RG7458)	Mucin 16	MMAE	Cleavable (*protease, Val-Cit*)	Genentech; Seattle Genetics linker technology	I	Ovarian cancer
Anti-NaPi2b (DNIB0600A, RG7599)	NaPi2b	MMAE	Cleavable (*protease, Val-Cit*)	Genentech	I II	Nonsquamous nonsmall cell lung cancer; ovarian cancer Platinum-resistant ovarian cancer
Anti-STEAP1 (DSTP3086S, RG7450)	STEAP	MMAE	Cleavable (*protease, Val-Cit*)	Genentech	I	Prostate cancer
ASG-15ME	SLITRK6	MMAE	Cleavable (*protease, Val-Cit*)	Astellas Pharma; Agensys; Seattle Genetics	I	Metastatic urothelial cancer
ASG-22ME	Nectin-4	MMAE	Cleavable (*protease, Val-Cit*)	Astellas Pharma; Agensys; Seattle Genetics	I	Metastatic urothelial cancer

Table 14.1 (continued)

ADC	Target	Drug	Linker	Lead sponsor(s)	Phase	Indication
ASG-5ME	SLC44A4	MMAE	Cleavable (*protease, Val-Cit*)	Astellas Pharma; Agensys; Seattle Genetics	I	Pancreatic cancer; Stomach cancer
BAY94-9343	Mesothelin	DM4	Cleavable (*Disulfide, SPDP*)	Bayer	I	Solid tumors; mesothelioma
Brentuximab Vedotin (SGN-35) (Adcetris®)	CD30	MMAE	Cleavable (*protease, Val-Cit*)	Seattle Genetics	I II III Approved	AML CD30+GCT; DLBCL CD30+PTCL HL; ALCL
DEDN6526A (RG-7636)	ETBR	MMAE	Cleavable (*protease, Val-Cit*)	Genentech	I	Malignant melanoma
Gemtuzumab ozogamicin	CD33	Cali-chDMH	Cleavable (*pH sensitive, AcBut*)	Pfizer	III	Relapsed AML
Glembatumu-mab vedotin (CDX-011)	GPNMB	MMAE	Cleavable (*protease, Val-Cit*)	Celldex Therapeutics	II	GPNMB overexpressing breast cancer
GSK2857916	BCMA	MMAF	Noncleavable (*maleimido-caproyl*)	GlaxoSmithKline	I	MM
HuMax®-TF-ADC	TF	MMAE	Cleavable (*protease, Val-Cit*)	Genmab	I	Solid tumors
IMGN-289	EGFR	DM1	Noncleavable (*thioether, SMCC*)	ImmunoGen	I	EGFR-positive tumors
IMGN-529	CD37	DM1	Non-cleavable (*thioether, SMCC*)	ImmunoGen	I	B-cell lymphoma; CLL; NHL
IMGN-853	Folate receptor	DM4	Cleavable (*disulfide, SPDP*)	ImmunoGen	I	Ovarian tumor; solid tumor
IMMU-132	Trop-2	SN-38	Cleavable (*pH-sensitive carbonate, CL2A*)	Immunomedics	I/II	Epithelial cancers
Indatuximab ravtansine (BT-062)	CD138	DM4	Cleavable (*disulfide, SPDP*)	Biotest; Immu-noGen TAP technology	II	MM

Table 14.1 (continued)

ADC	Target	Drug	Linker	Lead sponsor(s)	Phase	Indication
Inotuzumab ozogamicin (CMC-544)	CD22	Cali-chDMH	Cleavable (*pH sensitive, AcBut*)	Pfizer	II III	DLBCL ALL
Labetuzumab-SN-38 (IMMU-130)	CEACAM5	SN-38	Cleavable (*pH-sensitive carbonate, CL2A*)	Immunomedics	II	Colorectal cancer
Lorvotuzumab mertansine (IMGN-901)	CD56	DM1	Cleavable (*disulfide, SPDP*)	ImmunoGen	II	MM; solid tumors; SCLC
Milatuzumab-Dox	CD74	DOX	Cleavable (*protease, Phe-Lys, CL2E*)	Immunomedics	I/II	MM; CLL; NHL
MLN0264	Guanylyl cyclase	MMAE	Cleavable (*protease, Val-Cit*)	Millennium Pharmaceuticals	I	Advanced gastrointestinal malignancies
Pinatuzumab Vedotin (DCDT2980S, RG7593)	CD22	MMAE	Cleavable (*protease, Val-Cit*)	Genentech	II	NHL
PMSA-ADC	PMSA	MMAE	Cleavable (*protease, Val-Cit*)	Progenics	II	Prostate cancer
Polatuzumab vedo-tin (DCDS4501A, RG7596)	CD79b	MMAE	Cleavable (*protease, Val-Cit*)	Genentech	II	NHL
RG7598	Undisclosed			Genentech	I	MM
RG7600	Undisclosed			Genentech	I	Ovarian, pancreatic cancer
SAR3419	CD19	DM4	Cleavable (*disulfide, SPDP*)	Sanofi	I II	NHL DLBCL; ALL

Table 14.1 (continued)

ADC	Target	Drug	Linker	Lead sponsor(s)	Phase	Indication
SAR566658	Mucin 1	DM4	Cleavable (*disulfide, SPDP*)	Sanofi	I	Solid tumor
SC16LD6.5	SC-16	D6.5	undisclosed	Stem CentRx	I/II	SCLC
SGN-CD19A	CD19	MMAF	Noncleavable (*maleimido-caproyl*)	Seattle Genetics	I	ALL, NHL
SGN-CD33A	CD33	SGD-1882	Cleavable (*protease, Val-Ala*)	Seattle Genetics	I	AML
SGN-LIV1A	SLC39A6	MMAE	Cleavable (*protease, Val-Cit*)	Seattle Genetics	I	Breast cancer
Trastuzumab Emtansine (Kadcyla®)	Her2	DM1	Noncleavable (*thioether, SMCC*)	Genentech	Approved	HER2-positive metastatic breast cancer

AcBut 4-(4'-acetylphenoxy) butanoic acid, *ALCL* anaplastic large cell lymphoma, *ALL* acute lymphocytic leukemia, *AML* acute myeloid leukemia, *BCMA* B-cell maturation antigen, *CalichDMH N*-acetyl-gamma-calicheamicin dimethyl hydrazide, *CLL* chronic lymphocytic leukemia, *DLBCL* diffuse large B-cell lymphoma, *DM1/DM4* maytansinoid analogues, *DOX* doxorubicin, *EGFR* Epithelial growth factor receptor, *ENPP3* ectonucleotide pyrophosphatase/ phosphodiesterase family member 3, *ETBR* endothelin B receptor, *GCT* germ cell tumors, *GPNMB* glycoprotein NMB, *HL* Hodgkin lymphoma, *MM* multiple myeloma, *MMAE* monomethyl auristatin E, *MMAF* monomethyl auristatin F, *NaPi2b* sodium-dependent phosphate transport protein 2b, *NHL* Non-Hodgkin's lymphoma, *PMSA* prostate-specific membrane antigen, *PTCL* peripheral T-cell lymphoma, *RCC* renal cell carcinoma, *SC-16* scr-like kinase Fyn3, *SCLC* small cell lung cancer, *SMCC* succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate crosslinker, *SN-38* irinotecan metabolite, *SPDP* N-succinimidyl 3-(2-pyridyldithio)-propionate cross-linker, *STEAP* six-transmembrane epithelial antigen of prostate 1, *TF* Tissue factor

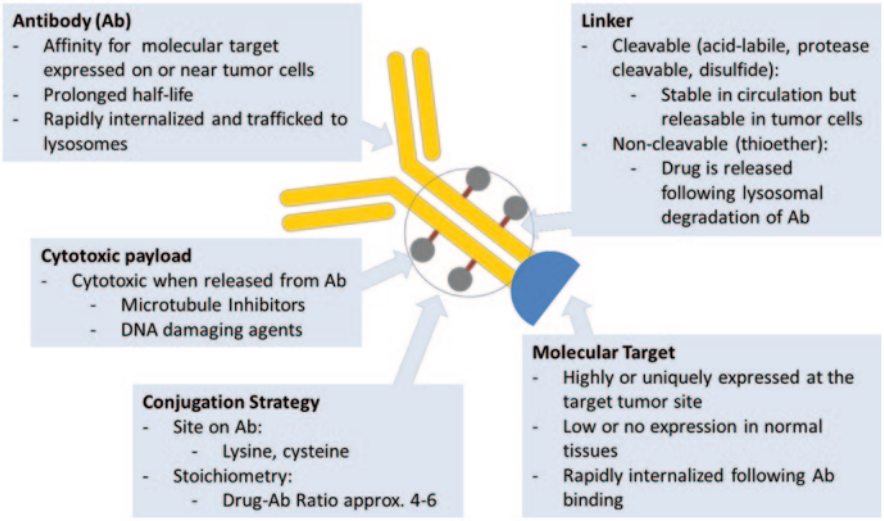

Fig. 14.1 Important components of an ADC. The main considerations in the design of ADC include the molecular target, the antibody, the details of chemical conjugation, the cytotoxic payload, and the linker between the antibody and cytotoxic payload

Teicher and Chari 2011; Fig. 14.2). The molecular target for one of the currently Food and Drug Administration (FDA)-approved ADCs (Adcetris®), CD30, fulfills this criteria very well. CD30 exhibits high uniform expression, low expression in normal tissues, and is rapidly internalized following antibody binding (Falini et al. 1995). However, many other ADCs do not fulfill the entire list of ideal criteria. For instance, the molecular target of Kadcyla®, HER2, does show high expression in breast cancer tissue and rapid internalization following antibody binding; however, it is also expressed in other tissues including the skin, heart, gastrointestinal (GI), respiratory, reproductive, and urinary tracts (Press et al. 1990). Nonetheless, the ADC is still well tolerated due to lower HER2 expression in vital organs compared to the target tumor cells. Another example of a less-than-ideal antigen expression is CD33, the molecular target of Mylotarg®. CD33 shows low expression density, however, its uniform expression on acute myeloid leukemia (AML) blast cells in the majority of patients, coupled with the lack of expression in hematopoietic stem cells, lymphoid cells, and nonhematopoietic cells (Dinndorf et al. 1986; Peiper et al. 1988) helps it overcome the low expression issue. New noninternalizing targets that are expressed in tumor extracellular matrix or neo-vasculature are also being identified. The advantage of these types of targets is that they are more easily accessed from the blood stream. Examples mainly include angiogenesis markers, such as the extradomain B of fibronectin (e.g., DEDN6526A by Genentech, Table 14.1; Perrino et al. 2014; Palumbo et al. 2011; Bernardes et al. 2012), or tissue-specific glycoproteins such as prostate-specific membrane antigen (PSMA; e.g., PMSA-ADC by Progenics, Table 14.1; Wang et al. 2011). These noninternalizing ADCs either rely on a labile linker that will release the drug in close proximity to the target cell sur-

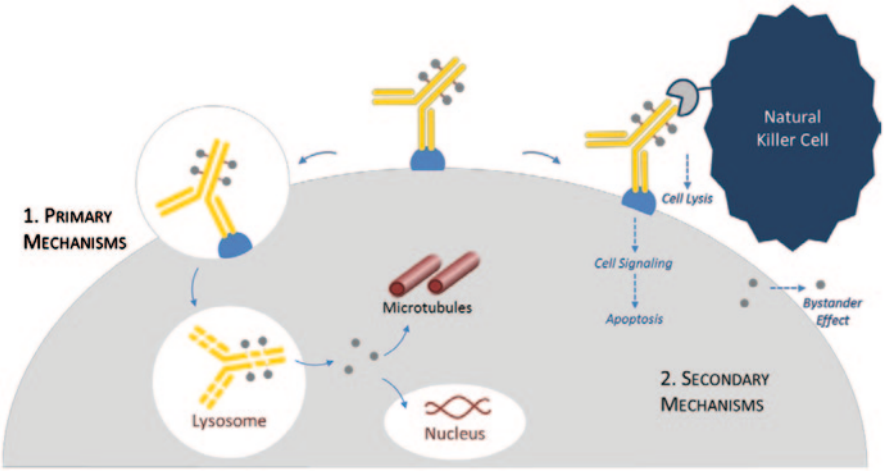

Fig. 14.2 Overview of ADC internalization and mechanisms of toxicity. Following binding to the cell surface receptor, the ADC is internalized by receptor-mediated endocytosis. The cytotoxic drug is released either by cleavage of the linker or degradation of the antibody, and then exerts its cytotoxicity (*1*). The two main classes of drugs used in ADCs either disrupt microtubule assembly (e.g., DM1, DM4, MMAE, MMAF) or cause DNA strand cleavage (e.g., calicheamicin analogs). Certain antibodies and isotypes (e.g., IgG1 and IgG3) also cause cell death via secondary mechanisms (*2*). Linkage of the drug to the antibody via cleavable linker may also lead to cell death in neighboring cells (bystander effect) (*2*)

face (e.g., disulfide linker by Perrino et al. 2014), or on the use of a drug with active toxicity outside the cell (e.g., photosensitizers by Palumbo et al. 2014).

Compared to the other important considerations in ADC design (chemical conjugation, linker, and cytotoxin), most of the diversity when comparing ADCs in clinical development is their molecular targets and thus the antibody (Table 14.1). The molecular targets range from tumor cell surface antigens (e.g., HER2 as in Kadcyla®) to surface receptors such as epithelial growth factor receptor (EGFR; IMGN-289) and folate receptor (IMGN-853), cell adhesion molecules such as Nectin-4 (ASG-22ME), and glycoproteins such as Mucin 1 (SAR566658) and GPNMB (CDX-011). Moving forward, the main hurdles in molecular targets are on answering key questions regarding the required tumor antigen density and heterogeneity, and the acceptable levels of expression in normal tissues and organs. Many ADC studies have indicated that the therapeutic effect correlates with antigen density; however, recent work has shown that the expression level may not be the main determinant for ADC efficacy. A study on an anti-CD79b ADC (DCDS4501A) showed that, in cell lines with CD79b expression levels above a minimum threshold, there was a nonlinear correlation of expression levels with in vitro activity (Dornan et al. 2009). Therefore, a minimum threshold may exist, which may be different for different targets. For the issue of off-target expression in healthy tissues, important considerations include whether or not the expression is in vital organs or regenerative tissues, and also the accessibility to the ADC. For instance, expression

in bone marrow may represent a manageable toxicity, since B cells and myeloid cells can regenerate in most cases. Another good example is PMSA, which is expressed in both prostate cancer cells and healthy prostate tissue. Prostatic toxicity is not a serious health concern though, and most prostate cancer patients would have their prostate removed. PMSA is also expressed on the apical membrane in kidney and small intestine. While toxicity in these organs would be problematic, the apical membrane of the cells is not easily accessible to ADCs which are constrained to the basolateral side (Horoszewicz et al. 1987; Israeli et al. 1993). Further understanding of the relationship between expression/specificity of molecular targets and efficacy/side-effect profiles will aid in the future development of ADCs.

14.1.2 Antibody Considerations

The antibody component is the important determinant of the targeting efficiency and pharmacokinetics (PK) of the ADC (Fig. 14.1). The main factors related to the antibody component of ADCs are the size, trafficking, intrinsic antibody effector functions, and Fc-receptor binding. Although some differences exist due to factors related to drug conjugation, ADCs typically have low clearance and long half-lives similar to the parent antibody. The long half-life is imparted by the Fc receptor-recycling pathway following binding of the Fc-region of the antibody (Janeway et al. 2001). As will be discussed later, the half-life and clearance of ADCs can differ slightly than the parent antibody component due to the drug–antibody ratio (DAR) and the additional metabolic pathway of drug cleavage.

One of the main considerations for the antibody design is its subclass (i.e., IgG1, IgG2, IgG3, or IgG4), which can affect its intrinsic effector function due to differences in the Fc region. IgG1 and IgG3 subclasses are typically more active in both antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), while IgG4 tends to lack effector functions (Janeway et al. 2001). Engagement of these secondary immune functions is considered beneficial for antitumor activity, but may have the unintended effect of reducing tumor localization. IgG1, the most common subclass in ADCs, is used for both Kadcyla®, which is highly active in ADCC (Junttila et al. 2011), and Adcetris®, which has modest ADCC activity (Oflazoglu et al. 2007). Mylotarg®, with an IgG4 variant, has no ADCC or CDC activity (Bross et al. 2001).

With regard to future improvements on the antibody component of the ADC, one approach is utilization of smaller antibody fragments (Wu and Senter 2005). These fragments, including “diabodies,” “minibodies,” and “nanobodies,” conceivably offer an advantage of higher tumor penetration due to their smaller size (Wu and Senter 2005), but have not yet been successful in the clinic. One perceivable drawback is a reduction in ADC half-life due to its smaller size. A second approach to improve antibody function, engineering strategies to allow for site-specific modification, will be discussed in the next section.

14.1.3 Chemical Conjugation Strategies

The site of conjugation and DAR both play important roles in the success of the ADC since they can affect the PK/PD, binding affinity, and aggregation state. The majority of the ADCs in the clinics take advantage of either an ε-amino group of lysine or a sulfhydryl group of cysteine (Lyon et al. 2012). In the case of lysine modification, the drug molecules are distributed across many lysine residues in the antibody with a range of DARs. Modification of cysteine residues involves reduction of disulfide bonds in the hinge region of the antibody and results in an evenly number DAR. Kadcyla® (3.5 DM1 molecules per antibody) and Adcetris® (~4 MMAE molecules per antibody) are produced via the lysine modification and cysteine coupling routes, respectively. Although conjugation of more drug molecules to the antibody component would result in delivery of a larger payload, it often results in higher aggregation of the final product, as well as poor PK and binding affinity compared to the parent antibody. With the drug conjugation, the half-life of the ADC tends to be shorter (several days) than the unmodified antibody (several weeks) (Chari 2008; Hamblett et al. 2004; Senter and Sievers 2012). Therefore, the average DAR is usually maintained at 3–4 in order to preserve the long half-life and high antibody-binding efficiency.

One of the other major drawbacks in the current chemical conjugation strategies is the generation of a heterogeneous final product, with multiple DARs at various sites. It has been calculated that conjugation through modification of lysine residues can distribute the drug molecules to approximately 40 different sites, resulting in $>10^6$ different ADC species (Wang et al. 2005). Conjugation via cysteine residues results in less heterogeneity, but can still yield >100 ADC species (Hamblett et al. 2004). Although none have yet reached the clinics, several different companies are focusing on site-specific modification methods to overcome this issue resulting in generation of a homogeneously loaded ADC. The major technologies include the use of engineered cysteine residues, incorporation of unnatural amino acids, and enzymatic conjugation as described below.

14.1.3.1 Engineered Cysteine Residues

One of the strategies to enable site-specific modification involves the introduction of cysteine residues into the antibody sequence by single-point mutation. This technology developed by Genentech, termed ThioMAb, first utilizes phage-display technology to select a suitable conjugation site. Next, cysteine residues (typically $n=2$) are introduced to the antibody amino acid sequence by single-point mutation. The drug is then conjugated to the engineered cysteines via a maleimide linker (Junutula et al. 2008; Shen et al. 2012).

14.1.3.2 Incorporation of Unnatural Amino Acids

Two different companies, Sutro Biopharma and Ambryx, are using nonnatural amino acid-based approaches to site-specifically modify ADCs. In this approach, unique functional groups not present in natural amino acids can be incorporated and specifically modified using bio-orthogonal chemistries. These techniques overcome limitations of maleimide and disulfide exchange chemistries seen with cysteine modification, and do not require further enzymatic processing steps. In Ambryx technology, a *p*-acetylphenylalanine group (pAcPhe) is incorporated, which introduces a keto group that can be selectively coupled to an alkoxy-amine derivatized drug to form a stable oxime bond linkage. Sutro Biopharma technology involves the introduction of a *p*-azidomethyl-L-phenylalanine (pAMF) group into the antibody, which can be reacted with the drug molecule via strain-promoted azide-alkyne cycloaddition (SPAAC) click chemistry (Zimmerman et al. 2014).

14.1.4 Enzymatic Conjugation

Innate Pharma is utilizing the enzymatic posttranslational modification to enable precise site-specific conjugation. This technology requires either the removal of *N*-linked glycans from the antibody, or the use of mutant aglycosylated variants generated by site-directed mutagenesis. Subsequently, cadaverine-derived drug substrates are conjugated to the antibody using bacterial transglutaminase (BTGase), resulting in the site-specific conjugation (Behrens and Liu 2014).

In addition to these approaches, many other site-specific technologies are being actively developed, including conjugation to native glycan residues (GlycoConnect™ by SynAffix), targeting the cysteine-rich hinge region (ThioBridge by PolyTherics and Stapled ADC technology by Igenica), and the "medtope-enabled" technology by Medtope Biosciences.

14.1.5 Linkers

Linker stability in the ADC must be sufficient enough in systemic circulation to reduce overall toxicity at off-target sites and maintain a prolonged half-life, but it must be rapidly cleaved at the target tumor site to release enough drug molecules to be efficacious. The linkers utilized for drugs in the clinics are classified as either cleavable or noncleavable (Table 14.1). Cleavable linkers take advantage of the different extracellular versus intracellular properties to release the conjugated drug inside the cells. For noncleavable linkers, the antibody portion of the ADC must be degraded in order to release the cytotoxic drug molecule.

The majority of ADCs currently in the clinics are produced using cleavable linkers, which include disulfide, pH-sensitive, and protease-sensitive linkers

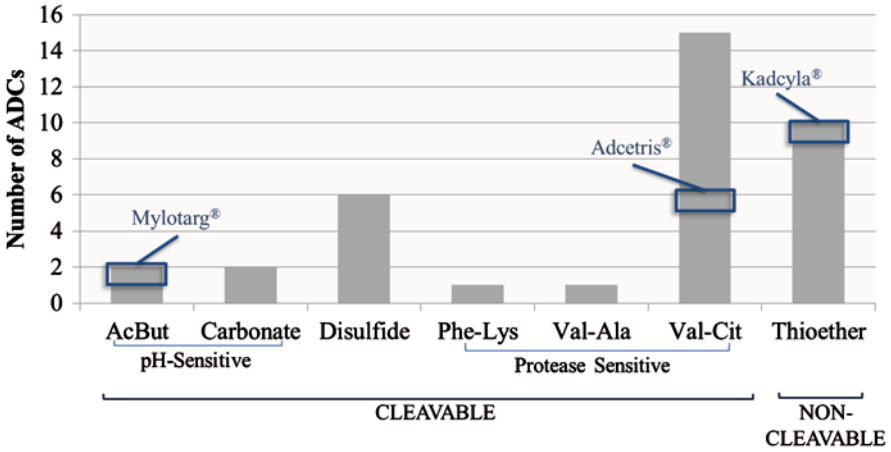

Fig. 14.3 Comparison of linkers used in ADCs in the clinic. The linkers used for ADCs in clinical trials shown in Table 14.1 were compared

(Table 14.1, Fig. 14.3). The disulfide linkers are used in several ADC (Table 14.1, BAY94-9343, IMGN-853, BT-062, IMGN -901, SAR3419, and SAR-566658), and exploit the higher concentration of thiols inside cells relative to the blood stream. These linkers are relatively stable in circulation, and can be further stabilized by introducing steric hindrance by flanking disulfide bonds with methyl groups (Sapra et al. 2011). Following RME, the ADCs are exposed to a mildly to moderately acidic environment in the endosomes and lysosomes (Fig. 14.2). Taking advantage of this condition, several ADCs have been made utilizing pH-sensitive linkers, such as 4-(4-acetylphenoxy)butanoic acid or CL2 A (Immunomedics), to conjugate the drug to the antibody via a hydrazone (e.g., Mylotarg®, CMC-544) or carbonate (IMMU-130 and IMMU-132) bond, respectively. The most common type of cleavable linkers used is the protease-sensitive type (Table 14.1). These linkers take advantage of the specific localization of lysosomal enzymes (e.g., cathepsin or plasmin) present at elevated levels in certain tumor tissues (Dubowchik and Firestone 1998). These proteases are also usually only active in the moderately acidic environment of the lysosome, offering an additional layer of specificity. The majority of ADCs with protease-sensitive linkages utilize a Seattle Genetics-developed maleimido-containing valine-citrulline (val-cit) linker, cleavable by cathepsin B. Immunomedics has also developed a protease-sensitive linker, CL2E (as in Milatuzumab-Dox, Table 14.1).

The ADCs generated with noncleavable linkers mainly utilize a thioether bond to link the drug to the antibody. ADCs with noncleavable linkers are found to be better tolerated, presumably due to a higher stability in systemic circulation. One main consideration of utilizing a noncleavable linker is whether or not the cytotoxic drug is still active despite being chemically modified. For example, Kadcyla®, linked via a noncleavable thioether linkage, releases the free drug as a lysine-MCC-DM1 analog which has similar potency to DM1 (LoRusso et al. 2011).

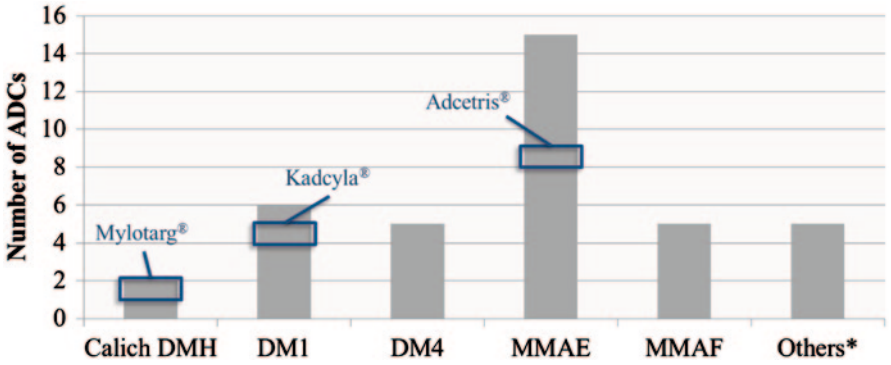

Fig. 14.4 Comparison of drugs used in ADCs in the clinic. The drugs used for ADCs in clinical trials shown in Table 14.1 were compared. (*Others include SGD1882 (pyrrolobenzodiazepine), SN38 (irinotecan metabolite), doxorubicin and D6.5 (a DNA-damaging agent.))

Recent studies by ImmunoGen, Inc. have also been focused on using a linker to overcome multidrug resistance (MDR), which has been linked with poor clinical responses in ADCs (Walter et al. 2003; Linenberger 2005). In order to evade the MDR1-resistance, a polyethylene glycol (PEG) spacer was used to link the cytotoxic drug, DM1, to the antibody. The results showed that the PEG-linked conjugates were more potent in MDR1-expressing cells, presumably due to the increased hydrophilicity of the PEG-linked DM1 which keep the drug inside the cell (Kovtun et al. 2010).

Despite the numerous advances in linker technology, there is no universal formula in their application to ADCs. Most studies have shown that the linker function is highly dependent on the main components of the ADC (the drug and antibody), and on the target site. Therefore, each antibody must be optimized on an individual basis to determine the most appropriate linker.

14.1.6 Cytotoxic Payloads

There are two main classes of drugs used in ADCs, antimitotic drugs that disrupt microtubule assembly and play an important role in mitosis (e.g., maytansine analogs DM1 and DM4, auristatin analogs MMAE and MMAF), and drugs that bind to the minor groove of DNA causing DNA double-strand cleavage (e.g., calicheamicin analogs) (Fig. 14.2). The antimitotic drugs, MMAE and DM1/DM4 are the most commonly used (Table 14.1, Fig. 14.4), and also have an additional layer of specificity due to their preferential destruction of highly proliferating cells and to the increased sensitivity of malignant cells to mitotic catastrophe. Both classes of ADC drugs have a much higher potency (100–1000-fold) compared to conventional chemotherapy agents such as doxorubicin and paclitaxanes (Pietersz et al. 1994; Tolcher et al. 1999).

Although ADCs are targeted toward the tumor site, it is estimated that only 1–2 % of the administered dose will accumulate there (Teicher and Chari 2011), indicating the requirement for high drug potency. Consequently, one of the main focuses on ADCs is on developing more potent compounds. One example is a pyrrolobenzodiazepine (PBD) dimer, SGD1882, utilized in Seattle Genetics' anti-CD33 ADC (SGN-CD33 A). PBDs are naturally occurring antitumor antibiotics (e.g., anthramycin, sibiromycin) produced by *Streptomyces* that initiate cell damage through DNA cross-linking, leading to cell death (Hartley and Hochhauser 2012; Smellie et al. 2003). Some PBD analogs are more potent than currently used drugs in ADCs and are also less sensitive to the P-glycoprotein (Pgp) resistance mechanism (Aird et al. 2008; Guichard et al. 2005). Pgp resistance has been linked with poor clinical responses in ADCs (Walter et al. 2003; Linenberger 2005), therefore overcoming this MDR mechanism is important. Another recent drug used in ADCs is α-amanitin, a cyclic octapeptide mushroom toxin. α-Amanitin strongly binds to RNA polymerase II, and through a series of events, induces cell death via apoptosis (Magdalan et al. 2010).

Taking another direction, some companies such as Immunomedics are working on ADCs using less toxic drugs, such as doxorubicin (Milatuzumab-Dox) and camptothecin analog SN-38 (IMMU-130 and IMMU-132), rationalizing that the use of a less-toxic drug with better targeting will improve the therapeutic index of the ADC.

Although there is a great deal of variability in the antigen/antibody and linker components of ADCs, the types of drug used are somewhat limited. Developing new drugs suitable for ADCs is a difficult task. In order to work with the ADC, the drug needs to have a high cytotoxicity; the target of the drug needs to be inside the cell; its size should be small to reduce the risk of immunogenicity and issues with aqueous solubility; it must be stable in circulation; and it should have an available linkage site in order to conjugate it to the antibody. Therefore, there is still plenty of room to grow in the identification of new, suitable drugs for ADCs.

14.2 Conclusions

After several decades of trials and errors, ADCs as the magic bullets for cancer treatment have become a reality, forming the newest class of cancer therapeutics. With two approved drugs performing well in the clinic and more than three dozen in various stages of clinical investigations, the future of ADC drug discovery and development look extremely bright. The paradigm of cancer treatment might shift to antigen identification and targeting from the current one based on the cancer origin of organ. This will facilitate the individualization or personalization of cancer therapy with greater efficacy and reduced side effects. It is expected that more indications will be approved for Adcetris® and Kadcyla® and two more ADCs be added to the approved list for cancer patient care before the current decade ends. Optimization of the payload, the targeting antibody, and the linker technology will

generate the next generation of ADCs, with greater drug-delivery efficiency, desirable adverse reaction profile, and reduced chance of drug resistance and cancer metastasis.

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Index

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