Pharm Res (2015) 32:3541–3571
DOI 10.1007/s11095-015-1704-4

EXPERT REVIEW

Antibody-Drug Conjugates: Design, Formulation and Physicochemical Stability

Satish K. Singh[1] • Donna L. Luisi[2] • Roger H. Pak[2]

Received: 19 December 2014 / Accepted: 23 April 2015 / Published online: 19 May 2015

ABSTRACT The convergence of advanced understanding of biology with chemistry has led to a resurgence in the development of antibody-drug conjugates (ADCs), especially with two recent product approvals. Design and development of ADCs requires the synergistic combination of the monoclonal antibody, the linker and the payload. Advances in antibody science has enabled identification and generation of high affinity, highly selective, humanized or human antibodies for a given target. Novel linker technologies have been synthesized and highly potent cytotoxic drug payloads have been created. As the first generation of ADCs utilizing lysine and cysteine chemistries moves through the clinic and into commercialization, second generation ADCs involving site specific conjugation technologies are being evaluated and tested. The latter aim to be better characterized and controlled, with wider therapeutic indices as well as improved pharmacokinetic-pharmacodynamic (PK-PD) profiles. ADCs offer some interesting physicochemical properties, due to conjugation itself, and to the (often) hydrophobic payloads that must be considered during their CMC development. New analytical methodologies are required for the ADCs, supplementing those used for the antibody itself. Regulatory filings will be a combination of small molecule and biologics. The regulators have put forth some broad principles but this landscape is still evolving.

KEY WORDS antibody-drug conjugate · formulation · linker · payload · physicochemical characteristics

Satish K. Singh, Donna L. Luisi and Roger H. Pak contributed equally to this work.

✉ Roger H. Pak
roger.pak@pfizer.com

[1] Pfizer, Inc., Pharmaceutical R&D, 700 Chesterfield Parkway West, Chesterfield, Missouri 63017, USA

[2] Pfizer, Inc., Pharmaceutical R&D, 1 Burtt Road, Bldg. K, Andover, Massachusetts 01810, USA

ABBREVIATIONS

AcBut	4-(4′-acetylphenoxy) butanoic acid
AcPAc	3-acetylphenyl acetic acid
ADA	Anti-drug antibody
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cell-mediated cytotoxicity
AF	Auristatin F
AUC	Area under the curve
bac	Bromoacetamido moiety
BLA	Biologics License Application
C2-keto-Gal	2-acetonyl-2-deoxy-galactose
Calich	*N*-acetyl gamma calicheamicin
CD	Circular dichroism spectroscopy
CDC	Complement-dependent cytotoxicity
CHO	Chinese hamster ovary cells
DAR	Drug antibody ratio
DM	Disulfide-containing maytansinoids
DMA	Dimethyl acid linker
DMH	Dimethyl hydrazide linker precursor
Doe	Dolaphenine
DP	Drug product
DS	Drug substance
DSC	Differential scanning calorimetry
DSI	Drug substance intermediate
DTNB	Dithionitrobenzoate
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ESI-MS	Electrospray ionization mass spectroscopy
EVA	Ethylvinyl acetate
Fab	Fragment antigen-binding
FcRn	Neonatal Fc receptor for IgG
FcγR	Fc gamma receptor for IgG
FTIR	Fourier transform infrared spectroscopy

GalNAz	*N*-acetyl-azido-galactosamine
GlcNAc	*N*-acetyl-D-glucosamine
HAHA	Human antibody-human antibody immune response
HC	Heavy chain of IgG
HDPE	High density polyethylene
HIC	Hydrophobic interaction chromatography
HIPS	Hydrazine-iso-Pictet-Spengler ligation
huFcγR	Human Fc gamma receptor for IgG
IND	Investigational New Drug
IV	Intravenous
LC	Light chain of IgG
LDPE	Low density polyethylene
mAb	Monoclonal antibody
MALDI-TOF	Matrix Assisted Laser Desorption/ Ionization Time of Flight
mc	Maleimidocaproyl linker
MCC	[*N*-maleimidomethyl]cyclohexane-1-carboxylate
MMAD	Monomethyl auristatin D
MMAE	Monomethyl auristatin E
MMAF	Monomethyl auristatin F
mTG	Microbial enzyme transglutaminase
MTX	Methotrexate
nAF	Oxyamine-auristatin F containing a short polyethylene glycol spacer
NEM	*N*-ethylmaleimide
nnAA	Non-native amino acids
OBP	Office of Biotechnology Products
ONDQA	Office of New Drug Quality Assessment
*p*abc	*p*-aminobenzyloxycarbonyl
*p*AcPhe	*p*-acetylphenylalanine
PBD	Pyrrolobenzodiazepine
PD	Pharmacodynamics
PEG	Polyethylene glycol
PETG	Polyethylene terephthalate glycol
PK-PD	Pharmacokinetic-pharmacodynamic
PTFE	Polytetrafluoroethylene
RP-HPLC	Reverse phase high performance liquid chromatography
scFv	Single-chain variable fragment
SEC-HPLC	Size exclusion column high performance liquid chromatography
SMCC	Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate
SPDP	*N*-succinimidyl-3-(2-pyridyldithio) propionate
TCEP	Tris(2-carboxyethyl)phosphine
TDC	THIOMAB drug conjugate
T-DM1	Ado-trastuzumab emtansine (Kadcyla)
UF/DF	Ultrafiltration/Diafiltration
vc	Valine-citrulline cleavable linker

INTRODUCTION

Antibody therapeutics have had considerable success for patients with solid tumors and hematological malignancies. In the last two decades, the US Food and Drug Administration has approved about a dozen antibody-based therapies to treat certain cancer types (1) and there are currently a large number of ongoing early and late stage clinical trials for additional therapies. Trastuzumab (Herceptin™) is one such example from Genentech/Roche Ltd. for solid tumors. Trastuzumab works by attaching to and blocking specific antigens (HER2/neu protein) which when activated promotes cell growth. Some breast cancer cells overexpress the HER2 gene and cause the cancer to grow and spread. When Trastuzumab attaches to the antigen it blocks the chemical signals that can stimulate uncontrolled growth. Even though there has been great success with antibody therapeutics due to their binding specificity to cells expressing their cognate antigen, antibodies alone can have some disadvantages which include, but are not limited to, low cytotoxicity and weak penetration into tumors (2). On the other hand, systemically administered anti-cancer cytotoxics, although highly potent, reach tumor cells randomly as they circulate. They also diffuse readily into both tumor cells and normal tissue. To achieve sufficient therapeutic effect at target, they may have to be dosed up to overcome lack of specificity to target. They are therefore associated with significant toxicity and non-target effects, limiting their utility and efficacy. One approach used to address these limitations of antibodies and small molecule cytotoxics is to combine them to create antibody-drug conjugates, utilizing the targeting ability of the antibody with the high cell-killing ability of the cytotoxic. Conjugation of the cytotoxic to the antibody reduces its systemic toxicity while still conjugated and in circulation. Antibody-drug conjugates are therefore highly potent targeted-therapy molecules, designed to specifically bind to cancer cells, minimize side effects and provide a wider therapeutic window (3,4). The construct created by combining the specificity of each antibody for its target antigen and the cytotoxicity of a small molecule drug, extends the therapeutic potential of both the antibody and the cytotoxic. With the first approval of an antibody-drug conjugate (ADC) being gemtuzumab ozogamicin (Mylotarg®) in 2001 and the more recent approvals of brentuximab vedotin in 2011 (Adcetris™) (5) and ado-trastuzumab emtansine in 2013 (Kadcyla™, T-DM1) (6) having validated the technology, drug companies

are actively pursuing ADCs with approximately 30 in clinical trials for solid tumors and hematological indications. Although gemtuzumab ozogamicin was subsequently withdrawn from the US market in 2010 (while currently undergoing further clinical trials), it is still approved and marketed in Japan.

ADCs are designed to selectively dispatch highly potent cytotoxic anticancer drugs to cancer cells while, ideally, leaving the healthy tissue relatively unaffected. Tumor-targeting monoclonal antibodies can be conjugated to cytotoxic anticancer drugs or payloads using a variety of linker chemistries with labile bonds. On injection, the antibody binds to specific antigens or receptors at the surface of the cancer cell. The cancer cell internalizes the entire ADC, the linker is then degraded and the cytotoxic payload is released. The exact mechanism of release will depend upon the type of linker used. These cytotoxic payloads can then kill the tumor cells through their established cytotoxic mechanisms. This targeted delivery maximizes its antitumor effect while minimizing damage to healthy tissue as would have occurred in traditional delivery of cytotoxic drugs.

The development of a successful ADC requires knowledge and understanding of the underlying biology as well as of the chemistry in play. The analytical complexity is significantly magnified relative to that for a mAb. Addressing manufacturing and regulatory issues involves consideration of biotherapeutics as well as small molecules perspectives. This review covers some of the design and development aspects of ADCs with a focus on biophysical and chemical stability of the ADC drug product We cover the basics of the design of ADCs by examining the requirements for each element of the ADC construct. The ADC construct has significant impact on its physicochemical stability and therefore the formulation and analytical challenges encountered. The review then concludes with a consideration of quality and some key regulatory aspects as they relate to ADCs. To our knowledge, these aspects have not been reviewed together before, and this review therefore provides a good introduction to a scientist embarking on developing ADCs for the first time. Some related reviews published recently include (7–9).

Targeting of anticancer drugs using antibodies is also being attempted in modalities such as immunotoxins and immunoliposomes but is outside the scope of this review. Some excellent references are available (2,10,11). Other technologies for improved delivery of anticancer drugs based on nanotechnology, colloidal systems, polymers *etc*. are covered in directed reviews (see *e.g.*, (12–15)).

DESIGN OF ADCS

The building blocks of an ADC comprise the monoclonal antibody, the linker and the payload (Fig. 1). Early attempts to create ADCs using murine or chimeric antibodies conjugated to chemotherapeutic agents did not achieve enough localization in the target tumor to be efficacious. The antibodies also resulted in immunogenicity, the antigens targeted were not selective enough resulting in off-target toxicity, and the drugs were not potent enough to be able to kill the tumors [summarized in (16)]. Some of these shortcomings were addressed in the development of gemtuzumab ozogamicin which comprises a humanized antibody and a highly potent cytotoxic derivative of calicheamicin. Early clinical results were promising (17) but some later trials showed no additional benefit of the ADC in combination with chemotherapy (18). Gemtuzumab ozogamicin was withdrawn from the U.S. market in 2010 (although it is still marketed in Japan and undergoing further clinical trials). The knowledge gained from these early experiences, as well as the greatly expanded understanding of the chemistry and biology underlying the design and function of ADCs has led to a surge in the number of programs under development.

The first design element of an ADC is the mAb. Selection of the mAb is based on the following requirements, which must be retained to a very large extent even after the conjugation process. Most if not all of these properties are superior in humanized or human mAbs in contrast to murine or chimeric mAbs.

Elements of ADC's:

- High specificity and binding affinity to target antigen on tumor cells
- Efficient receptor-mediated internalization and intracellular trafficking
- Low immunogenicity potential
- Long circulation time
- Potential desirability for effector function mediated antitumor activity towards the target cells such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC)
- Retention of above properties after conjugation

The second major design element of an ADC is the cytotoxic payload. The requirements for the payload are:

- High potency to be effective at concentrations achievable in the cell by the mAb-based delivery mechanism
- Selectivity in killing (proliferating) target cells
- Low immunogenic potential
- Compatibility with conjugation process (linker attachment site, process chemistry)
- Chemical stability in circulation
- Active in linked form (non-cleavable linker systems) or in free form (cleavable linker systems) based on design of ADC

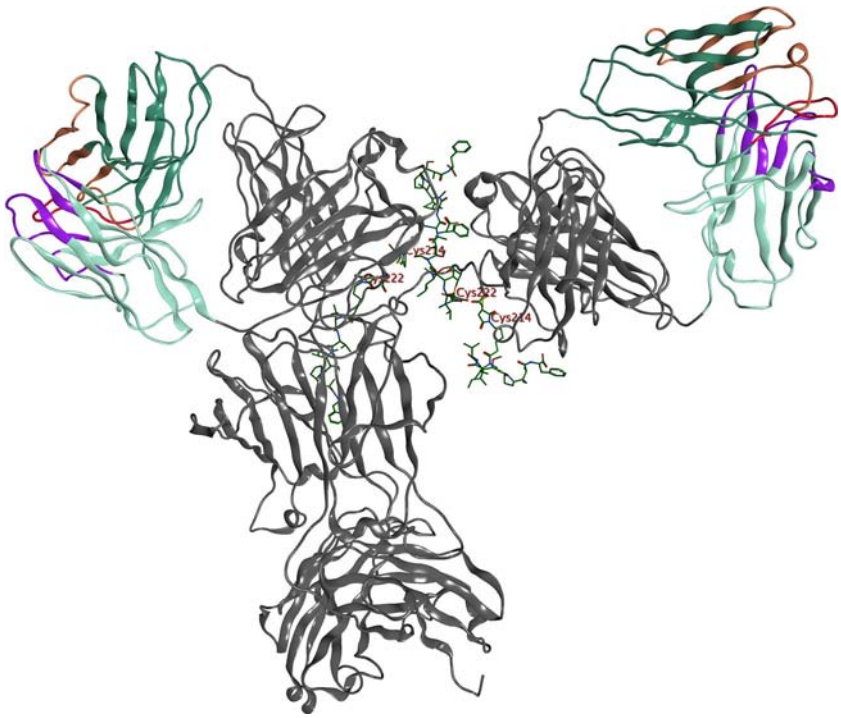


Fig. 1 A structural model of an antibody-drug conjugate showing 4 auristatins (mc-MMAF) attached to the upper hinge region (LC:C214 and HC:C222) on a human IgG1 mAb. The stick representations are provided for the linker and payload while the mAb is indicated by the *ribbon diagram*.

The third major design element of an ADC is the "linker". The requirements for the linker are:

- Presence of suitable site for attachment to antibody as well as payload
- Presence of complementary attachment site on antibody to achieve desired drug loading per antibody molecule
- Stability to process chemistry and product storage
- Stability in circulation
- Compatibility with desired mechanism of action (cleavable after internalization)

Each of these elements is reviewed in greater detail below.

Monoclonal Antibody

Antibody Type

Based on early experiences with immunogenicity from murine mAbs as well as the current state of the art in mAb technology, the majority of ADCs in development comprise humanized or human mAbs with a few chimerics. Also, IgG_1s are the most common but a few IgG_2 and IgG_4 (hinge stabilized) are also in development (19,20).

Recently approved brentuximab vedotin is based on a chimeric IgG_1 (cAC10) against CD30 receptors on tumor cells in Hodgkin's lymphoma and Anaplastic Large Cell Lymphoma. The package insert reports an incidence of persistent anti-drug antibody (ADA) (7%) and transient ADA (30%) in its Phase 2 trials. The ADA is directed against the antibody component in both persistent and transient cases. Of the persistently positive patients, 1% developed adverse reactions consistent with infusion reactions and discontinued treatment. Overall, patients with persistent positive ADA experienced a higher level of infusion related reactions. Sixty-two percent of ADA patients had neutralizing ADA. However, the impact of ADA on safety and efficacy is not known (21).

T-DM1 is based on the previously approved humanized anti-HER2 IgG_1 antibody trastuzumab and reports 5.3% of patients developing ADA at one or more post-dose timepoints (although true incidence may be masked by presence of the drug itself in the serum). Clinical significance of the ADA was not reported (22). In contrast, the human antibody-human antibody (HAHA) response was detected in only one patient out of 903 for trastuzumab (23). Although incidence of ADA cannot be compared across products due to differences in assays, considering the close relationship between these latter two products, it may be speculated that the (significantly) higher ADA with T-DM1 may be related to structural perturbations caused by the conjugation.

ADCs for oncology indications are not intended for chronic use and so the role of immunogenicity may be important only if function is impacted. Considering the state of the art in monoclonal antibody therapy, it is unlikely that a murine antibody would be brought forward. Therefore, the question of antibody selection boils down to sequence and structural features for reasons elaborated below (24).

Affinity

Monoclonal antibodies used in cancer therapy function through a variety of mechanisms; receptor down regulation, induction of apoptosis through inhibition of receptor-linked signaling pathways either by targeting the receptor or ligand, or effector functions (ADCC, CDC) (25,26). Internalization ability is generally not a necessary requirement in these modes of action. A majority of the current ADC approaches are, however, based on tumor cell-surface (antigen) targeting followed by internalization to deliver the payload inside the cell. An ADC design parameter that can impact the function is binding affinity. In general, the highest affinity is considered the most desirable but evidence suggests that a fast on-rate and a slow off-rate may not be the optimum (25). High binding affinity may lead to fast binding to the peri-vasculature with subsequent internalization and catabolization, and thus limit diffusion deeper into the tumor. Monoclonal antibodies, with long circulation half-lives as well as neonatal Fc receptor (FcRn) -mediated lysosomal retrieval, can achieve significant accumulation in the tumor cell without resorting to very high binding affinities (25,27). Goldmacher and Kovtun (19) also note that an intravenous (IV) administered ADC generates high initial blood concentrations (of the order of 0.1 μM or more) while typical K_d of the antibody ranges in the nM or lower. Thus, if the concentration of ADC at the tumor site is same as that in the blood, the antigen would be saturated, irrespective of the K_d. Schmidtt and Wittrup's mathematical model suggests that IgGs of K_d as low as 10^{-7} M can achieve substantial accumulation in tumors (27).

Isotype and Glycosylation

It is generally accepted that only careful *in vitro/in vivo* testing can determine which isotype fits a specific therapeutic purpose (28). However, for ADCs in oncology, antibodies of the IgG_1 isotype are the most common in the development pipelines due to expected additional benefit (in anti-tumor activity) of the immune effector functions ADCC and CDC, which are typically weak to negligible in IgG_2s or IgG_4s. [For reference, Kadcyla displays ADCC activity similar to trastuzumab, while Adcetris has minimal ADCC and no detectable CDC, although both are IgG_1s ((29,30)]. A contrary argument however suggests that binding to effector cells could interfere with targeting and internalization of the ADC by tumor cells, thus reducing its overall efficacy. It is unlikely that multiple isotypes would be taken to the clinic to compare efficacy and enable selection of the optimum isotype. There are few published studies on comparison of isotypes in the function of ADCs. McDonagh *et al.* (31) prepared a variety of anti-CD70 ADCs with IgG_1, IgG_2 and IgG_4 antibodies and variants. The IgG_1s and IgG_4s were engineered to remove Fc gamma receptor (FcγR) binding while the IgG_4 was also mutated to impair complement protein binding and to stabilize the hinge. Optimum conjugation conditions were found to be different for the different isotypes, ascribed to the differences in hinge-region sequence as well as disulfide bonding patterns. Conjugation to cysteines was shown to not impair FcγR binding (when not engineered away). All conjugates were found to have cytotoxicity activity *in vitro* in cell lines expressing the CD70 antigen, but potency varied depending on the cell line as well as isotype/engineered variant. Differences in pharmacokinetics of the ADCs were observed in mice as well as in maximum tolerated dose. Constructs which had improved AUC had somewhat better *in vivo* antitumor activity, although differences depended on the model. FcγR binding was not found to be correlated to improvement in therapeutic index. Thus, no general rule can be extracted from this study to address the question of preference of isotype, all the more so because of differences in human and mouse immune systems.

Although glycosylation plays an important role in the function (esp. effector function) and stabilization of antibodies (32), not much has been reported beyond this on any other roles in ADCs. Nevertheless, significant understanding of the molecular interactions between IgG-Fc and effector ligands has been generated. For maximal ADCC activity, a non-fucosylated IgG_1 format has been proposed to be optimal (33). An example of glycoengineering for enhanced ADCC is the recently approved antibody obinutuzumab (Gazyva™) which displays bisected non-fucosylated sugars (34). The PK of most commonly occurring mAb glycoforms is not strongly dependent on the glycoforms (35), which suggests that the ADCs would also be insensitive to the same. The two main receptors involved are the asialoglycoprotein and the mannose receptors. The asialogylcoprotein receptor recognizes complex N-linked glycan structures with missing sialic acid and terminating in galactose (G1F, G2F, G1F-GlcNac), whereas the mannose receptor recognizes high mannose and terminal *N*-acetylglucosamine (G0F) structures. Consistency of glycoforms is therefore important, especially since binding to the mannose receptor leads to faster clearance (36–38).

Scaffold

Along with isotype, there is the choice of the antibody scaffold to use in the ADC. Full-length antibodies remain the primary scaffold in the current development pipeline. Full-length antibodies have long circulation times which can be an advantage from a targeting perspective, allowing greater chance for reaching the tumor. However, a disadvantage of long residence time in circulation can be the enhanced risk of degradation of linker/payload, leading to non-specific toxicity. This leaves open the possibility of using antibody fragments, such as single-chain variable fragments (scFvs), fragment antigen-binding (Fab) or even other proteins to serve as carriers. Schmidt and Wittrup's model (27) suggests that small proteins

will have high penetration rates into tumors, which when combined with high binding affinity and slow off-rates, can result in substantial accumulation in tumors. But the model also suggests an uptake minimum around ~25 kDa which implies a lack of benefit from scFvs (~27 kDa), which furthermore have monovalent binding and thus lower avidity than a divalent mAb (or Fab_2). Small scFV fragments (~27 kDa) will have efficient penetration and distribution but also poor retention in tumors due to being monovalent and thus lower avidity than a mAb. Intermediate sized fragments, especially divalent fragments such as diabody (~55 kDa), or minibody (~80 kDa) would provide rapid tissue penetration, high tumor retention and also rapid elimination (due to lack of FcRn binding) (39). Monovalent fragments such as Fab (~50 kDa) may not have the tumor retention while bivalent Fab_2 (~110 kDa) approaches the size of a full mAb while lacking the (potential) added benefit of the effector function. On the other hand, even smaller constructs such as DARPins (~14 kDa) or affibodies (~7 kDa) with very high affinities may be interesting. To our knowledge, none of the programs that are in the clinic currently use anything other than full-length antibodies.

Conjugation Strategies: Site of Conjugation and Chemistries

A key characteristic of an ADC is the amount (number) of drug molecules per antibody known as the DAR. While the theoretical DAR, dependent on the chemistry chosen, may be very diverse (for example 8 for cysteine-linked; much higher for lysine-linked), the actual DAR of an ADC is determined both by the chemistry chosen and the process employed to perform the conjugation. Conventional conjugation (Fig. 2) approaches where the conjugation occurs at solvent-exposed reactive amino acids (primarily cysteine and lysine) results in a heterogeneous population of species with varying DARs as well as variable conjugation sites. This diversity can result in challenges in maintaining product consistency, while the individual species may theoretically also have distinct PK/PD, safety and efficacy/potency properties. To overcome these drawbacks, site-specific conjugation approaches have been developed using antibody engineering. These engineered antibodies and the associated conjugation approaches are also discussed in the subsequent sections.

Conventional Conjugation

Native Cysteine Conjugation. Thiol coupling to reduced cysteines from the interchain disulfides can result in a theoretical limit of 8 payload molecules per Ab (for an IgG_1 or IgG_2). Intrachain disulfides are less solvent exposed and more stable (40,41), and are generally not impacted by the reaction conditions used. However, complete coverage of the cysteines is generally not desirable due to greater toxicity and increased clearance associated with the high DARs without a concomitant improvement of efficacy. DARs of 4 have been found to be optimal (42), although this conclusion could be target and payload dependent. There is also a stronger perturbation in structure and enhanced hydrophobicity of the high DAR molecules (43,44) reducing their stability and increasing the likelihood of formation of aggregates over time. Reports on impact of partial reduction of the disulfides on effector function are mixed (41), but a high DAR is more likely to have an adverse impact. Actual DARs in a pool of the product could range from 0 to 8 with each DAR (other than 0 and 8) composed of various isomeric forms, each with a theoretically unique set of properties. However, proper control of the process conditions can be used to ensure product consistency and reduce the heterogeneity obtained in the pool. Sun *et al.* (45) show that partial reduction by dithiothreitol (DTT) followed by conjugation with valine citrulline-monomethyl auristatin E (vc-MMAE) (see Table I for linker and payload) or full reduction by DTT followed by partial oxidation with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and then conjugation, led to DARs of 2, 4 and 6, but with 60–90% isomeric homogeneity within the process used. Brentuximab vedotin produced using above technology has an average reported DAR of ~4 (46). Non-denaturing hydrophobic interaction chromatography (HIC) can be used in purification with success and ease of elution dependent on hydrophobicity of linker-payload and the DAR. Highly hydrophobic linker-payloads along with high DARs may require high levels of organics for elution (personal communication).

Lysine Conjugation. Lysine-based conjugation has also been reported to lead to DARs of between 0 and 8 (16) although theoretically significantly higher numbers are possible due to the abundant surface lysines in mAbs. However, a high degree of conjugation would significantly alter the structure and properties of the mAb due to loss of positive charges associated with lysines, and thus a possible loss of antibody functionality. An average DAR of 3.3 has been reported for T-DM1 where surface lysines are functionalized with a bifunctional linker SMCC (containing *N*-hydroxysuccinimidyl ester for an amide bond with lysine and maleimide for attachment to payload) (47). Wakankar *et al.* (48) report a DAR of 3.5 for T-DM1 with actual substitutions varying between 1 and 7. In lysine-conjugated-huN901-DM1, at least 47% of the lysine residues (out of 86) were found to be partially modified. Almost all the modified lysines are to be found in regions of high flexibility and solvent accessibility (49). The low overall degree of conjugation and thus the distribution of substitution over a large number of lysines residues (7 in light chain (LC), 13 in heavy chain (HC)) leads to large degree of heterogeneity in the population of isomers but consequently also low deviation from the parent structure (20,49). There is strong likelihood that the

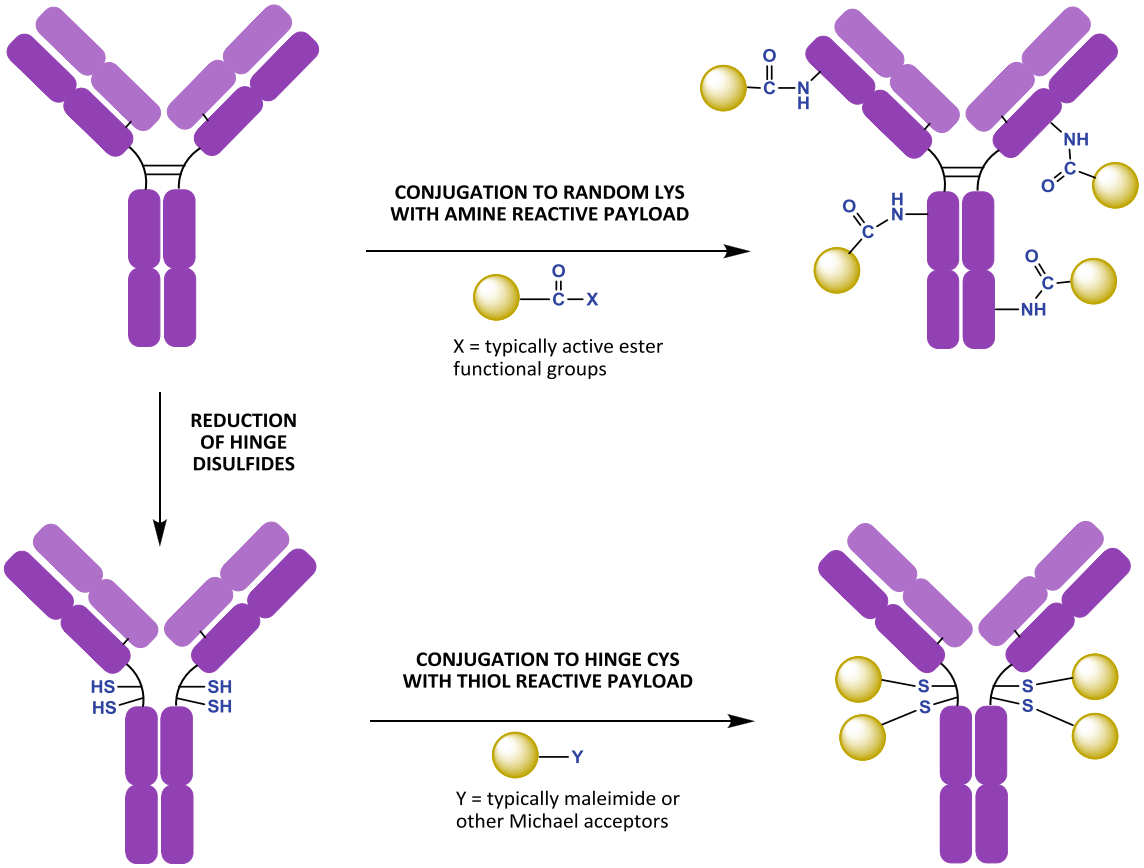


Fig. 2 Conventional conjugation approaches for payload-linker attachment to antibody protein using (*top*) active ester coupling to random lysines or (*bottom*) Michael addition donors to reduced disulfide bridged cysteines in the hinge region of the antibody.

conjugation reaction conditions determine the sites of conjugation. The importance of the DAR and population heterogeneity is emphasized by the observation from pharmacokinetic studies that high DAR species in T-DM1 (DAR 3 to 7) deconjugate faster *in vivo* than DAR1 or DAR 2 (50). Proper process controls can reduce the heterogeneity of the population. In the case of gemtuzumab ozogamicin, the calicheamicin payload (Calich) is attached directly to lysines *via* an amide bond. An overall DAR of 2–3 is reported but the product contains approximately 50% unconjugated mAb molecules while the rest are loaded at DAR of 4–6 (51). The related compound CMC-544 (inotuzumab ozogamicin) has a DAR of 5–7 with less than 10% unconjugated mAbs (52).

Site Specific Conjugation

The lysine and cysteine conjugation chemistries described above rely on non-specific electrophilic reactions with the resultant product profile controlled by process parameters. These strategies also results in a heterogeneous mixture with each subpopulation displaying different physicochemical and pharmacokinetic properties. The heterogeneity of these products and the presence of suboptimal molecules (fast clearing, higher toxicity and higher instability from the high DAR species, potentially sub-potent low DAR species) have led to the development of strategies for site-specific conjugation. Such an approach potentially enables identifying and using the optimal locations of conjugation that impart the desired stability, potency as well as PK-PD properties to the ADC. An optimal DAR may also enable a balance between potency and toxicity, enabling a wider window between therapeutic and toxic doses, *i.e.*, a wider therapeutic index. A low DAR molecule will likely require a higher dose for efficacy but will also be better tolerated. Since the approach uses a design strategy to include the conjugation sites in the antibody, the heterogeneity of the resulting population is limited, including limiting the fraction of unconjugated antibody.

Engineered Cysteine Conjugation. McDonagh *et al.* (53) maintained the use of thiol coupling but reduced the heterogeneity and number of conjugation sites by systematically eliminating 1, 2 or 3 of the 4 interchain solvent accessible disulfides bonds by cysteine to serine mutations in a chimeric anti-CD30 IgG_1 antibody cAC10. The subsequent conjugation to MMAE (by maleimido-caproyl-vc-*para*-aminobenzyloxycarbonyl-MMAE, mc-vc–*p*abc-MMAE) resulted in near quantitative homogeneous substitution at the remaining cysteines. Literature reports (53) show that reduction of the interchain

Table I Linker Abbreviations with Their Description and Corresponding Payloads

Abbreviation	Linker Name/Description	Linker Type	Payloads Used
AcBut	active ester-functionalized 4-(4′-acetylphenoxy) butanoic acid reacted with dimethyl hydrazide to form hydrazone linkage	Chemically labile (acid labile) linker	Calich
AcLys	acetyl-lysine linker	Reactive handle for transglutaminase enzymatic conjugation	Auristatins
AcPAc	Active ester-functionalized (3-acetylphenyl)acetic acid reacted with dimethyl hydrazide to form a hydrazone linkage	Chemically labile (acid labile) linker	Calich
AEVB	hydrazone of 5-benzoylvaleric acid-AE ester	Chemically labile (acid labile) linker	Auristatins
C2-keto-Gal	2-acetonyl-2-deoxy-galactose	Reactive functional group enzymatically transferred to *N*-acetylglucosamine (G0)	Alexa Fluor 488, Biotin
DMA	dimethyl acid	Noncleavable linker	Calich
DMH	dimethylhydrazide	Linker precursor to hydrazone linkage	Calich
Gal NAz	*N*-acetyl-azido-galactosamine	Reactive functional group enzymatically transferred to *N*-acetylglucosamine (G0)	Alexa Fluor 488, Biotin
Phe-Lys	phenylalanine-lysine	Enzymatically cleavable linker	Auristatins
mc	maleimidocaproyl	Noncleavable linker	Auristatins
MCC	(*N*-maleimidomethyl)cyclohexane-1-carboxylate	Noncleavable monofunctional linker resulting from SMCC reaction with mAb	Maytansinoids
*p*ABC	*para*-aminobenzylcarbonyl	Self-immolative spacer	Auristatins
*p*AcPhe	*para*-acetyl-phenylalanine	Unnatural amino acid used for selective conjugation with alkoxy-amine payloads	Auristatins
PEG	polyethylene glycol	Noncleavable spacer	Auristatins, Maytansinoids
SMCC	succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate	Noncleavable bifunctional reagent	Maytansinoids
SPDP	*N*-succinimidyl-3-(2-pyridyldithio)propionate	Disulfide-containing reducible linker	Maytansinoids

disulfides would not impact biosynthetic assembly of IgG, antigen affinity or PK properties. Removal of inter-heavy chain disulfides could impair effector functions. Replacement of the various cysteines with serine did not impact the antigen-binding. A reduction in efficacy and an increase in tolerability results in a net constant therapeutic index for DARs of 4 or 2. The site of conjugation was determined to be less critical for potency or tolerability compared to the stoichiometry, in agreement with Hamblett *et al.*, (42).

A variation of thiol-coupling after reduction of interchain disulfides was described in Klinguer-Hamour *et al.* (54). The technology involves reduction and subsequent relinkage using a bridging moiety that carries the payload. Conjugates formed with bridging moieties that included a polyethylene glycol (PEG, 11-mer) spacer, had reduced aggregation propensities compared to conventional conjugates. Miao *et al.* (55) also described an analogous technology called C-Lock™. Disulfide bridging technologies are specifically considered in a subsequent section on Linkers.

The first truly site-specific approach was developed by Junutula and coworkers (16,56) *via* the THIOMAB technology employing thiol coupling to engineered cysteines, without disruption of the interchain disulfides as in conventional cysteine conjugation. The THIOMAB technology involves selective introduction of unpaired cysteines at specific sites on the surface of the IgG so as not to impact the folding (after expression), structure or functionality (antigen binding) of the mAb (namely THIOMAB). Conjugation to these cysteines then generates THIOMAB drug conjugates (TDCs) with homogeneous and narrowly defined drug loading. THIOMAB constructs (at HC-Ala114Cys Kabat; DAR of 2) retained efficacy but showed improved safety profiles with respect to bone marrow and liver toxicity, in various mAbs despite slower clearance ((16,56–58)). Lower effective hydrophobicity of TDCs (as well as other low DAR molecules with hydrophobicity arising primarily from the payload) may lower the hepatotoxicity, since the liver plays a key role in metabolizing hydrophobic macromolecules (59). Junutula *et al.* (2010) also observe that the mode of toxicity of drug conjugates was predominantly target antigen independent. Liver and bone marrow toxicities are attributable to the high rate of fluid-phase pinocytosis and unrestricted access to blood-borne antibodies in these organs ((59)). TDCs seem to be able to reduce the DAR while maintaining efficacy and thus improving hepatoxicity. The overall benefit on bone marrow toxicity though is unclear.

The importance of the site selected for introduction of the cysteine residue (by replacing serine, valine, or alanine) in the THIOMAB has been illustrated by process and stability analyses. The LC-Val110Cys could not be as efficiently

conjugated as HC-Ala114Cys. Of 12 other sites (8 on LC and 4 on HC), 8 showed >90% conjugation efficiency (56), depending on solvent exposure (58). Similar DARs (1.7–1.9) were obtained when using THIOMAB constructs LC-Val205Cys, HC-Ala114Cys and HC(Fc)-Ser396Cys, and all constructs retained cell-binding and internalization properties of the native mAb (trastuzumab). However, *in vivo* efficacy and plasma stability studies with the TDCs showed that the LC-Val205Cys was the most active and stable, the Fc-Ser396Cys had little to no activity, while HC-Ala114Cys was moderate. Other Fc sites tested showed improved plasma stability suggesting that it was the site and not the domain that was critical.

The extent of solvent exposure of the introduced cysteine impacts not only its degree of substitution but also the *in vivo* stability when using the mc-vc-*p*abc linker. Loss of the conjugate occurs due to maleimide exchange with reactive thiols in plasma with albumin, free cysteine or glutathione (retro-Michael reaction) (58,60). However, not all sites are equally susceptible. Both HC-Ala114Cys and HC(Fc)-Ser396Cys are found in neutral charge environments but the extent of maleimide exchange was different, with HC(Fc)-Ser396Cys being the most susceptible. Conjugation site LC-Val205Cys on the other hand, is in a positively charged environment. In this case, the succinimide ring hydrolyzes rapidly and prevents the maleimide exchange to thiol-reactive plasma components from occurring (58). The retro-Michael reaction is clearly dependent on structural factors beyond solvent accessibility and local charge. Lower *in vivo* stability also correlated with increased liver toxicity, probably through uptake of albumin-drug conjugate, although it is not clear if these were dose limiting. Alley *et al.* (60) showed that attachment of drug to thiols using haloacetamide, by use of bromoacetamidocaproyl instead of the mc linker, circumvented the retro-Michael reaction, as discussed in detail later.

A modeling approach to help identify sites for introducing cysteine was proposed by Voynov, *et al.* (61). Fourteen IgG_1 cysteine variants were produced and analyzed for cross-linking or oligomerization propensity. The variants were designed so as to have at least one variant per immunoglobulin fold domain. The cross-linking propensity of the variants (of serine, threonine, asparagine or lysine) was related to the spatial-aggregation propensity (SAP) score of the substituted residue. [Calculated on a residue by residue basis, the SAP value of a particular region is a measure of the dynamic exposure of hydrophobic patches that is related to the aggregation propensity of that region (62)]. Sites with partial solvent exposure (SAP score 0.00 and −0.11) and away from hydrophobic patches (high SAP scores) are preferably modified with low impact on structure. A modification site with a high SAP patch in the vicinity is likely to result in structural abnormality (61). The model offers an interesting tool to rationally preselect sites for modification based on the consideration of risk for oligomerization of the cys-variants. However, production of the ADC requires further processing steps, while the actual impact of the selected site on toxicity, PK-PD and efficacy, based on the current state of the knowledge, can only be discerned by (pre)-clinical testing.

Enzymatic Conjugation. Conjugation in the THIOMAB constructs requires a reduction step to first remove any cysteine or glutathione adducts from cellular sources on the engineered cysteine. This step is necessary due to the presence of the mutant unpaired cysteine. Any concomitantly disrupted interchain disulfides are then allowed to re-oxidize (under mild oxidation conditions such as air or by induced oxidation using $CuSO_4$ or dehydroascorbic acid), prior to the conjugation step (56). Triple light-chain antibodies were detected and addressed by adjusting cell culture conditions (63,64). Some of these processing issues have been avoided by an enzymatic method for site-specific conjugation (65). The microbial enzyme transglutaminase (mTG) catalyzes the formation of a covalent bond between a glutamine side chain and a primary amine. Although mTG shows some promiscuity in its recognition sequence, it does not seem to recognize naturally occurring glutamines in the constant regions of glycosylated antibodies (66). However, it catalyzes bond formation for a variety of structurally diverse payloads as long as a four-carbon linker (no side chains) separates the primary amine group (acyl acceptor) from the rest of the molecule (67). Strop *et al.* (65) therefore introduced a glutamine tag (with LLQGA amino acid sequence) at various locations to design specific conjugation sites and examine impact of the site on conjugation efficiency, stability, PK and toxicity. Exploration of about 90 sites in an anti-EGFR IgG_1 mab identified 12 sites with good biophysical properties and high degrees of conjugation. A variety of structurally diverse probes and payloads could be conjugated by using appropriate amine-containing linkers. Two conjugation sites were examined in detail (both at the C-terminus of the anti-M1S1 antibody C16; C16-LC-AcLys-vc-MMAD or C16-HC-AcLys-vc-MMAD) after conjugation with AcLys-vc-MMAD. DARs were found to be 1.8–1.9 in the range of the expected 2.0. Detailed characterization of these mTG-based ADCs has been published. Mass spectrometric methods (intact; tryptic and Glu-C digests) showed the site-specificity of this technology to be better than 95% (based on a detection limit of 5% for the DAR 2 species) thus resulting in highly homogeneous ADCs (68). No species with loading greater than 2 were detected. Higher DAR ADCs were created by adding the glutamine tags to C16 HC and C16 LC simultaneously, showing mostly DAR 4 with a few DAR 3 resulting in an overall DAR of 3.8. Interestingly, a small fraction (~1.3%) of conjugates at Q295 were detected, arising from the presence of a small fraction of aglycosylated antibodies in the pool. Lack of glycosylation at N297 allows the 293EEQYN297 sequence to also be conjugated by mTG. This variant could be

eliminated by mutation Q295N, yielding better than 99.8% specificity (68).

Potency and efficacy of the transglutaminase-based ADCs in a mouse model was in proportion to the ~2-fold drug load compared to a standard cysteine conjugated C16-mc-vc-MMAD with DAR of 3.6. The PK of C16-LC-AcLys-vc-MMAD and C16-HC-AcLys-vc-MMAD was similar in mice (for the first week), but the C16-HC-AcLys-vc-MMAD showed significantly faster clearance in rats (83% in first 24 h). Stability differences were seen in DAR 1.0 species between mice and rats. Proteolysis was found to occur at the vc-pabc linkage. The difference in clearance was not attributable to FcRn interactions, suggesting that heavy chain C-terminal mutations may influence other cellular interactions that affect clearance (69).

Unnatural Amino Acids as Conjugation Sites. Site-specific conjugation has also been achieved by introducing unnatural amino acids in specific locations to serve as the conjugation sites. Axup *et al.* (70) report on the incorporation of *p*-acetylphenylalanine (*p*AcPhe) in to an IgG_1 and then using the keto group of *p*AcPhe to selectively conjugate alkoxy-amine derivatized drug of interest through a non-cleavable oxime bond. This was illustrated by synthesizing anti-Her2 (Fab or IgG) with nAF (oxyamine-auristatin F containing a short polyethylene glycol spacer). The *p*AcPhe was introduced at either position LC-K169X or LC-S202X or HC-A121X. Cell-based activity assays showed similar EC50s (1–10 nM) with LC-K169X being slightly lower. The conjugation kinetics are slow. Conjugation was performed in acetate buffer at pH 4.5 for 1–2 days at 37°C for the Fab or 4 days at 37°C for the IgG, with 20–30 fold molar excess of the AF. Detailed testing of the anti-Her2 IgG-nAF conjugate (HC-A121X) (DAR 2) showed no loss of activity after incubation in serum (3 days at 37°C), excellent potency in animal models, and PK similar to that of the naked mutant mAb. The impact of conjugation site on its PK was not studied. In a more recent and comprehensive report (71), the significant advantages of this site-specific technology was illustrated. *p*AcPhe was introduced into anti-5T4 and anti-Her2 antibodies in stable Chinese hamster ovary (CHO) cell lines with high titers (>1 g/L). ADCs were produced with both protease-cleavable and non-cleavable linkers. ADCs with non-cleavable linkers had improved *in vitro* and *in vivo* efficacy and pharmacokinetic stability in rodent models compared to cysteine-conjugated ADCs (both engineered cys and conventional cys). The improved *in vitro* efficacy and selectivity was attributed to stability of the linker before internalization and a more efficient intracellular drug release after internalization. For ADCs with cleavable linkers (vc-*p*abc), the site of insertion of the *p*AcPhe and therefore of the conjugation was found to significantly impact on stability in plasma. Single-dose rat toxicology study showed good tolerability of these ADCs up to 90 mg/kg (71). Behrens and Liu (69) judge the use of unnatural amino acids to be particularly well suited for scanning potential sites of conjugation due to the simplicity of the incorporation technology. Clinical aspects of the technology and risk related to the unnatural amino acids and such bio-orthogonal linkages remain to be studied. Conjugation kinetics can be reasonably expected to be related to the position of the substitution. Sutro Biopharma, Inc. report that azido-based unnatural amino acids and dibenzocyclooctyl -based linker-payload have rapid kinetics with conjugation complete in a few hours on certain sites under room temperature and with only 5× molar excess of linker-payload (72).

Glycan Conjugation. Technologies have been proposed to use these glycans for site specific conjugation since the glycan attachment site is generally removed from antigen and FcRn binding site. It must be noted that while the glycans are not directly involved in the effector function interactions, the conformation of the essential IgG Fc interface is dependent on the presence of the oligosaccharide. Effector functions can therefore be severely compromised or lost in aglycosylated or deglycosylated IgGs (33).

A glycan remodeling technology summarized in Klinguer-Hamour *et al.* (54) utilizes an enzyme to trim the glycans in an IgG followed by addition of a sugar carrying an azide, thiol or chloride moiety for further conjugation (73). Various technologies have been described for the addition of azide handle (74). Bicyclononyne attached to vc-MMAF was conjugated to the azide using copper-free click chemistry called strain-promoted alkyne-azide cycloaddition. The technology is proposed to be broadly applicable to all IgGs and can be extended by glycoengineering (*e.g.*, introducing new glycosylation sites or different sugar functionalities).

Another chemo-enzymatic approach is described by Boeggeman *et al.* (75) who use a mutant version of the enzyme galactosyltransferase to transfer a sugar residue with a reactive functional group (*e.g.*, C2-keto-Gal or Gal NAz) to *N*-acetylglucosamine. The N-linked (Asn297) glycoprotein is first enzymatically trimmed down to G0, purified, and then tagged with C2-keto-Gal from its UDP-derivative to the free GlcNAc residues on the mAb by the modified galactosyltransferase. Identification of the galactosidase enzyme for reproducible and complete trimming to G0 was critical. This remodeling of the glycans was shown by ELISA to not impact binding to antigen. A fluorescent probe was introduced using a C_5-aminooxyacetamide linker. A conjugation level of 2 was achieved (equivalent to a DAR 2).

Cytotoxic Payload

The second design aspect, the cytotoxic payload, is what makes the ADC a powerful therapeutic. The payload is the active species that kills a tumor cell typically through (1) interference with the tumor cell mitotic cycle by binding to tubulin, or (2) damaging DNA *via* cleavage or alkylation. Early work

with ADC payloads relied on clinically utilized drugs such as vinca alkaloids (4-deacetylvinblastine, tubulin inhibitor) (76), methotrexate, doxorubicin (77) and taxanes (paclitaxel, microtubule stabilizer) (78). However, development of these early ADCs was hampered by less than ideal payload potencies, heterogeneity and lack of specific targeted cytotoxicity. From a targeting standpoint, the fact that only a very small percentage of the injected dose gets localized to the tumor site requires that the payloads be highly potent.

The current payloads, either approved or in clinical development that are the focus of most ADC development, are all natural product-derived molecules that typically have picomolar or better potencies. The calicheamicins and duocarmycins are DNA binding cytotoxic agents while the auristatins (monomethyl auristatin E, MMAE; monomethyl auristatin F, MMAF) and maytansines (DM1, DM4) are tubulin inhibitors (79,80).

Calicheamicins

The calicheamicin family of antitumor antibiotics (81) are perhaps the most potent payloads with subpicomolar potencies. These cytotoxic semi-synthetic natural products were isolated from the soil microorganism *M. echinospora calichensis*.and modified to incorporate a linker handle. The calicheamicin analog used in gemtuzumab ozogamicin is the *N*-acetyl-γ-calicheamicin (Calich). In Fig. 3, the calicheamicin structure displays three general functional areas. The larger region includes four monosaccharide units and a hexasubstituted benzene ring. This aryltetrasaccharide functionally binds tightly to the minor groove of double-helix DNA (82) with sequence specificity toward 5′-TCCT-3′ and 5′-TTTT-3′ (83). The next functional area contains a highly strained enediyne unit within a 10-member bicyclic ring. This rigid enediyne moiety is attached to an allylic disulfide trigger group. When the disulfide is cleaved under reducing conditions, the resulting nucleophilic thiolate can undergo a rapid *1,4*-addition to give a dihydrothiophene. The strained enediyne will spontaneously undergo a cycloaromatization reaction generating a highly-reactive *1,4*-benzenoid diradical that eventually leads to DNA double strand cleavage (83) and ultimately cell death. Finally, the third region on the opposing side of the disulfide bond is the linker functionality. Two types of linkers have been synthesized onto Calich, one containing a hydrazone cleavage site (AcBut) and one without (DMA). Both react with Lys on the monoclonal antibody through an *N*-succinimidyl ester functionality (84).

Auristatins

The auristatin family of anti-mitotic cytotoxins were derived from the natural product dolastatin 10, a potent pentapeptide with unique amino acids (Fig. 4a), found in the Indian Ocean sea hare *Dolabella auricularia* (85,86). Many of the dolastatin analogs were investigated as anticancer, antimalarial (87) and antifungal (88) agents due to their ability to interact with the protein tubulin and inhibit its polymerization into microtubules thus causing cells in culture to accumulate in

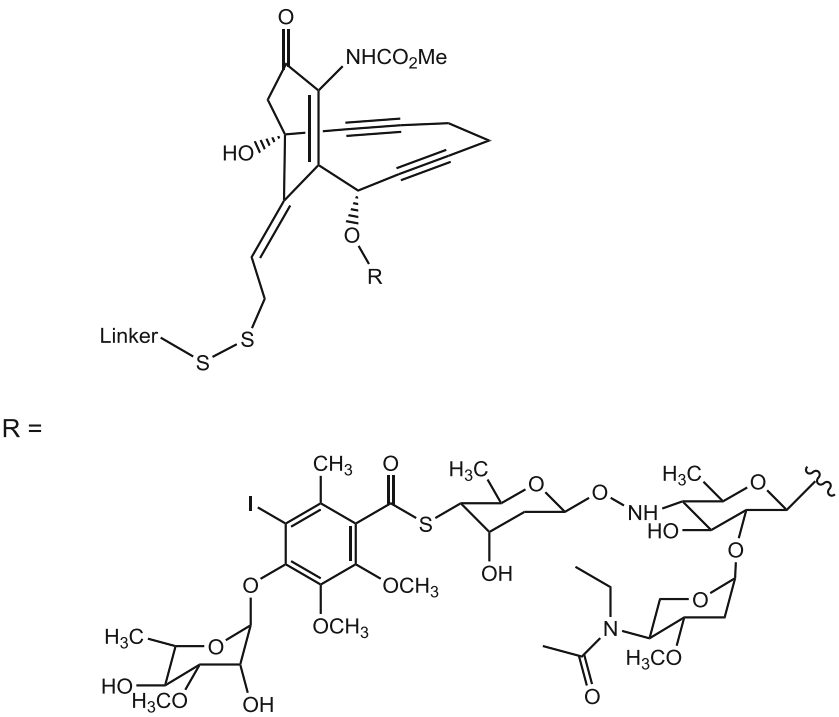


Fig. 3 Chemical structure of *N*-acetyl-γ-calicheamicin payload (Calich).

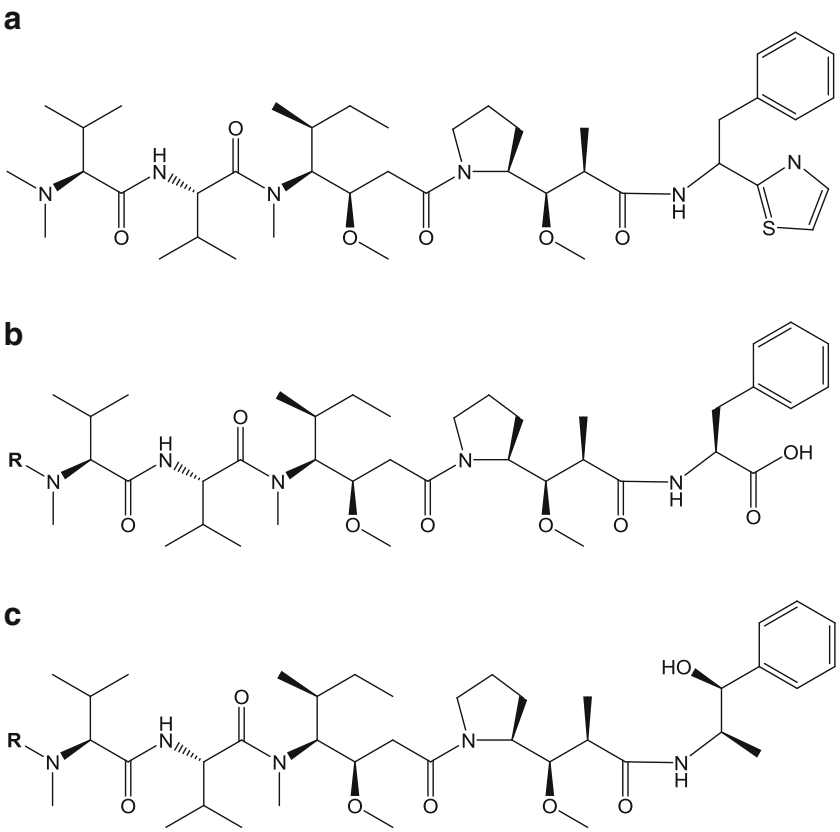
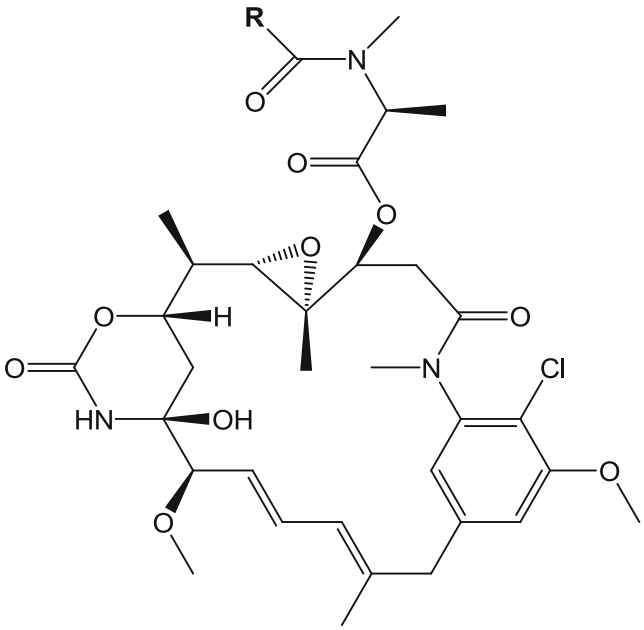


Fig. 4 Chemical structures of auristatin payloads, (**a**) dolastatin 10, (**b**) monomethylauristatin F (MMAF) and (**c**) monomethylauristatin E (MMAE).

metaphase arrest. During studies to determine the mechanism of action on tubulin, it was found that the third amino acid (dolaisoleucine) was the most critical for interaction and that chiral centers were important for cytotoxicity (89). Since the total synthesis of dolastatin 10 was accomplished, multiple analog series have been synthesized. The auristatin series are closely related structural analogs of dolastatin 10 lacking the thiazole moiety on the C-terminal dolaphenine (Doe) residue. While a number of more polar amino acids at the C- or N-termini significantly decreased activity, the substitution of a phenylalanine for the Doe C-terminal residue coupled with the monomethyl-amine terminus yielded what is called MMAF (Fig. 4b) which retained good potency. MMAE (Fig. 4c) is analogous to MMAF but with a C-terminal 3-hydroxyphenethylamide (86,90). Seattle Genetics, Inc. has been pursuing the development of these auristatin analogs for use in their ADC technologies and brentuximab vedotin uses the MMAE payload. Multiple companies have licensed such technology from Seattle Genetics for use in their own in-house ADC projects and several ADCs using an auristatin are in clinical trials (91) [for example: Glembatumumab vedotin (Celldex Therapeutics, Inc.), anti-CD79b (Genentech/Roche), anti-PSMA (Progenics Pharmaceuticals, Inc.), anti-AGS-16 (Agensys, Inc.), anti-GCC (Millenium/Takeda Co.), anti-5T4 (Pfizer, Inc.), and anti-EGFR (Abbvie, Inc.)].

Maytansinoids

The maytansinoids are anti-mitotic tubulin inhibitors derived from maytansine, a benzoansamacrolide antibiotic first isolated from the bark of the African shrub *Maytenus ovatus* (92) and subsequently found in the soil microorganism *Nocardia* sp (93). These cytotoxic agents bind to the same site on tubulin as the vinca alkaloids but are 100- to 1000-times more potent than clinically used anticancer drugs in killing cancer cell lines *in vitro* (94,95). Several Phase I and Phase II clinical trials were performed with maytansine but due to its poor therapeutic window, development of this agent was discontinued. Kupchan *et al.* (96) investigated the structural requirements for cytotoxic and antimitotic activity with a library of structural analogs. They determined that the C-3 ester is necessary for activity but variations in the ester group are acceptable. Blockage of the C-9 carbinolamide *via* etherification reduces tubulin polymerization but does not strongly affect antitubulin activity (Fig. 5). Chari *et al.* (95,97) from ImmunoGen, Inc. were able to develop new, potent analogs of maytansine designed for conjugation to an antibody and release upon internalization within tumor cells. Two such analogs, $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)-maytansine (DM1) and $N^{2'}$-deacetyl-$N^{2'}$-(4-mercapto-4-methyl-1-oxopentyl)-maytansine (DM4), were synthesized and developed for further use as payloads on ADCs in the clinic. Ado-trastuzumab emtansine



DM1: R = $-CH_2-CH_2-SH$
DM3: R = $-CH_3-CH_2-CH(CH_3)-SH$
DM4: R = $-CH_2-CH_2-C(CH_3)_2-SH$

Fig. 5 Chemical structures of maytansinoid analog payloads, $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)-maytansine (DM1), $N^{2'}$-deacetyl-$N^{2'}$-(4-mercapto-1-oxopentyl)-maytansine (DM3) and $N^{2'}$-deacetyl-$N^{2'}$-(4-mercapto-4-methyl-1-oxopentyl)-maytansine (DM4).

(T-DM1) uses the DM1 payload while the Phase II anti-CD19 conjugate, coltuximab ravtansine (previously called SAR3419), uses the DM4 payload (98).

Duocarmycins

The class of duocarmycin analog payloads were based on the natural product duocarmycin isolated from *Streptomyces* sp. bacteria collected at the foot of Mt. Fuji, Japan in 1988 (99). These analogs bind to AT-rich regions of the minor groove of DNA with subsequent irreversible alkylation, disrupting the DNA and leading to cell death. This unique mode of action exerts its effect at any stage of the cell cycle, unlike tubulin binders which are only effective in the mitotic state of the cell (100). The reactivity begins when the duocarmycin binds to the DNA causing a conformational change to the otherwise stabilized cyclopropane moiety. Disruption of the vinylogous amide brings the cyclopropane into conjugation with the cyclohexadienone ring system and selectively activates it for adenine N3 alkylation of the DNA strand (101). This provides for the important benefit of multi-drug resistance, even for *P*-glycoprotein efflux pump expressing cell lines. These molecules, even though based on natural products, can be fully chemically synthesized and are thus not dependent upon isolation from natural sources. A novel prodrug analog of duocarmycin was conjugated to a fully human anti-CD70 antibody to form the MDX-1203 ADC and used in a Phase I clinical trial (102,103).

Other natural product payloads investigated include pyrrolobenzodiazepines (PBDs, DNA crosslinking) (104), the cyclic octapeptide analogs of the mushroom amatoxins (RNA polymerase II inhibitor) (105), epothilones and anthracyclins. Additionally, there have been a few non-natural product-derived synthetic drugs investigated in preclinical studies but none have found much success, possibly due to their less than ideal potencies. Some future directions in payload discovery and development involve investigating more potent chemotypes and identifying novel tumor cell-killing mechanisms beyond those already being pursued (personal communication). Additionally, improvements to solubility and attenuation of hydrophobicity effect on parent antibody biophysical structure while not affecting binding activity or pharmacokinetics would be advantageous (8).

Linker

The final design aspect of ADCs is the linker between the antibody and the toxic payload. This relatively short spacer moiety is a key component of an ADC structure. See linker reviews by (82,106,107). One important characteristic of an ideal ADC linker is that it should remain stable *in vivo* at neutral pH in the serum and also stay inert to any endogenous reactive molecules (*e.g.,* thiols, glutathione), enzymes (*e.g.,* cathepsins, proteases) or other biomolecules (albumin, immunoproteins). Additionally, the linker is required to release the toxic payload at the intended targeted site (such as within tumor cell lysosomal compartments). The stability of the linker and subsequent targeted release play important roles in the efficacy and safety/tolerability of ADCs. Premature release of the cytotoxic payload into the systemic circulation could prove damaging to normal tissue and result in clinically relevant side effects. Loss of conjugated drug on the tumor-targeting antibody also has a negative effect on potency. Therefore, choice of linker is an important consideration when designing an ADC.

The linkers are bifunctional, conjugating the relatively small molecule payload to the larger biomolecule antibody *via* orthogonal chemistries—one functional group to react only with the payload and another functional group to conjugate to the antibody. The ideal linker should involve simple, cost effective, high yielding chemistry for attachment to the toxic payload (typically organic solvent based) while providing gentle, aqueous-based bioconjugation to the antibody.

Most linkers fall into the broad categories of cleavable or non-cleavable. Cleavable linkers rely on specific proteases, pH environment within lysosomal compartment or reduction by endogenous thiols such as glutathione in the cytoplasm for their function for releasing. Non-cleavable linkers on the other hand, rely on proteolytic cleavage of the antibody itself in

order to release the payload, which will still be attached to a portion of the linker and the amino acid residue at the site of conjugation.

Gemtuzumab ozogamicin and CMC-544 (a phase III ADC) use a cleavable linker to conjugate the toxic payload Calich to an anti-CD33 mAb for gemtuzumab ozogamicin (108) or to an anti-CD22 mAb for CMC-544 (52). The dimethyl hydrazide (DMH)-functionalized Calich payload, Calich-DMH, is reacted with the acetyl group of the active ester-functionalized 4-(4′-acetylphenoxy)butanoic acid moiety to form the AcBut linkage (Fig. 6). The active ester is used to conjugate to the lysine residues of the antibody with a resulting amide bond (106). During development of the gemtuzumab ozogamicin conjugate, it was shown that a hydrolytic cleavage site was necessary for potent and selective cytotoxicity from *in vitro* and *in vivo* rodent studies - this in spite of the presence of a reducible disulfide bond on the calicheamicin structure (84). This disulfide is stabilized by two adjacent methyl groups which likely prevent premature release of the payload due to endogenous circulating thiols mentioned above. It was found that stabilizing this disulfide bond improves the therapeutic index of the resulting ADC. Hinman *et al.* (81), using a number of linker analogs, found that the serum stability of the disulfide was proportional to the steric bulk placed adjacent to the disulfide and that the two methyl groups provided adequate balance between stability in circulation and release in lysosomes. To modulate (109) the stability of the hydrazone moiety, a broad variety of aromatic aldehyde and ketone precursors were used to make hydrazone linkers and were screened in an *in vitro* assay (108). In this assay, the hydrazone linker conjugates were incubated for 24 h at pH 4.5 and pH 7.4 at 37°C to approximate the pH in lysosomes and in circulation, respectively. The hydrazone linker made from the AcBut was hydrolyzed at 97% at pH 4.5 but only 6% at pH 7.4. This stability difference plus *in vitro* cytotoxicity, selectivity and *ex vivo* testing were the reasons this AcBut linker was chosen for gemtuzumab ozogamicin. Similarly, for CMC-544, a number of linkers were investigated including acid-labile linkers (AcBut and (3-acetylphenyl)acetic acid (AcPAc)) with Calich-DMH and an acid-stable amide linker, *N*-acetyl γ-calicheamicin dimethyl acid (Calich-DMA) (110) (Fig. 6). The AcBut and AcPAc hydrazone linkers hydrolyze at similar rates under acidic conditions while the amide linker is not expected to hydrolyze significantly under those same conditions. Any internalized amide-linked conjugate would likely require more extensive proteolytic degradation than the "quick" release of the acid-labile linkers. This is supported by the fact that the acid-labile linked AcPAc conjugate was 4- to 8-fold more potent than the amide-linked conjugate in $CD22^+$ B-cell lymphoma (BCL) cytotoxicity assays and that the AcBut linker conjugate was the most efficacious in a BCL tumor-mouse model. The AcBut linked conjugate was chosen for further development (CMC-544) as it caused greater and longer lasting inhibition of Ramos B-cell lymphoma growth than AcPAc linked conjugate (85% vs 28% of mice being tumor-free after 50 days) (110).

Seattle Genetics brentuximab vedotin provides another example of a cleavable linker system on an ADC. In order to improve plasma stability while still affording drug release after tumor cell internalization, a protease-cleavable peptide linker

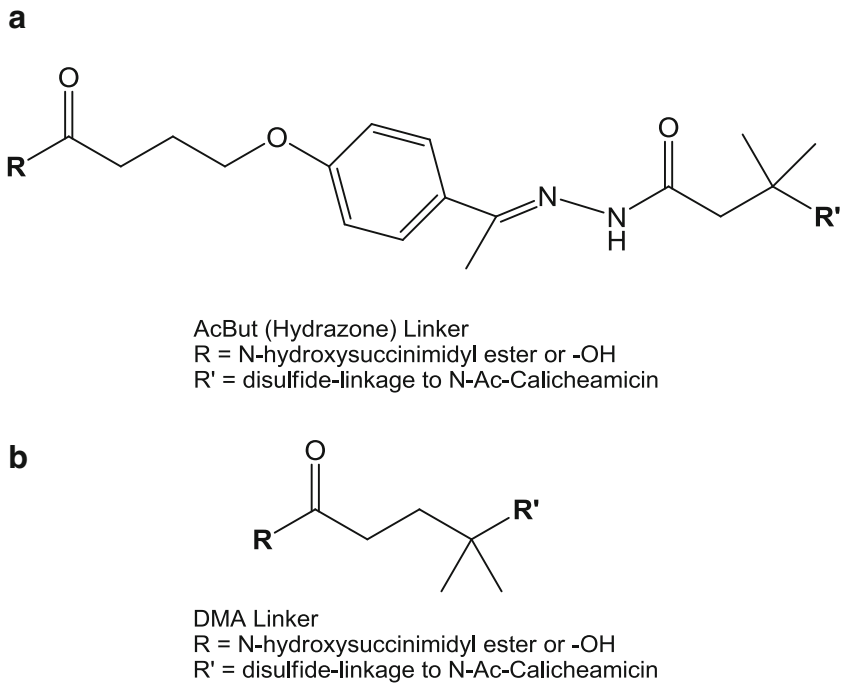


Fig. 6 Chemical structures of (**a**) 4-(4′-acetylphenoxy) butanoic acid (AcBut) and (**b**) dimethyl acid linker (DMA) linkers.

was developed for use with the MMAE payload (46). This vc linker is composed of the dipeptidyl substrate, valine-citrulline, linked through the amine terminus to a self-immolative spacer *para*-aminobenzoyl carbonyl (*p*abc) (Fig. 7). The vc linkage is stable in plasma but is very rapidly hydrolyzed by lysosomal enzymes, in particular, Cathepsin B. Upon cleavage of the vc, the *p*abc group undergoes spontaneous [1, 6]-fragmentation and releases the MMAE payload in native form (90). Initial studies compared the vc linker with an acid-labile hydrazone of 5-benzoylvaleric acid-AE ester (AEVB). Antibody conjugates of maleimido-vc-MMAE and maleimido-AEVB were made using a chimeric mAb cBR96 and *in vitro* plasma stabilities studies (mouse and human plasma) were performed. The hydrazone-linked AEVB was released from human plasma with a half-life of 2.6 days while the vc-linked MMAE was much more stable with a projected half-life of 230 days. While both conjugates were potent by *in vitro* cytotoxic assay, the mAb-AEVB conjugate was more toxic *in vivo* with a maximum tolerated dose (MTD) of 15 mg/kg compared to 30 mg/kg for the mAb-vc-MMAE conjugate.

Another example of a cleavable linker system is the disulfide based linker. Scientists at ImmunoGen (111), working with the maytansinoid payloads, set out to identify disulfide based cleavable linkers that would leverage the 1000-fold difference in endogenous thiols (*i.e.*, glutathione) from human circulating blood (typically micromolar range) to the cytoplasm of cells (typically millimolar range) (95). Several conjugates were made with huC242 antibody, a humanized IgG_1 that binds CanAg, a tumor specific antigen, varying the degree of steric hindrance (0, 1, or 2 methyl groups) on the geminal carbon to the disulfide bond on the linker side and on the maytansinoid side of the disulfide bond (111). The rates of reduction of the disulfide linkages in conjugates were measured at 37°C, pH 6.5, *in vitro* and it was found that the steric hindrance on the linker side produced greater increases in stability than steric hindrance on the maytansinoid side while methyl groups on both sides of the disulfide produced the greatest stability toward reduction. Based on CD-1 mice pharmacokinetic studies, the rate of loss of maytansinoid from conjugate during circulation in plasma correlates with the level of steric hindrance at the disulfide bond which suggests that release of payload is likely occurring through reductive cleavage. The corollary to this is that the rate of conjugate clearance from plasma increases as the stability of the disulfide linker increases. *In vivo* efficacy studies using HT29 and COLO205 tumor models, however, indicate that there are more factors than linker stability involved with *in vivo* efficacy.

An example of an ADC with a non-cleavable linker is T-DM1. The antibody is conjugated at lysines *via* the succinimidyl ester reactive group on the bifunctional SMCC linker and the DM1 payload is attached through its thiol moiety to the maleimide functionality on SMCC. Interestingly, when comparing this non-cleavable linker to disulfide linkers (reducible/cleavable linkers), the serum concentrations of T-DM1 retained elevated concentrations compared to those ADCs with reducible linkers. Additionally, in a target independent study in rats, toxicity was lower for the T-DM1 with SMCC than a reducible SPP ADC comparator (112). It was hypothesized that in order to release the payload from the non-cleavable linker, that portions of the antibody would need to be catabolized resulting in amino acid-capped-linker-payload. Shen *et al.* (113) performed a preclinical study that identified the major catabolites of T-DM1 to include DM1, [N-maleimidomethyl] cyclohexane-1-carboxylate-DM1 (MCC-DM1), and Lys-MCC-DM1. These species of ADC linker-payload catabolites were also detected in patient plasma samples.

Next Generation Linker Technologies

Ambrx, Inc. has proprietary bacterial (ReCODE) or yeast/mammalian (EuCODE) expression systems which incorporate

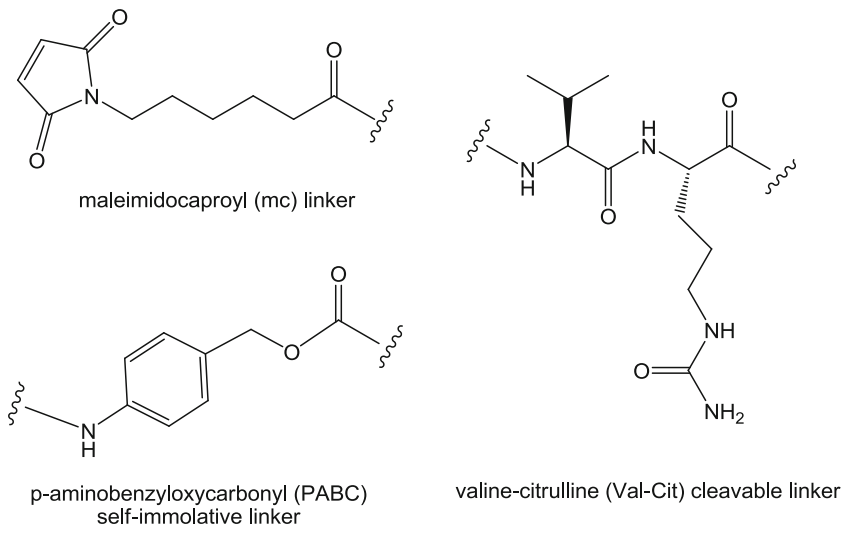


Fig. 7 Chemical structures of non-cleavable maleimidocaproyl (mc) linker, *p*-aminobenzyl-oxycarbonyl (PABC) self-immolative linker and cleavable valine-citrulline (vc) linker.

non-native amino acids (nnAA), such as *p*-acetylphenylalanine (*p*AcPhe), into expressed proteins through the ribosomal use of an Ambrx-engineered tRNA synthetase. These site specific incorporated nnAAs react bioorthogonally with custom hydroxylamine linkers to yield site-specific antibody-drug conjugates (NDCs) (109). In two model systems using *p*AcF engineered anti-5T4 and anti-HER2 antibodies, hydroxylamine-functionalized monomethyl auristatin D, with either cleavable or noncleavable linkers, were conjugated site-selectively to the incorporated nnAAs of the mAbs. The NDCs using non-cleavable linkers proved more stable and displayed improved efficacy over the conventional ADCs with maleimide linkers attached at cysteines resulting from interchain disulfide reduction or cysteines that were engineered at surface-exposed positions (70,71). Though promising, the full potential of these NDCs have yet to be determined in clinical trials.

Allozyne, Inc. is another company focusing on site-specific engineering of nnAAs (114). Their Biociphering^{TM} platform originated in the labs of David Tirrell at Cal Tech (115). This technology focuses on the incorporation of azido- or alkynyl-analogs of certain amino acids onto tumor-targeting mAbs and conjugation of the resulting site-specifically incorporated reactive groups to custom bioorthogonal payload linkers using Staudinger Ligation (or "click" chemistry) to create "AzAbs". The click reaction involves the copper catalyzed cycloaddition of an azide with an alkyne to produce a triazole. It is claimed that the aromatically stabilized linkage is more stable than maleimide, thioester or oxime chemistries. However, published data is sparse. Allozyne is pursuing undisclosed ADC targets for oncology (116).

Redwood Bioscience (now part of Catalent, Inc.) is developing SMARTag^{TM} technology which utilizes site-selective aldehyde placement and aldehyde specific chemistry to control site of conjugation and payload stoichiometry (117). This bioconjugation chemistry called "aldehyde tagging" was initially designed by the Carolyn Bertozzi group from UC Berkeley (118). A formylglycine-generating enzyme (FGE) recognition sequence consisting of five amino acids (CxPxR) is inserted specifically at intended conjugation sites. The FGE selectively alters the cysteine residue in the sequence to an aldehyde bearing formylglycine residue (fGly). This formyl moiety reacts with aldehyde specific payload linker reagents. One recent report identifies a hydrazine-iso-Pictet-Spengler (HIPS) ligation of a fluorophore to an anti-HER2 antibody as model system for ADCs. The HIPS ligation reagent was found to be superior to aminooxy compounds due to faster reaction kinetics and improved hydrolytic stability of the resulting oxacarboline linkage in buffer or serum stability (119). Agarwal *et al.* (120) claimed that in an *in vivo* efficacy study of SMARTag^{TM} HER2 ADC, no free drug was observed over 28 days. However, no analytical details on limits of detection were revealed. Drake *et al.* (121) studied a panel of ADCs with the aldehyde tags at different locations on the antibody (trastuzumab) selected to minimize perturbation of native structure but accessible to conjugation. There was no impact on thermal stability compared to unconjugated antibody and only a slight reduction on FcRn off-rate. When compared to a T-DM1-like lysine conjugated ADC, the aldehyde tagged ADCs showed improved PK and lesser toxicity in rodents.

Disulfide bridging linker technologies from Polytherics Ltd, Thiologics, Ltd. or Igenica, Inc. are another approach at site specific conjugation which occurs at the disulfide bridges between light and heavy chains. This category of linkers include Polytherics Ltd.'s ThioBridge^{TM} technology, Thiologics, Ltd.'s *3,4*-substituted maleimide linkers and Igenica, Inc.'s SNAP technology. Polytherics' Thiobridge^{TM} linker is composed of a thiol-specific bis-sulfone moiety that is capable of sequential addition-elimination across a disulfide bond (122). In the case of an ADC (123), this would entail the interchain disulfides of the hinge region (heavy-heavy) or between the light and heavy chains. The resulting conjugate contains a three-carbon bridge at the site of the reduced disulfide bond. This presumably would keep the interchain stabilized as well as provide attachment for the toxic payload. Thiologics' *3,4*-substituted maleimide linkers work in a similar fashion yet with different reactive chemistries that yield a rigid two-carbon bridge without the introduction of new chiral centers (124). Dihalo- or dithiophenolic-maleimide linkers have been used *in situ* to attach PEG polymers across the disulfide bond of somatostatin (125) or across the disulfide of an antibody fragment with nitroxide spin labels for diagnostic continuous-wave electron paramagnetic resonance or "spinostics" (126). Castaneda *et al.* (124) used this rigid linker to improve interchain stability for a doxorubicin-Her2 Fab conjugate. Igenica, Inc.'s SNAP ADC technology (previously called Stapled ADC) provides payload attachment at each of the disulfide bonds in a mAb (127,128). To date, however, none of these 3 disulfide bridging linker technologies have been fully developed for clinical use.

ANALYTICAL METHODS FOR PHYSICOCHEMICAL STABILITY

Critical to the efficacy and safety of the ADC is stability of the linker, the chemical stability and potency of the drug species (once it has been conjugated), and the DAR. Approaches and methods that have been used for analytical characterization of ADCs have been reviewed in detail by Wakankar *et al.*, (129). Some of the key analytical methods, focusing on stability indicating assays for formulation development purposes, are briefly outlined here along with some examples of their application.

Drug Antibody Ratio (DAR)

The drug antibody ratio of an ADC can be monitored by a number of methods. The simplest and most predominant method is the spectroscopic analysis by UV/Vis of the ADC (129). This method only requires that the UV/Vis spectrum of the antibody and the payload have different maximum absorbance wavelengths in order to calculate the concentration of both and thereby derive the DAR. A number of different ADCs have applied this technique such as calicheamicin analogs (81), immunoconjugates of doxorubicin (130), monomethyl auristatin conjugates (42) and maytansinoid conjugates (97).

Hydrophobic interaction chromatography (HIC) can also directly measure the DAR as well as drug loading distribution. HIC separates individual species based upon the degree of conjugation and then employs UV absorbance for detection. This separation not only allows for direct determination of the various conjugated species present but also allows for isolation and purification of the various species, if required for detailed characterization. (The possible utility for use of HIC in purification has been discussed earlier) . The DAR is calculated based on the weighted average of the different species. The HIC method is not as easily applied to ADCs obtained from lysine conjugation since the higher heterogeneity of substitution (range of drug loading and sites), complicates the chromatographic separation (131). HIC is best applied to cysteine or site-specific conjugates where distinct well-defined species are present. In the case of MMAE conjugated to the anti-CD30 mAb, peaks corresponding to two, four and six DAR were identified (42). To assess the impact of high drug payloads and ionic strength to the overall stability of auristatin ADCs, the HIC method was used to separate fractions and the various DAR species were then collected and incubated (132). After incubation in low and high ionic strength buffers, aggregate content was measured by size exclusion chromatography (SEC) and it was determined that the aggregate content in the presence of high ionic strength was higher for certain DAR species (DAR 2 and DAR 4).

Biophysical Analysis

Despite the size difference between a four-chain antibody and the cytotoxic drugs these small hydrophobic payloads can have a significant effect on the ADCs stability, solubility and solution properties (129). Biophysical techniques commonly used for monoclonal antibodies can also be used to assess the impact of conjugation, but the presence of the bound drug may complicate the characterization in certain cases.

It has been shown that the folding and assembly of the antibody is not affected by the conjugation process (56,133). This is likely to be process dependent but more often, attaching small hydrophobic payloads to an antibody has been shown to cause subtle changes in secondary and tertiary stuctures. These changes can be studied by spectroscopic techniques such as circular dichroism (CD), intrinsic fluorescence, second-derivative UV spectroscopy and Fourier transform infrared spectroscopy (FTIR). However, many of the cytotoxic drugs used in ADCs exhibit a strong near-UV absorbance which can interfere with the characterization of the tertiary structure when using near-UV CD. Far-UV CD and FTIR on the other hand can be used to study the effect of the conjugated drug(s) on the overall secondary structure of the mAb (43,44).

Thermal analysis methods can also be used to study the impact of the conjugation on the conformational stability of the antibody. The most common is differential scanning calorimetry (DSC) (43,44,48,132). Differential scanning fluorimetry has also been used for studying antibodies but to date no reports have been published with the use of this technique with ADCs. It is possible that the dye used as the reporting fluorophore could interact with the hydrophobic payload, confounding the signal.

Particle Characterization

The conjugation of hydrophobic payloads to a mAb can lead to aggregate formation by way of increasing the hydrophobic nature of the mAb. The conjugation of drug can also disrupt local secondary and tertiary structure resulting in unfavorable conformational changes. Just as with other protein products, the presence of aggregates and/or particulates is detrimental to the formulation development of ADCs. The standard tools used to examine particulates for unconjugated antibodies are also applicable for ADCs, and are reviewed in (134–136). To our knowledge, there are no specific reports of particulates in ADC products. Particulates assessment and characterization would be required for the ADC products as well as of their diluted infusion solutions, as discussed above [see also USP<1787>; (137)].

Analysis of Residual or Dissociated Free Drug in Drug Product

An important aspect of the ADC physicochemical stability is the measure of free drug or unconjugated drug that has either dissociated from the antibody upon storage or is a residual amount that remained in the final drug product. The quantity of free drug poses concerns for both differential toxicity and safety.

The unconjugated drugs are typically small and hydrophobic which makes separation and quantification by reverse-phase high performance liquid chromatography (RP-HPLC) possible. For example, RP-HPLC was used for quantitative determination of free methotrexate (MTX) in the MTX-antibody conjugates on stability (138). It was demonstrated

that no detectable amount of free MTX in hydroxlyamine-treated conjugates was present on stability in contrast to the presence of increasing amounts of MTX in samples produced at lower pH values. In an evaluation of an acid-sensitive linker, RP-HPLC was used to monitor the release of adriamycin from monoclonal antibodies in mildly acidic conditions and it was found to be rapidly released by hydrolysis in a pH range of 4 to 5.5, conditions typically found in endosomal and lysosomal vesicles (139).

Free drug may also be evaluated by competitive enzyme-linked immunosorbent assays (ELISA). For example, Tolcher *et al.* (140) determined the amount of non-protein-bound maytansinoid in a protein free extract of patient plasma. The free maytansinoid concentrations of two patients (dosed at the highest ADC levels in this study) could be detected in the plasma up to 48 h following treatment, 52 and 56 nmol/L immediately following cantuzumab mertansine infusion and declined to 4.6 and 5.5 nmol/L by 48 h. Based on this data, the free drug at all time points was less than 1% of the total circulating drug (free drug plus antibody-conjugated drug). To quantify the amount of free calicheamicin in gemtuzumab ozogamicin, Buckwalter *et al.* (141), employed a competitive ELISA method that used specific rabbit anti-calicheamicin antibody. Levels of unconjugated anti-CD33 antibody, total calicheamicin derivatives, and unconjugated calicheamicin derivatives were then used to characterize the pharmacokinetics of the drug.

In Vitro Activity

An *in vitro* cell assay is used to evaluate the potency of ADCs upon release and storage as a quality parameter. There are various cell viability and apoptosis assays that can be performed *in vitro* to monitor the effects of the antibody-drug conjugate on the targeted cell. One example is a luminescent cell viability assay that can determine the number of viable cells in a culture after exposure to the ADC of interest based on the quantitation of the ATP present, an indication of metabolically active cells (142). One can also evaluate the effects of the ADC of interest on cellular health by a fluorescence-based method. The method uses a fluorescent dye that reacts to the reducing capability of living cells thereby allowing one to generate a quantitative measure of viability and cytotoxicity (143,144).

PHYSICOCHEMICAL STABILITY

Formulation approaches and challenges for an ADC are similar to that of a conventional antibody or antibody fragment. Just as with antibody therapeutics there is a need to improve the physiochemical stability of an ADC. Therefore there is a need to understand how the ADC and the parent antibody differ in terms of physical and chemical stability and how to go about formulating both molecules. Various aspects of the approach are discussed in the next section.

Physical Stability

Conjugation in the ADCs involves modification of residues and the addition of bulky hydrophobic payloads. Impact on structure and stability is therefore expected. Higher order structural analysis shows perturbation but no significant global change for cys and lys conjugations. As discussed in a previous section, the greater the degree of substitution, the greater the destabilization of the ADC.

Wakankar *et al.* (48) showed that the melting temperature of the trastuzumab antibody CH2 domain decreased when only the linker was attached (T-MCC) and then decreased further upon conjugation to the drug DM1 to form (lysine-conjugated) T-DM1. It was also noted that there was reversibility of the first transition for the trastuzumab antibody and the conjugated antibody (T-DM1) but, in the case of the linker intermediate T-MCC, it was only observed to be partially reversible. The greatest impact of conjugation was on the CH2 domain which agrees with the analysis that the lysines in the CH2 domain are associated with greater flexibility and are therefore are also more likely to be conjugated (48). Although lysine conjugation removes a positive charge from the mAb (also see below), the "light smear" of substitution is not expected to perturb the structure significantly (49). In the case of T-DM1, T-MCC and trastuzumab, the thermal stability did not completely correlate to aggregation behavior. T-MCC had highest rates of aggregation under thermal stress (at 40°C), followed by T-DM1 while trastuzumab had very negligible rates—which also explains the lack of reversibility of the first transition in T-MCC in the DSC analysis. Unlike trastuzumab or T-DM1, the majority of T-MCC aggregation was driven by the unconjugated maleimidyl moiety reacting with side chains of nucleophilic amino acid residues (cysteine, serine or tyrosine) on other molecules leading to formation of covalent aggregates. More significantly, the T-DM1 also showed higher rates of thermal aggregation than the trastuzumab which is in line with the expectation of destabilization on conjugation.

Qualitatively similar results were reported for the cysteine conjugates by Beckley *et al.* and Guo *et al.* (43,44). The conjugated mAbs were less stable to thermal aggregation compared to the parent mAb. Conjugation after reduction of disulfide bonds did not measurably perturb the secondary and tertiary structure, but did reduce the stability of the structure as seen by reduction of melting points, loss of reversibility of CH2 melt, lower energetics of melting and less cooperative melting. Again, the CH2 domain was more significantly impacted compared to the CH3 and Fab. Two regions in the Fc (in the CH2 and in the CH2-CH3 interface) were found to have

greater conformational dynamics in the ADC (cysteine conjugated vc-MMAE/mc-MMAF) compared to the parent mAb by hydrogen-deuterium exchange MS (145), providing orthogonal confirmation of the DSC results. Interestingly, the increase in conformational dynamics was caused by the breakage of the interchain disulfide bonds than by the conjugation (145). On the other hand, molecular modeling calculations suggest a substantial decrease in the conformational energy of the mAb upon conjugation—more so compared to impact of breakage of disulfide bonds only (44). The local surface around the conjugation sites also becomes more hydrophobic in the ADC, reducing the colloidal stability. The combination of lower conformational stability as well as lower colloidal stability compared to the mAb, results in a greater tendency of the ADC to aggregate (44). This is in agreement with the findings of Beckley *et al.* (43) and Adem *et al.* (132) who also showed that higher DAR species are more prone to aggregation due to the greater extent of destabilization (more disulfides broken) and even lower colloidal stability. The higher DAR species, due to their greater hydrophobicity, also enhance the risk for interfacial aggregation (44). Adem *et al.* (132) also report higher fragmentation rates for high DAR (6,8) species, which is related to the greater loss of interchain disulfide bonds.

Conjugation to lysines has less impact on thermostability than to cysteines as may be expected by the nature of the perturbation involved. Two different antibodies using the same chemistry and resultant DARs may also have different extents of destabilization. The extent of destabilization of an antibody for a specific type of conjugation (*e.g.*, lysine amine or cysteine thiol) is also dependent on the linker (for the same DAR) (146); however, the impact of linker could be overwhelmed by that of the payload for large hydrophobic payloads. Acchione *et al.* (146) also examined impact of coupling to sugar residues on the N-linked glycans using PEG (4-mer) and hydrazide linker. These conjugates had the least impact on thermal stability.

The impact of lysine conjugation on charge profile of an ADC is more complex than a simple loss of a positive charge for one payload added. Even a DAR of 1 can result in multiple charged isoforms depending upon the site of conjugation through impact on local (structural) environment, potential loss of salt bridges, perturbation of electrostatic network and influence on pKa. Higher DARs could also arise from conjugation of partially buried lysines which can alter the conformation and expose buried charged residues. Thus a greater heterogeneity in the electrostatic properties of conjugates is observed compared to that expected solely on the basis of lysines residues modified (147). Maeda *et al.* (148) also found a large number (>20) of peaks in the electropherogram (by capillary isoelectric focusing) for an IgG_4 ADC compared to that of an (unrelated) IgG_4 mAb ((8) peaks). Since change in charge (shifts in pI between 0.5 and 1 units) can alter PK and tissue distribution of a protein (35), an understanding of the charge heterogeneity after conjugation is important. Proper control of the process to limit the range of sites will reduce the range of charge heterogeneity seen.

In summary, ADCs show reduced conformational stability compared to their parent mAb. They tend to be more hydrophobic due to the payload, thus reducing their colloidal stability also. This effect is proportional to the DAR, and is broadly independent of the chemistry involved. Structural perturbation is mainly limited to the domains that carry the conjugation with distant domains being less impacted. Lysine conjugation reduces net charge but also creates a heterogeneous mixture of a variety of charged isoforms. All these properties must be considered when designing the formulation for the ADC.

Chemical Stability

Chemical Stability of the Antibody

ADCs can inherit the chemical liabilities of their parent mAb. Protein chemical liabilities have been well studied and understood (149), and will not be discussed here. It is clearly important that the chemical modifications of the mAb as a consequence of chemical liabilities, do not impact the binding properties and activity of the mAb/ADC. Chemical hotspots are preferably addressed in the mAb design/selection phase itself, to prevent their carry-over into the ADC. There exists the possibility that the structural and conformational impact of conjugation results in exposure of buried (in the mAb) chemical hotspots making them active. However, as discussed above, the impact on structure and conformation is small so the risk of this effect is also small.

Chemical Stability of the Payloads and Linkers

The chemical stability of the conjugated linker-payload affects the conjugation, purification, formulation and analytical development of an ADC. Conjugation and purification requires conditions that do not affect the molecular structure of the payload or linker. Formulation requires the stabilization of physical and chemical degradation pathways for both the antibody protein and small molecule linker-payload for long-term shelf storage. Analytical methods require sample preparation, matrices and solvent systems that have the potential to affect the sample being tested and, thus an understanding of the chemical stability is crucial (129). There is much in the literature regarding chemical stability of monoclonal antibody therapeutics (149–151). This section will focus on the chemical stability of the linker-payloads under *in vitro* conditions.

The chemical properties of the linker-payload as well as the site of conjugation on the antibody are major determinants in the chemical stability of the linker-payload (129). Changes

that affect the molecular structure of the payload and/or integrity of the linker attachment to the antibody potentially could lead to increased toxicity or decreased efficacy and could effectively change the therapeutic window of the ADC. Preserving the intact conjugate and preventing the release of free toxic payload during shelf storage is of critical importance in the formulation and development of the ADC drug product.

Calicheamicins. The calicheamicin payload-linkers contain hydrazone and disulfide moieties that are susceptible to *in vitro* chemical degradation under certain conditions. These ADCs have been characterized with several different analytical techniques including UV, IR, HPLC, and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS) (152). The hydrazone linker, found in gemtuzumab ozogamicin which is designed to hydrolytically release at lower pH in the lysosome, is acid-labile and known to hydrolyze in the presence of succinic acid during MALDI-MS or acetic acid in the ionizing solvent in ESI-MS during analytical testing (152). Calich is also susceptible to UV photolysis whether as the free drug or as a payload conjugated on an antibody. Low intensity UV irradiation of the free derivative Calich-DMA afforded no parent molecular ions (theoretical $M+Na^+$ m/z 1500) but ions that corresponded to some degradation product ($M+Na^+$ m/z 1322, likely a related sodium ion adduct of structure with MW 1299). This degradation product is consistent with loss of an H_2S group although no structure has been assigned. When Calich-DMA-hCTM01 mAb conjugate was irradiated with UV, the mass centroid of the broad MALDI-MS peak decreased in MW with increasing UV exposure. Presumably, the Calich-DMA payload undergoes UV photolysis leading to loss of payload from the antibody.

Auristatins. Senter *et al.* have developed the use of auristatin payload-linkers for use in ADCs and examined chemical stability as one criteria in their designs (60,90,153). Two auristatin payloads, AE and MMAE, were found to be stable for 16 days in buffered saline at 37°C. Additionally, *in vitro* liver lysosomal extracts or proteases did not appear to metabolize or degrade the auristatin payload molecular structures (90). In the course of designing their linker-payloads, they compared the pH-sensitive linkers against the protease-cleavable linkers. AE was synthesized with a pH-sensitive hydrazone linker at the *C*-terminus of the pentapeptide by condensing a maleimidocaproyl hydrazide with an AE ketoester. The resulting hydrazone of 5-benzoylvaleric acid AE ester (AEVB) was relatively stable at pH 7.2 ($t_{1/2}$>60 h) but was labile at pH 5.0 ($t_{1/2}$ 3 h). The AEVB was conjugated *via* the maleimide functionality to the cBR96 mAb and was also found to be stable at pH 7.2 ($t_{1/2}$ 183 h) and released payload at pH 5.0 ($t_{1/2}$ 4.4 h). In comparison, the protease-cleavable dipeptide linker was attached at the *N*-terminus of MMAE through the self-immolative *p*abc spacer to form vc-*p*abc-MMAE. Since peptide bonds are extremely stable to simple hydrolysis, the dipeptidyl vc linker, as expected, was found to be quite stable toward hydrolysis in an *in vitro* plasma stability study (133). In this study, a cAC10-vc-MMAE ADC was incubated over 10 days in human or dog plasma at 37°C and less than 2% of the total drug was released as analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Additionally, no other drug-related molecular species was seen. Over the same time period, free MMAE also did not show any degradation products while incubated in human plasma at 37°C, showing the robust nature of the chemical structure of the auristatin payload.

The chemical stability of the *p*abc spacer moiety was first reported by Katzenellenbogen *et al.* (154) who designed this spacer for use in small molecule prodrug therapies. They reported the *in vitro* stability of a prodrug with this *p*abc spacer to have a $t_{1/2}$~40 h at 25°C (0.05 M Bistris, pH 6.9). The *p*abc spacer was found to be stable under physiological conditions similar to vc-*p*abc-Dox conjugates from Bristol-Myers Squibb (155,156). Presumably, differences in the solvent accessibility and/or contact surfaces of the payload with the larger antibody conjugates compared to small molecule prodrugs may be attributed for the longer stability in payload release for ADCs using *p*abc.

The reactive moiety on the linker also has a role to play in the chemical stability of the payload-linker. Alley *et al.* (60) compared the stabilities of mc-MMAF and bac-MMAF on mab 1F6-C4v2. The mc-MMAF conjugation to thiols on the reduced mAb resulted in thioether-succinimidyl ring linkages while the bac-MMAF conjugation resulted in thioether-acetamido linkages. Thioether-succinimidyl ring linkages (aka maleimide linkages) are known to undergo retro-Michael addition and succinimidyl ring-opening hydrolysis (157,158). Deconjugated maleimide payload-linkers in the presence of excessive amounts of circulating reactive thiols (*i.e.,* serum albumin, glutathione) are prone to adduct formation. For the 1FV-C4v2-mc-MMAF conjugate in an *in vivo* assay, payload was found to release with a half-life of 7 days. This correlated with an *in vivo* mouse study of cAC10-vc-MMAE with a release half-life of 6 days (153). The acetamido linkage was more stable *in vivo* with no drug release during the 14 day assay. This increased stability is presumably due to lack of a deconjugation pathway by the thioether-acetamido linkage. It should be noted that these are *in vivo* studies and may involve more than just chemical stability. Baldwin *et al.* (159) investigated the retro reaction of maleimide-thiol conjugates using an *in vitro* system consisting of phosphate buffer at pH 7.4 with and without various thiol compounds (*e.g.,* glutathione). They used model systems of 4-mercaptophenylacetic acid, *N*-acetylcysteine or 3-mercaptoproprionic acid reacted with *N*-ethylmaleimide and followed the kinetics of deconjugation

with ^{1}H-NMR and HPLC. These model systems showed half-lives of retro reaction from 19 to 337 h and were dependent on the concentration of thiol reductant.

Maytansinoids. The chemical stability of the majority of the relevant maytansinoid payload analogs revolves around the *N*-acyl-*N*-methyl-L-alanyl ester moieties at the C3 ring position (see structures of DM1 and DM4). It was found during the synthesis of various analogs that this C3 ester linkage is susceptible to elimination under mild basic conditions (pH >9) (95) to form the α,β-unsaturated maysine derivative. Interestingly, the C3 ester was found to be stable to a wide panel of commercially available esterases and lipases in terms of enzymatic hydrolysis. Under oxidative stress conditions (substoichiometric concentrations of hydrogen peroxide at 37°C, pH 5.5 or pH 7.4 buffer) (160), the ansa macrolide ring exhibited no signs of oxidation. When the C3 ester of the payload contains a free mercapto or disulfide moiety, it is susceptible to typical thiol chemistries: electrophilic addition, metal-catalyzed oxidation (mercapto) or thiol exchange, reduction (disulfides). Although there are a number of chiral centers on this ansa macrolide payload, no evidence of stereoisomerization has been reported (to our knowledge) while utilized on a maytansinoid ADC.

The chemical stability of the linkers used for maytansine-loaded ADCs depends on the type of linker chemistry: a disulfide linkage (*i.e.,* SPDP) or a maleimide linkage (*i.e.,* SMCC) (see Fig. 8). The currently marketed T-DM1 utilizes the non-cleavable thioether (SMCC) linker. This maleimide linker was thought to be susceptible to the retro-Michael addition or the succinimidyl-ring opening hydrolysis seen in other bioconjugates (157,158). A third degradation pathway was postulated by Fishkin *et al.* (160) where the thioether-succinimide linkage undergoes chemical oxidation followed by sulfoxide elimination under mild aqueous conditions. In this study, Fishkin *et al.* used a model compound, DM1 conjugated to *N*-ethylmaleimide (DM1-NEM). They found that the DM1-NEM was fairly stable in PBS, pH 7.4 at 37°C (≤5% sulfoxide formation after 40 h) but more susceptible in *ex vivo* human plasma (37°C, ~20% cleavage and sulphonate formation at 40 h). More surprisingly, the unoxidized DM1-NEM showed no apparent retro-Michael reaction in the presence of 1000 eq. of DTT in PBS for 12 h at 37°C. Under the same conditions, the sulfoxide species yielded 82% free DM1 cleavage product. They found that steric hindrance adjacent to the thioether can attenuate the oxidative degradation pathways for free payload release. One cautionary note is that low levels of autooxidation may have occurred during acetone extraction and sample concentration during HPLC analysis of unoxidized DM1-NEM.

Indeed, care must be taken during sample preparation for payload-linker analysis. This is not only during drug substance and drug product analytical method development, but also during pharmacokinetic analysis. Dere *et al.* (161) described the development of pharmacokinetic assay development for T-DM1. They used orthogonal methods such as ELISA for identifying payload conjugated to antibody and LC-MS-MS for free DM1 payload catabolites. In the sample preparation for the LC-MS/MS assay, extra care was taken to identify various chemically transformed and biotransformed DM1-related species. They noted that ~0.4% of DM1 in PK samples is releasable in the form of disulfide linkage or by thioether oxidation and sample prep included a reduction and capping step to capture all forms of releasable DM1.

FORMULATION CONSIDERATIONS

Issues that are faced when developing a formulation for a monoclonal antibody are also relevant for the ADC and possibly exacerbated based on the impact to the structure as discussed earlier. Proteins can be susceptible to degradation when exposed to factors such as temperature, pH, agitation

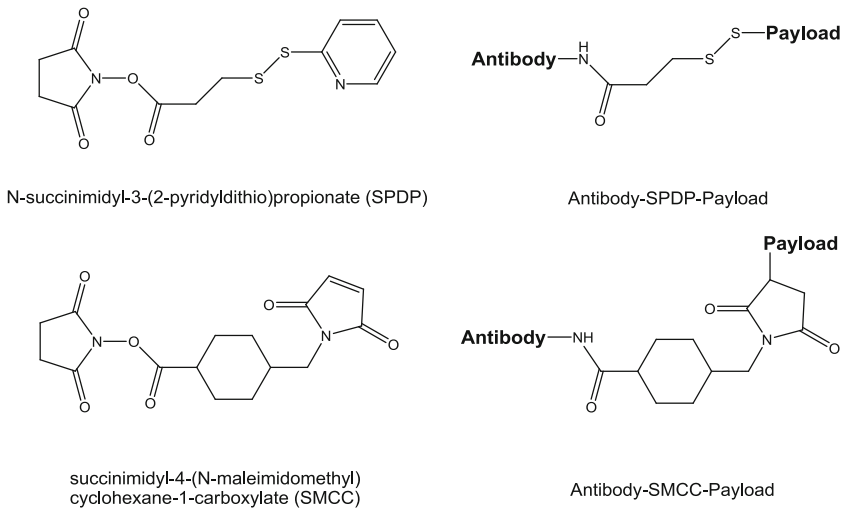


Fig. 8 Chemical structures of *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) linkers before and after typical conjugation reactions with antibody and drug payload.

and light (149,162). When developing a formulation parameters such as protein concentration, pH, buffering species, excipients, solid *versus* liquid dosage form need to be considered. However, an ADC presents some additional challenges to formulation development. Consideration has to be given not only to the overall stability of the mAb/ADC, but also the chemical stability of the payloads and linkers. The conditions that render the optimal stability for the hydrophilic mAb may not be well suited for the hydrophobic drug-linker. The chemical stability of the payloads and linkers are discussed in detail in the previous section. This section will focus on the concerns for developing a formulation for the ADC drug product. For reference, the current commercial products are all lyophilized dosage forms that contain a buffer, stabilizer (*e.g.,* trehalose or sucrose), and surfactant.

An important aspect for ADC's is the increased propensity for self-association driven by the hydrophobic payload(s). Self-association can lead to the formation of aggregates which can impact the potency of the drug, and increase the risk for immunogenicity upon administration (162). High solubility in general is a desirable property of antibody formulations. However, conjugation alters the hydrophilic-hydrophobic balance of the molecule. While antibodies are most soluble in aqueous buffered solutions the hydrophobic linkers and payloads prefer polar organic solvents (163). After conjugation, this duality is incorporated into the construct. The degree of conjugation can cause issues with solubility as the more highly loaded species have the greater tendency to precipitate or aggregate (164,165). Using a hydrophilic linker to balance the hydrophobic payload as part of the design of the ADC has helped mitigate this effect. Highly water-soluble hydrophilic linkers with either a negatively charged α-sulfonic acid group or a polar polyethylene glycol chain enabled an increase in solubility of antibody-maytansinoid conjugates (166,167). These hydrophilic linkers allowed for a higher DAR than the hydrophobic N-Succinimidyl 3-[2-pyridyldithio]-propionate (SPDP) and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) linkers (Fig. 8), and were shown to be either equal or more cytotoxic. BR96 immunoconjugate aggregation was inhibited by the use of highly branched, hydrophilic methoxytriethylene glycol linkers. This enabled the synthesis of conjugates with molar ratios up to 16 mol of doxorubicin (168). Solubility of a highly hydrophobic ADC can generally not be significantly impacted by formulation approaches.

If the conjugation of the payload is through a surface-exposed lysine, then the reduction in net surface charge can also cause solubility issues due to modification of the electrostatic properties (147). It has been well documented in mAbs that electrostatic surface potential distribution can have an effect on the solubility, thermal stability and viscosity behavior (169–171). Solubility in these situations can in some cases be modulated by changes in ionic strength, pH, and by the addition of certain excipients (*e.g.,* arginine).

The key to identifying a stabilizing formulation for an ADC would be to identify the optimal pH and buffer species that balances the various routes of degradation for the mAb, and for the linker-payload if these too are susceptible. Subsequently, formulating to inhibit aggregation can generally follow the same principles as for a mAb alone, for reviews see (172–174). Typical general formulation excipients/additives that can be included for stabilization of structure and the prevention of aggregation include sugars and polyols, amino acids, amines, polymers, and surfactants (172). More specifically, the examination of higher order structure using various techniques as discussed earlier shows that ADCs are both conformationally (reduced structural stability) as well as colloidally (increased hydrophobicity) destabilized compared to the mAb. Since the degree of destabilization is related to DAR, the higher DAR species in a pool will drive the overall stability of the product (44). Formulation efforts would therefore require structure stabilizers (*e.g.,* sucrose) in low ionic strength buffers, along with a surfactant to reduce impact of hydrophobicity and interfacial stress (44). [This is confirmed by Adem *et al.*, (132) who show that cys-conjugated MMAE ADC when formulated in high ionic strength buffer, resulted in significantly faster rates of aggregation (and fragmentation) compared to low ionic strength buffer. The predominant aggregating and fragmenting species were of high DAR (6,8)].

The question of optimal dosage form arises next. The ADCs that have gained approval (gemtuzumab ozogamicin, brentuximab vedotin, T-DM1) are all lyophilized products. While this is a small data set, it is quite likely that the lyophilized dosage form will be the preferred model for ADCs. A properly designed lyophilized formulation can enhance the stability of the product significantly while eliminating risk of degradation (physical or chemical). Although liquid dosage forms for mAbs are common, the risk for free drug (payload) formation would seriously preclude the development of a liquid product for the ADC. From a handling safety perspective, opinions differ whether accidental exposure risk to a healthcare provider is greater from a broken vial of liquid or lyophilized powder.

Development of lyophilized dosage forms is well understood in the industry and will not be covered here. The general rules for stabilization of proteins in lyophilized form apply [see *e.g.,* (175)]. A few particular aspects of ADCs are worth mentioning. ADC doses are currently low, of the order of a few mg/kg (*e.g.,* brentuximab vedotin 1.8 mg/kg; T-DM1 3.6 mg/kg; gemtuzumab ozogamicin 9 mg/m^2 or approx. 18–27 mg total) (21,22,51), which in turn implies that the amount in a vial will be low and/or relatively dilute solutions will be lyophilized (*e.g.,* estimated as brentuximab vedotin 5 mg/mL; ado-trastuzumab emtansine 20 mg/mL). At these low protein concentrations, proteins do not contribute to cake structural stability. This would imply that crystallizing additives such as mannitol would be required to obtain good cake

structure while running economical lyophilization cycles. Use of sucrose or trehalose added as cryoprotectants to the ADC drug substance (as discussed earlier) is compatible with lyophilization of drug product. Crystallizing additives may then be added at the DP stage, prior to lyophilization. It is likely that with site-specific conjugates, and for low DARs, the doses will be higher, particularly as the therapeutic index widens. At a load of about 30–50 mg/mL the ADC may itself aid in lyophilization by contributing to cake structure and eliminating the need to add a crystallizing excipient.

PROCESS CONTROL

The process of mAb (drug substance intermediate, DSI) production is well understood and widely reported. On the other hand, the process for production of ADCs (drug substance, DS and drug product, DP) requires some special considerations, however, the focus of this section will be on the production of DP. The linkers and payloads are generally chemically synthesized or produced from semi-synthetic processes (and outside the scope of this review). However, these latter intermediates as well as the ADCs, have to be produced and handled in facilities specifically designed for high potency drugs. The facility design has to be supplemented with procedures for containment, protection of personnel, protection of product, environmental monitoring as well as (equipment) cleaning. Some of these processing challenges have been discussed by Ducry (176). We discuss below, some related process and use aspects which can have a significant impact but which have not been specifically examined in relation to ADCs.

Cleaning Requirements

As a consequence of the cytotoxic nature of the ADC product, special containment and handling procedures and facilities are required for the protection of the product as well as the operators. Payloads are produced synthetically or semi-synthetically while the linkers are chemically synthesized. The conjugation reaction is carried out in glass or stainless steel reactor to enable use of organic solvents. The facility for production of ADCs is designed to handle highly potent compounds using closed vessels, isolators, rooms with appropriate air locks and pressure differentials, personnel protective equipment, and cleaning, decontamination and spill clean-up procedures. The ADC drug product has to be produced in similar high containment facilities including lyophilizers with appropriate ventilation as well as cleaning controls. High containment facilities often use peroxide based techniques (*e.g.,* vaporized hydrogen peroxide or hydrogen peroxide gas plasma) to decontaminate/sterilize the isolators or glove boxes where the cytotoxic material is handled for weighing, pumping, filtration *etc.* Such systems are qualified and have limits on residual peroxide. It is known that peroxides (and other sanitizing agents) can be transferred through the vapor phase into open products (177) resulting in oxidative degradation of proteins. Wang *et al.* (177) also showed that the nature of the impact of sanitizing agent varied with NaOCl causing instantaneous oxidation while H_2O_2 caused slow oxidation. Therefore it is essential that a risk assessment be performed and the tolerance limits for peroxide contamination in the ADC product be established, to ensure that worse-case exposure in the isolators does not lead to degradation of the product. Of course, proper process design with appropriate aeration cycles and venting times is essential to reduce the contamination levels to acceptable limits in all these technologies. Procedures for handling of components (*e.g.,* bags of stoppers) or cleaning of conveyer belts should preclude use of oxidative sanitizing agents for the same reason. In a contamination event, it is unlikely that the whole product batch would be contaminated uniformly, resulting in erratic release and stability behavior leading to difficulty in establishing cause in later investigations. While the above precautions around sanitizing agents are generally applicable, use of high containment facilities for ADCs makes it particularly important that the development scientists are aware of this risk.

Storage and Transport of Intermediates

Due to the type of facilities required to produce ADC drug substance and drug product (as discussed above), it is generally quite likely that transport of the mAb intermediate to the conjugation facility and then subsequent transport of the ADC drug substance to the drug product facility would be required. Long-term storage of the mAb and the ADC drug substance in container/closure systems that enable these logistics must be considered. The established scale of bioreactor facilities in many companies is such that the mAb itself is produced in significantly larger amounts than required per batch of the ADC DS. The mAb has to therefore be stored frozen in long-term storage and metered out for ADC production. Frozen storage of mAbs has been extensively discussed in the literature and the formulation knowledge as well as technology is well established (178,179). It is preferable that the mAb is formulated in a buffer and cryoprotectant system that is compatible with the subsequent conjugation step. A surfactant is preferably avoided at this stage. Histidine buffer is commonly used for stabilizing mAbs. It is a weak nucleophile and can interfere in amino conjugations. Phosphate buffers have also been used to formulate mAbs and are generally compatible with most conjugation reaction systems. Common cryoprotectants include sucrose or trehalose, which also tend to be quite compatible with most conjugation chemistries and therefore do not require removal prior to conjugation. Sucrose based systems can be stored for the

long-term at −20°C ± 5°C. However, trehalose or high concentration mannitol systems are preferably stored at −40°C or lower, due to the risk for crystallization of the cryoprotectant and subsequent aggregation of the mAb (180) (181). This latter aspect holds true for the ADC DS also. Once the conjugation is completed, the final ultrafiltration/diafiltration UF/DF step is used to convert the ADC into the formulation intended for the DP. This is likely to be the preferred buffer with a cryo/lyoprotectant and a nonionic surfactant.

The choice of the container closure system for the mAb is determined by the combination of temperature of storage as well as the logistics of the conjugation step. Similarly, storage of ADC drug substance has to take into consideration the batch-size as well as operational flow at the DP facility. Celsius® bags made of ethylvinyl acetate (EVA) are available in two sizes that can accommodate between 2 L and 16 L and can be frozen and thawed in a reproducible manner using an active freeze/thaw unit (182). Smaller-sized bags of the same material are available but must undergo passive processing. EVA bags are preferably stored above −55°C due to the risk for brittle fracture below this temperature. Bags made of low density polyethylene (LDPE) are also available and can be used with passive (simple freezer) systems. More flexible options are offered by storage in polymer bottles such as polytetrafluoroethylene (PTFE), high density polyethylene (HDPE), polycarbonate (PC) or polyethylene terephthalate glycol-modified (PETG) with various sizes being available in pre-sterilized configuration (except PTFE). PTFE, HDPE and PC bottles have a very wide operational temperature range (down to −90°C) while PETG has a brittleness temperature of −40°C. Use of bottle makes it especially important that proper procedures be defined for each step such as filling, freezing, re-torqueing of cap after freezing, handling and storage, to ensure reproducibility and sterility and maintain integrity. Thawing is preferably performed at room temperature (to enable rapid thaw) so as to prevent cryo-concentration and ionic strength (as well as potentially pH) driven variations to cause degradation (178,183,184).

Stability of ADCs in IV Infusion Bags

Most ADCs are intended for oncology indications and are expected to be administered *via* intravenous infusion in a health-care provider setting. Dilution of the ADC drug product into an infusion solution (*e.g.,* normal saline 0.9% NaCl, 5% dextrose, Lactated Ringers, *etc.*) is likely to enable the infusion at a defined rate. An intravenous push or bolus injection is not allowed with any current ADC product for safety reasons since an infusion can be terminated if an adverse event is observed. The brentuximab vedotin prescribing information (21) recommends dilution of the reconstituted drug product so as to achieve a concentration between 0.4–1.8 mg/mL in the infusion solution. The infusion solution should be dosed immediately or stored at 2-8°C and used within 24 h of reconstitution. Freezing of the infusion solution is contraindicated. T-DM1 infusion solution is prepared by dilution of reconstituted solution only into normal saline diluent (22). Use of 5% dextrose as a diluent is not allowed, in accordance with the instructions for parent mAb Herceptin. The infusion solution is recommended to be used immediately or stored at 2-8°C for a maximum of 4 h prior to use. Again, freezing is contraindicated. The difference in use period of the prepared infusion solution may relate to the amount of data provided for microbiological quality verification (185). For both of the above products, addition of other drugs into the infusion solution is not allowed. Co-administration *via* the same bag or infusion system is generally disallowed due to lack of knowledge of chemical or physical compatibility with other drugs. On the other hand, it is noted through *in vitro* studies that DM1 in Kadcyla is mainly metabolized by CYP3A4 enzyme and to a lesser extent by CYP3A5 enzyme. Therefore, it is recommended that concomitant therapy (also unfortunately called co-adminstration but more appropriately called concomitant therapy or use) with strong inhibitors of CYP3A4 should be avoided as it would potentially increase the exposure to DM1 and toxicity, although the PK Is not expected to be impacted. A similar risk is associated for Adcetris ((29,30).

The content of the Dosing and Administration Section of the Prescribing Information is defined by the FDA (186). Short-term stability studies are required to document the suitability of the dosing and administration instructions provided in the examples above. These studies are designed based on the clinical dosing protocol and cover the range of dilution concentrations expected in the clinic. Development studies help to identify infusion solutions that are suitable or contraindicated (186–188). Prepared infusion solutions covering the targeted concentration range are then held for 24 or 48 h at 2–8°C and/or room temperature and analyzed to document the use period of the infusion solution. Impact of light has to be incorporated (189). While such studies are fairly routine, some particular aspects are worth mentioning in the context of ADCs.

Dose levels for ADCs can be quite low, especially during Phase I clinical studies. This may require significant levels of dilution of the ADC drug product leading to risk of adsorptive losses combined with difficulties in analysing and assessing the quality parameters at the low concentrations. Hydrophobicity of the payload can enhance adsorption onto plastics which may be ameliorated to some extent by the addition of surfactants. However, dilution for dosing reduces the level of stabilizing excipients and excipients that prevent surface adsorption, particularly the surfactant, and reduces the stability of the ADC increasing the risk for aggregation and particle formation (188,190). Dosage and administration confirmation studies can be designed to determine the highest dilution levels that may be reproducibly used for dose administration. High

salt concentrations can enhance hydrophobic interactions and impact solubility. Although normal saline is a relatively low ionic strength solution, non-specific electrostatic screening can potentially lead to reduced solubility and aggregate and particulate formation, especially of high DAR species. Apart from the above physical liabilities, chemical liabilities may or may not be enhanced in the infusion solution, depending on the properties of the components of the ADC. No specific reports are available but the critical attributes of the ADC (binding ability and affinity of mAb, chemical stability of payload, amount of free payload, DAR) have to be examined. It is known that certain payloads (*e.g.*, calicheamicin) are sensitive to light. In these cases, the dose preparation and administration instructions have to be qualified with appropriate precautionary instructions (*e.g.,* preparation under shielded fluorescent lighting and shrouding of infusion bag with a UV light protective cover [see *e.g.*, (51)].

QUALITY AND REGULATORY ASPECTS

The complexity of the ADC construct requires that all components are appropriately characterized and controlled prior to assembly. The payload and linker are individually controlled as (small molecule) APIs with the same requirements for characterization of structure, purity, impurity profile, degradation *etc.* (191). Impurities above 0.1% threshold would have to be identified and above 1% would have to be qualified per International Conference on Harmonisation guidance ICH Q3A(R2) (192). How would the ICH Q3B(R2) (193) be applied to the drug product (*i.e.,* to the ADC DS or the final product)? For oncology products, ICH S9 may be used to assess and justify the risk from impurities (194). The blanket application of the ICH Q3A qualification and identification limits for impurities (from linker and payload) should however be re-considered in light of the fact that only a fraction of the linker and payload APIs are present in the final product. The fate of these impurities, whether they are carried over or cleared during conjugation, must also be considered. Conjugatable impurities can potentially be carried over into the final drug substance and therefore the safety risk they pose must be understood and limits set. However, the likelihood that a conjugatable impurity is "more" toxic than its cytotoxic parent is small. Impurities with genotoxic potential may require stricter controls, but considering the generally life-threatening indications and nonchronic usage of ADCs, limits higher than the threshold for toxicological concern (of 1.5 μg/day exposure) may be used as allowed for in the European Medicines Agency EMA guidance (195). Further discussion on the risk assessment strategy is available in a forthcoming publication by Jones *et al.* (196).

Assays, specifications, preferred storage conditions and shelf-life has to be set for each component - the linker, the payload and of the intermediate if these linker and payload are reacted and stored prior to the conjugation step. Changes to the linker and payload processes can potentially be managed, but the impact (or lack thereof) on the quality of the ADC has to be shown (197).

The current practice for ADCs are to understand and control the parent mAb per current standards for mAb products including identity, purity, degradation products, size and charge heterogeneity, and process-related impurities. A binding or potency assay to reflect biological activity is a requirement, typically a binding ELISA. Furthermore, if the mAb has ADCC or CDC activity, additional assays may be required for the mAb, the ADC or both.

The ADC requires a host of additional assays, including the DAR, drug load distribution, free payload, free antibody, size heterogeneity and process-related impurities. The presence of free antibody is theoretically a concern due to potential for competition for receptor occupancy with the ADC but the likelihood of saturating the antigen sites before encountering toxicity is very small. A potency assay for the cytotoxicity of the payload would be expected. The assays required for release and stability depend on the ADC product and process, and typically include free payload, aggregates, size heterogeneity and other degradation products (where present). Some of these parameters may have to be monitored on stability also. The presence/generation of free payload could have an impact on safety. Setting specifications for this and other quality attributes may be a challenge since manufacturing experience at licensure is generally limited and a direct link between the possible range of quality parameters and clinical outcomes is difficult to accomplish. A possibility may be to use preclinical or *in vitro* models to test impact of conjugation-related quality parameters and help guide specifications.

The ADC must also be well-characterized by a variety of methods. Characterization should explore the structural and functional elements of the ADC. Demonstration that conjugation does not impact antigen binding is a critical component of the characterization. Comparability studies for ADCs should follow a strategy similar to that for any biologic, with side-by-side characterization of pre- and post-change lots. Process-related impurities must be characterized and controlled, while any co-solvents need to be tested and demonstrated to be cleared per ICH Q3C.

Wakankar *et al.* (129) have provided a review of the analytical methods for physicochemical characterization of ADCs, while challenges around the bioanalytical aspects, including immunogenicity assays, have been reviewed by Xiao *et al.* (198). Analytical characterization and regulatory aspects have also been reviewed by Harris *et al.* (197).

An important regulatory aspect of the ADC is the designation of regulatory starting materials. Most payloads are derived from synthetic or semi-synthetic (chemically modified fermentation product) processes. Linkers may be chemically

synthesized. In this case, the identification of the point at which the materials are designated as starting materials and need to be under proper current Good Manufacturing Practices (cGMP) and change management controls as well as reporting requirements, becomes important (ICHQ7; also see ICH Q11) (199,200). Actual designation is best done in discussion with the regulatory agency (201). It is also recommended to us as many combinations of linker/payload and mAb as possible during clinical development to create a database of quality parameters that get tested in clinic.

The review of an ADC CMC section submission at the US FDA is a collaborative effort that includes reviewers from the Office of Biotechnology Products (OBP) and Office of New Drug Quality Assessment (ONDQA) along with the Biotech Manufacturing Assessment Branch (191). The OBP focuses on the characterization, comparability, impurities, testing and specifications for the mAb, DS and DP. The ONDQA focuses on the starting materials and intermediates and on characterization, testing and specifications of linker, payload, DS and DP. The IND (Investigational New Drug) Module 3S is preferably organized as 3 separate DS sections/folders, one each for the mAb (DSI), linker/payload intermediates, and for the final DS (191). An interesting divergence in the traditional approaches to the validation section (3.S.3.5) arises in that biologics (*i.e.,* mAb and therefore the ADC) usually provide process validation data in a BLA (Biologics License Application) while small molecules (*i.e.,* linker and payload) provide a validation protocol in the NDA. A risk-based strategy to the process validation should be considered in terms of whether to use validated components in the validation of the ADC.

DISCUSSION AND CONCLUSIONS

This review highlights some of the product developmental aspects of antibody-drug conjugates. With the recent approval of two ADCs, these therapeutic modalities are becoming an increasing part of many major biopharmaceutical drug pipelines. Three distinct elements (payload, linker and mAb) have to combine synergistically to lead to a successful ADC. A number of new technologies are being explored in this field within these three elements. As the understanding grows, the development is beginning to move away from empiricism to rational design, to improve safety, efficacy and thus therapeutic index, as well as PK-PD. Quality and regulatory aspects are also evolving but the key to development is a deep understanding of the physicochemical characteristics studied by a variety of techniques. Advances in ADC design, improved stability *in vitro* and *in vivo*, and appropriate targeting of diseased tissue is allowing the clinical application of ADCs to accelerate.

However, there are many facets of the technology that require further development and understanding. The ability to understand the impact of isotype and the site and type of conjugation on PK-PD or efficacy without resorting to animal studies would remove some of the empiricism. Furthermore, the relevance of animal models to human response is not always clear. The same goes for the development of novel linkers and payloads and their impact on structure and functional properties of the ADCs, including specificity, toxicity, solubility and stability. Improved process understanding may allow broader strategies for product control beyond operating the process in a narrow range. A tighter control on heterogeneity through improvements in site-specific conjugation technologies will help improve safety and efficacy. Current products and a number of those in development are lyophilized presentations. Can excipients be identified or developed to allow a liquid product? Continuing advances in characterization methods will be required as novel combinations are created, tested and produced. Finally, the question remains if the technology can find utility beyond oncology.

ACKNOWLEDGMENTS AND DISCLOSURES

All authors are employees of Pfizer, Inc and hold financial interest in Pfizer, Inc. We acknowledge Sandeep Kumar of Pfizer for the model in Fig. 1 and to our many colleagues in Biotherapeutics Pharmaceutical Science who helped review the manuscript.

REFERENCES

1. Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. Nat Rev Cancer. 2012;12(4):278–87.
2. Sapra P, Shor B. Monoclonal antibody-based therapies in cancer: advances and challenges. Pharmacol Ther. 2013;138(3):452–69.
3. Glennie MJ, van de Winkel JG. Renaissance of cancer therapeutic antibodies. Drug Discov Today. 2003;8(11):503–10.
4. Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. Nat Biotechnol. 2005;23(9):1137–46.
5. de Claro RA, McGinn K, Kwitkowski V, Bullock J, Khandelwal A, Habtemariam B, *et al.* U.S. Food and Drug Administration approval summary: brentuximab vedotin for the treatment of relapsed Hodgkin lymphoma or relapsed systemic anaplastic large-cell lymphoma. Clin Cancer Res Off J Am Assoc Cancer Res. 2012;18(21):5845–9.
6. FDA approves new treatment for late-stage breast cancer. [press release]. Washington, DC USA, 2/26/2013 2013.
7. Jain N, Smith SW, Ghone S, Tomczuk B. Current ADC linker chemistry. Pharm Res. 2015. doi:10.1007/s11095-015-1657-7.
8. McCombs JR, Owen SC. Antibody drug conjugates: design and selection of linker, payload and conjugation chemistry. AAPS J. 2015;17(2):339–51.
9. Perez HL, Cardarelli PM, Deshpande S, Gangwar S, Schroeder GM, Vite GD, *et al.* Antibody-drug conjugates: current status and future directions. Drug Discov Today. 2014;19(7):869–81.

10. Govindan SV, Goldenberg DM. Designing immunoconjugates for cancer therapy. Expert Opin Biol Ther. 2012;12(7):873–90.
11. Janthur WD, Cantoni N, Mamot C. Drug conjugates such as Antibody Drug Conjugates (ADCs), immunotoxins and immunoliposomes challenge daily clinical practice. Int J Mol Sci. 2012;13(12):16020–45.
12. Yordanov G. Advanced strategies for drug delivery in nanomedicine. Progr Colloid Interf Sci. 2014;4:3–36. Colloid and Interface Chemistry for Nanotechnology.
13. Arias JL. Drug delivery strategies in targeting cancer: current concepts and future developments. Curr Drug Targets. 2011;12(8):1094–5.
14. Basile L, Pignatello R, Passirani C. Active targeting strategies for anticancer drug nanocarriers. Curr Drug Deliv. 2012;9(3):255–68.
15. Alexander-Bryant AA, Vanden Berg-Foels WS, Wen X. Bioengineering strategies for designing targeted cancer therapies. Adv Cancer Res. 2013;118:1–59.
16. Panowksi S, Bhakta S, Raab H, Polakis P, Junutula JR. Site-specific antibody drug conjugates for cancer therapy. mAbs. 2014;6(1):34–45.
17. Sievers EL, Linenberger M. Mylotarg: antibody-targeted chemotherapy comes of age. Curr Opin Oncol. 2001;13(6):522–7.
18. Ravandi F. Gemtuzumab ozogamicin: one size does not fit all–the case for personalized therapy. J Clin Oncol Off J Am Soc Clin Oncol. 2011;29(4):349–51.
19. Goldmacher VS, Kovtun YV. Antibody-drug conjugates: using monoclonal antibodies for delivery of cytotoxic payloads to cancer cells. Ther Deliv. 2011;2(3):397–416.
20. Adair JR, Howard PW, Hartley JA, Williams DG, Chester KA. Antibody-drug conjugates - a perfect synergy. Expert Opin Biol Ther. 2012;12(9):1191–206.
21. Adcetris. Prescribing information2013 February 2014. Available from: http://www.ich.org/prodcts/guidelines/quality/article/quality-guidelines.html.
22. Kadcyla. Prescribing information2013 February 2014. Available from: http://www.gene.com/download/pdf/kadcyla_prescribing.pdf.
23. Herceptin. Prescribing information2014 May 2014. Available from: http://www.gene.com/download/pdf/herceptin_prescribing.pdf.
24. Clark M. Antibody humanization: a case of the 'Emperor's new clothes'? Immunol Today. 2000;21(8):397–402.
25. Ritchie M, Tchistiakova L, Scott N. Implications of receptor-mediated endocytosis and intracellular trafficking dynamics in the development of antibody drug conjugates. mAbs. 2013;5(1):13–21.
26. Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. Nat Biotechnol. 2005;23(9):1147–57.
27. Schmidt MM, Wittrup KD. A modeling analysis of the effects of molecular size and binding affinity on tumor targeting. Mol Cancer Ther. 2009;8(10):2861–71.
28. Salfeld JG. Isotype selection in antibody engineering. Nat Biotechnol. 2007;25(12):1369–72.
29. Adcetris. Adcetris Assessment report EMA/702390/2012 Available from: www.ema.europa.eu.
30. Kadcyla. Kadcyla Assessment report EMA/749228/2013 Available from: www.emea.europa.eu.
31. McDonagh CF, Kim KM, Turcott E, Brown LL, Westendorf L, Feist T, *et al.* Engineered anti-CD70 antibody-drug conjugate with increased therapeutic index. Mol Cancer Ther. 2008;7(9):2913–23.
32. Jefferis R. Isotype and glycoform selection for antibody therapeutics. Arch Biochem Biophys. 2012;526(2):159–66.
33. Jefferis R. Antibody therapeutics: isotype and glycoform selection. Expert Opin Biol Ther. 2007;7(9):1401–13.
34. Ratner M. Genentech's glyco-engineered antibody to succeed Rituxan. Nat Biotechnol. 2014;32(1):6–7.
35. Putnam WS, Prabhu S, Zheng Y, Subramanyam M, Wang YM. Pharmacokinetic, pharmacodynamic and immunogenicity comparability assessment strategies for monoclonal antibodies. Trends Biotechnol. 2010;28(10):509–16.
36. Jones AJ, Papac DI, Chin EH, Keck R, Baughman SA, Lin YS, *et al.* Selective clearance of glycoforms of a complex glycoprotein pharmaceutical caused by terminal N-acetylglucosamine is similar in humans and cynomolgus monkeys. Glycobiology. 2007;17(5):529–40.
37. Alessandri L, Ouellette D, Acquah A, Rieser M, Leblond D, Saltarelli M, *et al.* Increased serum clearance of oligomannose species present on a human IgG1 molecule. mAbs. 2012;4(4):509–20.
38. Goetze AM, Liu YD, Zhang Z, Shah B, Lee E, Bondarenko PV, *et al.* High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. Glycobiology. 2011;21(7):949–59.
39. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. Nat Biotechnol. 2005;23(9):1126–36.
40. Liu H, Chumsae C, Gaza-Bulseco G, Hurkmans K, Radziejewski CH. Ranking the susceptibility of disulfide bonds in human IgG1 antibodies by reduction, differential alkylation, and LC-MS analysis. Anal Chem. 2010;82(12):5219–26.
41. Liu H, May K. Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. mAbs. 2012;4(1):17–23.
42. Hamblett KJ, Senter PD, Chace DF, Sun MM, Lenox J, Cerveny CG, *et al.* Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. Clin Cancer Res Off J Am Assoc Cancer Res. 2004;10(20):7063–70.
43. Beckley NS, Lazzareschi KP, Chih HW, Sharma VK, Flores HL. Investigation into temperature-induced aggregation of an antibody drug conjugate. Bioconjug Chem. 2013;24(10):1674–83.
44. Guo J, Kumar S, Prashad A, Starkey J, Singh SK. Assessment of physical stability of an antibody drug conjugate by higher order structure analysis: impact of thiol- maleimide chemistry. Pharm Res. 2014;31(7):1710–23.
45. Sun MM, Beam KS, Cerveny CG, Hamblett KJ, Blackmore RS, Torgov MY, *et al.* Reduction-alkylation strategies for the modification of specific monoclonal antibody disulfides. Bioconjug Chem. 2005;16(5):1282–90.
46. Senter PD, Sievers EL. The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. Nat Biotechnol. 2012;30(7):631–7.
47. Junutula JR, Flagella KM, Graham RA, Parsons KL, Ha E, Raab H, *et al.* Engineered thio-trastuzumab-DM1 conjugate with an improved therapeutic index to target human epidermal growth factor receptor 2-positive breast cancer. Clin Cancer Res Off J Am Assoc Cancer Res. 2010;16(19):4769–78.
48. Wakankar AA, Feeney MB, Rivera J, Chen Y, Kim M, Sharma VK, *et al.* Physicochemical stability of the antibody-drug conjugate Trastuzumab-DM1: changes due to modification and conjugation processes. Bioconjug Chem. 2010;21(9):1588–95.
49. Wang L, Amphlett G, Blattler WA, Lambert JM, Zhang W. Structural characterization of the maytansinoid-monoclonal antibody immunoconjugate, huN901-DM1, by mass spectrometry. Prot Sci Publ Prot Soc. 2005;14(9):2436–46.
50. Bender B, Leipold DD, Xu K, Shen BQ, Tibbitts J, Friberg LE. A mechanistic pharmacokinetic model elucidating the disposition of trastuzumab emtansine (T-DM1), an Antibody-Drug Conjugate (ADC) for treatment of metastatic breast cancer. AAPS J. 2014;16(5):994–1008.

51. Mylotarg. Prescribing information 2010 [cited 2014 March]. Available from: http://www.drugs.com/pro/mylotarg.html.
52. DiJoseph JF, Armellino DC, Boghaert ER, Khandke K, Dougher MM, Sridharan L, *et al.* Antibody-targeted chemotherapy with CMC-544: a CD22-targeted immunoconjugate of calicheamicin for the treatment of B-lymphoid malignancies. Blood. 2004;103(5):1807–14.
53. McDonagh CF, Turcott E, Westendorf L, Webster JB, Alley SC, Kim K, *et al.* Engineered antibody-drug conjugates with defined sites and stoichiometries of drug attachment. Prot Eng Des Sel PEDS. 2006;19(7):299–307.
54. Klinguer-Hamour C, Strop P, Shah DK, Ducry L, Xu A, Beck A. World antibody-drug conjugate summit, October 15–16, 2013, San Francisco, CA. mAbs. 2014;6(1):18–29.
55. Miao D, editor Panel screenings, powerful modular ADC technology platforms from target to IND. IBC Bioconjugates; 2013 June 26–28; Coronado, CA.
56. Junutula JR, Raab H, Clark S, Bhakta S, Leipold DD, Weir S, *et al.* Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. Nat Biotechnol. 2008;26(8):925–32.
57. Boswell CA, Mundo EE, Zhang C, Bumbaca D, Valle NR, Kozak KR, *et al.* Impact of drug conjugation on pharmacokinetics and tissue distribution of anti-STEAP1 antibody-drug conjugates in rats. Bioconjug Chem. 2011;22(10):1994–2004.
58. Shen BQ, Xu K, Liu L, Raab H, Bhakta S, Kenrick M, *et al.* Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. Nat Biotechnol. 2012;30(2):184–9.
59. Damle NK. Antibody-drug conjugates ace the tolerability test. Nat Biotechnol. 2008;26(8):884–5.
60. Alley SC, Benjamin DR, Jeffrey SC, Okeley NM, Meyer DL, Sanderson RJ, *et al.* Contribution of linker stability to the activities of anticancer immunoconjugates. Bioconjug Chem. 2008;19(3):759–65.
61. Voynov V, Chennamsetty N, Kayser V, Wallny HJ, Helk B, Trout BL. Design and application of antibody cysteine variants. Bioconjug Chem. 2010;21(2):385–92.
62. Chennamsetty N, Voynov V, Kayser V, Helk B, Trout BL. Prediction of aggregation prone regions of therapeutic proteins. J Phys Chem B. 2010;114(19):6614–24.
63. Gomez N, Ouyang J, Nguyen MD, Vinson AR, Lin AA, Yuk IH. Effect of temperature, pH, dissolved oxygen, and hydrolysate on the formation of triple light chain antibodies in cell culture. Biotechnol Prog. 2010;26(5):1438–45.
64. Gomez N, Vinson AR, Ouyang J, Nguyen MD, Chen XN, Sharma VK, *et al.* Triple light chain antibodies: factors that influence its formation in cell culture. Biotechnol Bioeng. 2010;105(4):748–60.
65. Strop P, Liu SH, Dorywalska M, Delaria K, Dushin RG, Tran TT, *et al.* Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. Chem Biol. 2013;20(2):161–7.
66. Jeger S, Zimmermann K, Blanc A, Grunberg J, Honer M, Hunziker P, *et al.* Site-specific and stoichiometric modification of antibodies by bacterial transglutaminase. Angew Chem. 2010;49(51):9995–7.
67. Ohtsuka T, Sawa A, Kawabata R, Nio N, Motoki M. Substrate specificities of microbial transglutaminase for primary amines. J Agric Food Chem. 2000;48(12):6230–3.
68. Farias SE, Strop P, Delaria K, Galindo Casas M, Dorywalska M, Shelton DL, *et al.* Mass spectrometric characterization of transglutaminase based site-specific antibody-drug conjugates. Bioconjug Chem. 2014;25(2):240–50.
69. Behrens CR, Liu B. Methods for site-specific drug conjugation to antibodies. mAbs. 2014;6(1):46–53.
70. Axup JY, Bajjuri KM, Ritland M, Hutchins BM, Kim CH, Kazane SA, *et al.* Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. Proc Natl Acad Sci U S A. 2012;109(40):16101–6.
71. Tian F, Lu Y, Manibusan A, Sellers A, Tran H, Sun Y, *et al.* A general approach to site-specific antibody drug conjugates. Proc Natl Acad Sci U S A. 2014;111(5):1766–71.
72. Sutro. Sutro Biopharma Technology 2013 [cited 2014 08Feb]. Available from: http://www.sutrobio.com/tech/index.html.
73. Synaffix connecting molecules 2014 [cited 2014 07Feb]. Available from: www.synaffix.com.
74. van Hest JC, van Delft FL. Protein modification by strain-promoted alkyne-azide cycloaddition. Chembiochem Eur J Chem Biol. 2011;12(9):1309–12.
75. Boeggeman E, Ramakrishnan B, Pasek M, Manzoni M, Puri A, Loomis KH, *et al.* Site specific conjugation of fluoroprobes to the remodeled Fc N-glycans of monoclonal antibodies using mutant glycosyltransferases: application for cell surface antigen detection. Bioconjug Chem. 2009;20(6):1228–36.
76. Starling JJ, Maciak RS, Hinson NA, Nichols CL, Briggs SL, Laguzza BC, *et al.* In vivo antitumor activity of a panel of four monoclonal antibody-vinca alkaloid immunoconjugates which bind to three distinct epitopes of carcinoembryonic antigen. Bioconjug Chem. 1992;3(4):315–22.
77. Trail PA, Willner D, Lasch SJ, Henderson AJ, Hofstead S, Casazza AM, *et al.* Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. Science. 1993;261(5118):212–5.
78. Safavy A, Georg GI, Vander Velde D, Raisch KP, Safavy K, Carpenter M, *et al.* Site-specifically traced drug release and biodistribution of a paclitaxel-antibody conjugate toward improvement of the linker structure. Bioconjug Chem. 2004;15(6):1264–74.
79. Flygare JA, Pillow TH, Aristoff P. Antibody-drug conjugates for the treatment of cancer. Chem Biol Drug Des. 2013;81(1):113–21.
80. Gerber HP, Koehn FE, Abraham RT. The antibody-drug conjugate: an enabling modality for natural product-based cancer therapeutics. Nat Prod Rep. 2013;30(5):625–39.
81. Hinman LM, Hamann PR, Wallace R, Menendez AT, Durr FE, Upeslacis J. Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. Cancer Res. 1993;53(14):3336–42.
82. Dosio F, Brusa P, Cattel L. Immunotoxins and anticancer drug conjugate assemblies: the role of the linkage between components. Toxins. 2011;3(7):848–83.
83. Smith AL, Nicolaou KC. The enediyne antibiotics. J Med Chem. 1996;39(11):2103–17.
84. Hamann PR, Hinman LM, Beyer CF, Lindh D, Upeslacis J, Flowers DA, *et al.* An anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Choice of linker. Bioconjug Chem. 2002;13(1):40–6.
85. Bai RL, Pettit GR, Hamel E. Structure-activity studies with chiral isomers and with segments of the antimitotic marine peptide dolastatin 10. Biochem Pharmacol. 1990;40(8):1859–64.
86. Pettit GR. The dolastatins. Progress in the chemistry of organic natural products. Prog Chem Org Nat Prod. 1997;70:1–19.
87. Fennell BJ, Carolan S, Pettit GR, Bell A. Effects of the antimitotic natural product dolastatin 10, and related peptides, on the human malarial parasite Plasmodium falciparum. J Antimicrob Chemother. 2003;51(4):833–41.
88. Pettit RK, Pettit GR, Hazen KC. Specific activities of dolastatin 10 and peptide derivatives against Cryptococcus neoformans. Antimicrob Agents Chemother. 1998;42(11):2961–5.
89. Bai R, Roach MC, Jayaram SK, Barkoczy J, Pettit GR, Luduena RF, *et al.* Differential effects of active isomers, segments, and analogs of dolastatin 10 on ligand interactions with tubulin.

Correlation with cytotoxicity. Biochem Pharmacol. 1993;45(7):1503–15.
90. Doronina SO, Toki BE, Torgov MY, Mendelsohn BA, Cerveny CG, Chace DF, *et al.* Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nat Biotechnol. 2003;21(7):778–84.
91. Seattle-Genetics. 2014.
92. Kupchan SM, Komoda Y, Court WA, Thomas GJ, Smith RM, Karim A, *et al.* Maytansine, a novel antileukemic ansa macrolide from Maytenus ovatus. J Am Chem Soc. 1972;94(4):1354–6.
93. Higashide E, Asai M, Ootsu K, Tanida S, Kozai Y, Hasegawa T, *et al.* Ansamitocin, a group of novel maytansinoid antibiotics with antitumour properties from Nocardia. Nature. 1977;270(5639):721–2.
94. Remillard S, Rebhun LI, Howie GA, Kupchan SM. Antimitotic activity of the potent tumor inhibitor maytansine. Science. 1975;189(4207):1002–5.
95. Widdison WC, Wilhelm SD, Cavanagh EE, Whiteman KR, Leece BA, Kovtun Y, *et al.* Semisynthetic maytansine analogues for the targeted treatment of cancer. J Med Chem. 2006;49(14):4392–408.
96. Kupchan SM, Sneden AT, Branfman AR, Howie GA, Rebhun LI, McIvor WE, *et al.* Structural requirements for antileukemic activity among the naturally occurring and semisynthetic maytansinoids. J Med Chem. 1978;21(1):31–7.
97. Chari RV, Martell BA, Gross JL, Cook SB, Shah SA, Blattler WA, *et al.* Immunoconjugates containing novel maytansinoids: promising anticancer drugs. Cancer Res. 1992;52(1):127–31.
98. ImmunoGen, Inc. Announces activity reported with SAR3419 plus rituxan in difficult-to-treat patient population [press release]. December 9, 2013 2013.
99. Takahashi I, Takahashi K, Ichimura M, Morimoto M, Asano K, Kawamoto I, *et al.* Duocarmycin A, a new antitumor antibiotic from Streptomyces. J Antibiot. 1988;41(12):1915–7.
100. Boger DL. The duocarmycins: synthetic and mechanistic studies. Acc Chem Res. 1995;28(1):20–9.
101. Wolfe AL, Duncan KK, Lajiness JP, Zhu K, Duerfeldt AS, Boger DL. A fundamental relationship between hydrophobic properties and biological activity for the duocarmycin class of DNA-alkylating antitumor drugs: hydrophobic-binding-driven bonding. J Med Chem. 2013;56(17):6845–57.
102. Thevanayagam L, Bell A, Chakraborty I, Sufi B, Gangwar S, Zang A, *et al.* Novel detection of DNA-alkylated adducts of antibody-drug conjugates with potentially unique preclinical and biomarker applications. Bioanalysis. 2013;5(9):1073–81.
103. Clinicaltrials.gov. Study of MDX-1203 in subjects with advanced/recurrent clear cell renal Cell Carcinoma (ccRCC) or relapsed/refractory B-cell non-Hodgkin's lymphoma (B-NHL) 2014 [March 26, 2014]. Clinical trials database]. Available from: http://clinicaltrials.gov/ct2/show/NCT00944905?term=mdx-1203&rank=1.
104. Sutherland MSK, Walter RB, Jeffrey SC, Burke PJ, Yu C, Kostner H, *et al.* SGN-CD33A: a novel CD33-targeting antibody-drug conjugate using a pyrrolobenzodiazepine dimer is active in models of drug-resistant AML. Blood. 2013;122(8):1455–63.
105. Moldenhauer G, Salnikov AV, Luttgau S, Herr I, Anderl J, Faulstich H. Therapeutic potential of amanitin-conjugated anti-epithelial cell adhesion molecule monoclonal antibody against pancreatic carcinoma. J Natl Cancer Inst. 2012;104(8):622–34.
106. Nolting B. Linker technologies for antibody-drug conjugates. Meth Mol Biol (Clifton, NJ). 2013;1045:71–100.
107. Ducry L, Stump B. Antibody-drug conjugates: linking cytotoxic payloads to monoclonal antibodies. Bioconjug Chem. 2010;21(1):5–13.
108. Hamann PR, Hinman LM, Hollander I, Beyer CF, Lindh D, Holcomb R, *et al.* Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Bioconjug Chem. 2002;13(1):47–58.
109. Ambrx. 2014 [February 28, 2014]. Ambrx company website]. Available from: www.ambrx.com.
110. DiJoseph JF, Popplewell A, Tickle S, Ladyman H, Lawson A, Kunz A, *et al.* Antibody-targeted chemotherapy of B-cell lymphoma using calicheamicin conjugated to murine or humanized antibody against CD22. Cancer Immunol Immunother CII. 2005;54(1):11–24.
111. Kellogg BA, Garrett L, Kovtun Y, Lai KC, Leece B, Miller M, *et al.* Disulfide-linked antibody-maytansinoid conjugates: optimization of in vivo activity by varying the steric hindrance at carbon atoms adjacent to the disulfide linkage. Bioconjug Chem. 2011;22(4):717–27.
112. Lewis Phillips GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, *et al.* Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. Cancer Res. 2008;68(22):9280–90.
113. Shen BQ, Bumbaca D, Saad O, Yue Q, Pastuskovas CV, Khojasteh SC, *et al.* Catabolic fate and pharmacokinetic characterization of trastuzumab emtansine (T-DM1): an emphasis on preclinical and clinical catabolism. Curr Drug Metab. 2012;13(7):901–10.
114. Allozyne. 2014 [February 28, 2014]. Allozyne company website]. Available from: www.allozyne.com.
115. Nairn NW, Shanebeck KD, Wang A, Graddis TJ, VanBrunt MP, Thornton KC, *et al.* Development of copper-catalyzed azide-alkyne cycloaddition for increased in vivo efficacy of interferon beta-1b by site-specific PEGylation. Bioconjug Chem. 2012;23(10):2087–97.
116. Aijun Wang NWN, Marelli M, Grabstein K. Protein engineering with non-natural amino Acids. In: Kaumaya PP, editor. Protein Engineering: InTech; 2012.
117. Redwood. Redwood Bioscience; 2014 [February 25, 2014]. Redwood Bioscience company website]. Available from: www.redwoodbioscience.com.
118. Carrico IS, Carlson BL, Bertozzi CR. Introducing genetically encoded aldehydes into proteins. Nat Chem Biol. 2007;3(6):321–2.
119. Rabuka D, Rush JS, deHart GW, Wu P, Bertozzi CR. Site-specific chemical protein conjugation using genetically encoded aldehyde tags. Nat Protoc. 2012;7(6):1052–67.
120. Agarwal P, Kudirka R, Albers AE, Barfield RM, de Hart GW, Drake PM, *et al.* Hydrazino-Pictet-Spengler ligation as a biocompatible method for the generation of stable protein conjugates. Bioconjug Chem. 2013;24(6):846–51.
121. Drake PM, Albers AE, Baker J, Banas S, Barfield RM, Bhat AS, *et al.* Aldehyde tag coupled with HIPS chemistry enables the production of ADCs conjugated site-specifically to different antibody regions with distinct in vivo efficacy and PK outcomes. Bioconjug Chem. 2014;25(7):1331–41.
122. Balan S, Choi JW, Godwin A, Teo I, Laborde CM, Heidelberger S, *et al.* Site-specific PEGylation of protein disulfide bonds using a three-carbon bridge. Bioconjug Chem. 2007;18(1):61–76.
123. Thayer AM. Building antibody-drug conjugates. Chem Eng News. 2014;13–20.
124. Castaneda L, Maruani A, Schumacher FF, Miranda E, Chudasama V, Chester KA, *et al.* Acid-cleavable thiomaleamic acid linker for homogeneous antibody-drug conjugation. Chem Commun. 2013;49(74):8187–9.
125. Schumacher FF, Nobles M, Ryan CP, Smith ME, Tinker A, Caddick S, *et al.* In situ maleimide bridging of disulfides and a new approach to protein PEGylation. Bioconjug Chem. 2011;22(2):132–6.

126. Schumacher FF, Sanchania VA, Tolner B, Wright ZV, Ryan CP, Smith ME, *et al.* Homogeneous antibody fragment conjugation by disulfide bridging introduces 'spinostics'. Sci Rep. 2013;3:1525.
127. Igenica. 2014 [March 9, 2014]. Igenica Company Website]. Available from: www.igenica.com.
128. Cukier-Meisner E. Appreciating in private. BioCentury. 2014.
129. Wakankar A, Chen Y, Gokarn Y, Jacobson FS. Analytical methods for physicochemical characterization of antibody drug conjugates. mAbs. 2011;3(2):161–72.
130. Willner D, Trail PA, Hofstead SJ, King HD, Lasch SJ, Braslawsky GR, *et al.* (6-Maleimidocaproyl)hydrazone of doxorubicin–a new derivative for the preparation of immunoconjugates of doxorubicin. Bioconjug Chem. 1993;4(6):521–7.
131. Stephan JP, Kozak KR, Wong WL. Challenges in developing bioanalytical assays for characterization of antibody-drug conjugates. Bioanalysis. 2011;3(6):677–700.
132. Adem YT, Schwarz KA, Duenas E, Patapoff TW, Galush WJ, Esue O. Auristatin antibody drug conjugate physical instability and the role of drug payload. Bioconjug Chem. 2014;25(4):656–64.
133. Francisco JA, Cerveny CG, Meyer DL, Mixan BJ, Klussman K, Chace DF, *et al.* cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. Blood. 2003;102(4):1458–65.
134. Zolls S, Tantipolphan R, Wiggenhorn M, Winter G, Jiskoot W, Friess W, *et al.* Particles in therapeutic protein formulations, Part 1: overview of analytical methods. J Pharm Sci. 2012;101(3):914–35.
135. Singh SK, Afonina N, Awwad M, Bechtold-Peters K, Blue JT, Chou D, *et al.* An industry perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. J Pharm Sci. 2010;99(8):3302–21.
136. Singh SK, Toler MR. Monitoring of subvisible particles in therapeutic proteins. Meth Mol Biol (Clifton, NJ). 2012;899:379–401.
137. USP<1787>. Measurement of subvisible particulate matter in therapeutic protein injections. In: USP, editor. Washington, DC, USA 2014.
138. Hudecz F, Garnett MC, Khan T, Baldwin RW. The influence of synthetic conditions on the stability of methotrexate-monoclonal antibody conjugates determined by reversed phase high performance liquid chromatography. Biomed Chromatogr BMC. 1992;6(3):128–32.
139. Greenfield RS, Kaneko T, Daues A, Edson MA, Fitzgerald KA, Olech LJ, *et al.* Evaluation in vitro of adriamycin immunoconjugates synthesized using an acid-sensitive hydrazone linker. Cancer Res. 1990;50(20):6600–7.
140. Tolcher AW, Ochoa L, Hammond LA, Patnaik A, Edwards T, Takimoto C, *et al.* Cantuzumab mertansine, a maytansinoid immunoconjugate directed to the CanAg antigen: a phase I, pharmacokinetic, and biologic correlative study. J Clin Oncol Off J Am Soc Clin Oncol. 2003;21(2):211–22.
141. Buckwalter M, Dowell JA, Korth-Bradley J, Gorovits B, Mayer PR. Pharmacokinetics of gemtuzumab ozogamicin as a single-agent treatment of pediatric patients with refractory or relapsed acute myeloid leukemia. J Clin Pharmacol. 2004;44(8):873–80.
142. Ingle GS, Chan P, Elliott JM, Chang WS, Koeppen H, Stephan JP, *et al.* High CD21 expression inhibits internalization of anti-CD19 antibodies and cytotoxicity of an anti-CD19-drug conjugate. Br J Haematol. 2008;140(1):46–58.
143. Sutherland MS, Sanderson RJ, Gordon KA, Andreyka J, Cerveny CG, Yu C, *et al.* Lysosomal trafficking and cysteine protease metabolism confer target-specific cytotoxicity by peptide-linked anti-CD30-auristatin conjugates. J Biol Chem. 2006;281(15):10540–7.
144. Law CL, Cerveny CG, Gordon KA, Klussman K, Mixan BJ, Chace DF, *et al.* Efficient elimination of B-lineage lymphomas by anti-CD20-auristatin conjugates. Clin Cancer Res Off J Am Assoc Cancer Res. 2004;10(23):7842–51.
145. Pan LY, Salas-Solano O, Valliere-Douglass JF. Conformation and dynamics of interchain cysteine-linked antibody-drug conjugates as revealed by hydrogen/deuterium exchange mass spectrometry. Anal Chem. 2014;86(5):2657–64.
146. Acchione M, Kwon H, Jochheim CM, Atkins WM. Impact of linker and conjugation chemistry on antigen binding, Fc receptor binding and thermal stability of model antibody-drug conjugates. mAbs. 2012;4(3):362–72.
147. Boylan NJ, Zhou W, Proos RJ, Tolbert TJ, Wolfe JL, Laurence JS. Conjugation site heterogeneity causes variable electrostatic properties in Fc conjugates. Bioconjug Chem. 2013;24(6):1008–16.
148. Maeda E, Urakami K, Shimura K, Kinoshita M, Kakehi K. Charge heterogeneity of a therapeutic monoclonal antibody conjugated with a cytotoxic antitumor antibiotic, calicheamicin. J Chromatogr A. 2010;1217(45):7164–71.
149. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of protein pharmaceuticals: an update. Pharm Res. 2010;27(4):544–75.
150. Manning MC, Patel K, Borchardt RT. Stability of protein pharmaceuticals. Pharm Res. 1989;6(11):903–18.
151. Cleland JL, Powell MF, Shire SJ. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. Crit Rev Ther Drug Carrier Syst. 1993;10(4):307–77.
152. Siegel MM, Tabei K, Kunz A, Hollander IJ, Hamann RR, Bell DH, *et al.* Calicheamicin derivatives conjugated to monoclonal antibodies: determination of loading values and distributions by infrared and UV matrix-assisted laser desorption/ionization mass spectrometry and electrospray ionization mass spectrometry. Anal Chem. 1997;69(14):2716–26.
153. Sanderson RJ, Hering MA, James SF, Sun MM, Doronina SO, Siadak AW, *et al.* In vivo drug-linker stability of an anti-CD30 dipeptide-linked auristatin immunoconjugate. Clin Cancer Res Off J Am Assoc Cancer Res. 2005;11(2 Pt 1):843–52.
154. Carl PL, Chakravarty PK, Katzenellenbogen JA. A novel connector linkage applicable in prodrug design. J Med Chem. 1981;24(5):479–80.
155. Dubowchik GM, Firestone RA. Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin. Bioorg Med Chem Lett. 1998;8(23):3341–6.
156. Dubowchik GM, Mosure K, Knipe JO, Firestone RA. Cathepsin B-sensitive dipeptide prodrugs. 2. Models of anticancer drugs paclitaxel (Taxol), mitomycin C and doxorubicin. Bioorg Med Chem Lett. 1998;8(23):3347–52.
157. Lewis MR, Shively JE. Maleimidocysteineamido-DOTA derivatives: new reagents for radiometal chelate conjugation to antibody sulfhydryl groups undergo pH-dependent cleavage reactions. Bioconjug Chem. 1998;9(1):72–86.
158. Lin D, Saleh S, Liebler DC. Reversibility of covalent electrophile-protein adducts and chemical toxicity. Chem Res Toxicol. 2008;21(12):2361–9.
159. Baldwin AD, Kiick KL. Tunable degradation of maleimide-thiol adducts in reducing environments. Bioconjug Chem. 2011;22(10):1946–53.
160. Fishkin N, Maloney EK, Chari RV, Singh R. A novel pathway for maytansinoid release from thioether linked antibody-drug conjugates (ADCs) under oxidative conditions. Chem Commun. 2011;47(38):10752–4.
161. Dere R, Yi JH, Lei C, Saad OM, Huang C, Li Y, *et al.* PK assays for antibody-drug conjugates: case study with ado-trastuzumab emtansine. Bioanalysis. 2013;5(9):1025–40.
162. Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int J Pharm. 1999;185(2):129–88.

163. Myers AG, Cohen SB, Kwon BM. A study of the reaction of calicheamicin.Gamma.1 with glutathione in the presence of double-stranded DNA. J Am Chem Soc. 1994;116(4):1255–71.
164. Hollander I, Kunz A, Hamann PR. Selection of reaction additives used in the preparation of monomeric antibody-calicheamicin conjugates. Bioconjug Chem. 2008;19(1):358–61.
165. Safavy A, Bonner JA, Waksal HW, Buchsbaum DJ, Gillespie GY, Khazaeli MB, *et al.* Synthesis and biological evaluation of paclitaxel-C225 conjugate as a model for targeted drug delivery. Bioconjug Chem. 2003;14(2):302–10.
166. Zhao RY, Wilhelm SD, Audette C, Jones G, Leece BA, Lazar AC, *et al.* Synthesis and evaluation of hydrophilic linkers for antibody-maytansinoid conjugates. J Med Chem. 2011;54(10):3606–23.
167. Kovtun YV, Audette CA, Mayo MF, Jones GE, Doherty H, Maloney EK, *et al.* Antibody-maytansinoid conjugates designed to bypass multidrug resistance. Cancer Res. 2010;70(6):2528–37.
168. King HD, Dubowchik GM, Mastalerz H, Willner D, Hofstead SJ, Firestone RA, *et al.* Monoclonal antibody conjugates of doxorubicin prepared with branched peptide linkers: inhibition of aggregation by methoxytriethyleneglycol chains. J Med Chem. 2002;45(19):4336–43.
169. Chari R, Jerath K, Badkar AV, Kalonia DS. Long- and short-range electrostatic interactions affect the rheology of highly concentrated antibody solutions. Pharm Res. 2009;26(12):2607–18.
170. Yadav S, Laue TM, Kalonia DS, Singh SN, Shire SJ. The influence of charge distribution on self-association and viscosity behavior of monoclonal antibody solutions. Mol Pharm. 2012;9(4):791–802.
171. Dominy BN, Minoux H, Brooks 3rd CL. An electrostatic basis for the stability of thermophilic proteins. Proteins. 2004;57(1):128–41.
172. Wang J, Dang V, Zhao W, Lu D, Rivera BK, Villamena FA, *et al.* Perchlorotrityl radical-fluorophore conjugates as dual fluorescence and EPR probes for superoxide radical anion. Bioorg Med Chem. 2010;18(2):922–9.
173. Wang W. Protein aggregation and its inhibition in biopharmaceutics. Int J Pharm. 2005;289(1–2):1–30.
174. Philo J. A critical review of methods for size characterization of non-particulate protein aggregates. Curr Pharm Biotechnol. 2009;10:359–72.
175. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophilized protein formulations: some practical advice. Pharm Res. 1997;14(8):969–75.
176. Ducry L. Challenges in the development and manufacturing of antibody-drug conjugates. Meth Mol Biol (Clifton, NJ). 2012;899:489–97.
177. Wang W, Cui TY, Wang YJ, Martin-Moe S. Oxidation of protein by vaporized sanitizing agents. PDA J Pharm Sci Technol / PDA. 2004;58(3):121–9.
178. Singh SK, Kolhe P, Wang W, Nema S. Large-scale freezing of biologics. A practioner's review Part One: Fundamental aspects. BioProcess Int. 2009;7(9):32–44.
179. Singh SK, Kolhe P, Wang W, Nema S. Large-scale freezing of biologics. A practioner's reveiw. Part 2: practical advice. BioProcess Int. 2009;7(10):34–42.
180. Singh SK, Kolhe P, Mehta AP, Chico SC, Lary AL, Huang M. Frozen state storage instability of a monoclonal antibody: aggregation as a consequence of trehalose crystallization and protein unfolding. Pharm Res. 2011;28(4):873–85.
181. Zhou R, Schlam RF, Yin S, Gandhi RB, Adams ML. Scale considerations for selection of saccharide excipients for liquid formulations. J Pharm Sci. 2011;100(4):1605–6.
182. Kolhe P, Mehta AP, Lary AL, Chico SC, Singh SK. Large-scale freezing of biologics (Part III). Understanding frozen-state protein and solute concentration changes in Celsius bags. BioPharm Int. 2012;25(10):40–8.
183. Bhatnagar BS, Bogner RH, Pikal MJ. Protein stability during freezing: separation of stresses and mechanisms of protein stabilization. Pharm Dev Technol. 2007;12(5):505–23.
184. Kolhe P, Amend E, Singh SK. Impact of freezing on pH of buffered solutions and consequences for monoclonal antibody aggregation. Biotechnol Prog. 2010;26(3):727–33.
185. Metcalfe JW. Microbiological quality of drug products after penetration of the container system for dose preparation prior to patient administration. Am Pharm Rev. 2009;12(1):84–9.
186. FDA U. Guidance for Industry. Dosage and administration section of labeling for human prescriptiopn drug and biological products - content and format. 2010.
187. Demeule B, Palais C, Machaidze G, Gurny R, Arvinte T. New methods allowing the detection of protein aggregates: a case study on trastuzumab. mAbs. 2009;1(2):142–50.
188. Sreedhara A, Glover ZK, Piros N, Xiao N, Patel A, Kabakoff B. Stability of IgG1 monoclonal antibodies in intravenous infusion bags under clinical in-use conditions. J Pharm Sci. 2012;101(1):21–30.
189. Baertschi SW, Clapham D, Foti C, Jansen PJ, Kristensen S, Reed RA, *et al.* Implications of in-use photostability: proposed guidance for photostability testing and labeling to support the administration of photosensitive pharmaceutical products, part 1: drug products administered by injection. J Pharm Sci. 2013;102(11):3888–99.
190. Kumru OS, Liu J, Ji JA, Cheng W, Wang YJ, Wang T, *et al.* Compatibility, physical stability, and characterization of an IgG4 monoclonal antibody after dilution into different intravenous administration bags. J Pharm Sci. 2012;101(10):3636–50.
191. Mikisinski SP, Shapiro M. Regulatory considerations for antibody-drug conjugates2012 February 2014. Available from: http://www.fda.gov/downloads/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/UCM341177.pdf.
192. Impurities in new drug substances. 2006.
193. Q3B(R2) I. Impurities in new drug products. 2006.
194. Nonclinical evaluation for anticancer pharmaceuticals. 2009.
195. EMEA. Guideline on the limits of genotoxic impurities. EMEA/CHMP/QWP/251344/2006. 2006.
196. Jones MT, Gong HH, Lee DH, Zheng J, Kott L, Miller S, et al. Risk assesment for small molceule impurities in Antibody Drug Conjugaes (ADC). BioPharm Int. 2014;27(tbd):tbd.
197. Harris J, Jacobson FS, Jochheim CM, Amphlett G, Francissen K, McLeod L. Uniting small molecule and biologic drug perspectives. Analytical characterization and regulatory considerations for antibody-drug conjugates. BioProcess Int. 2011;9(8):12–22.
198. Xiao YQ, Halford A, Hayes RN. Meeting challenges for analysis of antibody-drug conjugates. BioProcess Int. 2012;25(10):60–3.
199. Good manufacturing practice guide for active pharmaceutical ingredients. 2000.
200. Development and manufacture of drug substances (chemical entities and biotechnological/biologic entities). 2012.
201. Shapiro MA, Chen X-H. Regulatory considerations when developing assays for the characterization and quality control of antibody-drug conjugates. Am Lab. 2012;44(8):1–7.