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Typical Antibody–Drug Conjugates

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1.1 Introduction

1.1.1 A Simple Concept

Ever since cancer patients were first treated with cytotoxic agents with the goal of eradicating the tumor tissue, oncologists have looked to widen the therapeutic window for these agents. The goal of combination chemotherapy, pioneered by Emil “Tom” Frei and others [1], was to increase antitumor efficacy of cytotoxic drug therapy, without substantially increasing overall toxicity to the patient, by using agents with nonoverlapping dose-limiting toxicities. However, such modalities have proven only partially effective at the maximum achievable doses, limited by the severe side effects of the cytotoxic agents used. Attaching cytotoxic effector molecules to an antibody to form an antibody–drug conjugate (ADC) provides a mechanism for the selective delivery of the cytotoxic payload to cancer cells via the specific binding of the antibody moiety to cancer-selective cell surface molecules. This simple concept was thought to be a particularly attractive solution to the challenge of finding a way to increase the therapeutic window of the cytotoxic agent (Figure 1.1). Furthermore, conjugation of a small molecular weight cytotoxic agent to a large hydrophilic antibody protein is expected to restrict penetration of the cytotoxic compound across cellular membranes of antigen-negative normal cells, providing an additional mechanism by which the therapeutic index of the small molecule cytotoxin is widened, beyond that of targeted delivery. Thus, from the perspective of a medicinal chemist, an ADC is a prodrug that can only be activated within tumor cells and is excluded from normal cells by virtue of conjugation to a protein. In addition, giving the in vivo distribution properties of an antibody to the small molecular weight cytotoxic agent has the potential to reduce its systemic toxicity.
1.1.2 Turning Antibodies into Potent Anticancer Compounds

There is another way to look at the simple concept of an ADC. Ever since the advent of monoclonal antibody technology [2], a focus of cancer research has been to develop antibodies for anticancer therapy. Indeed, four monoclonal antibodies, rituximab, trastuzumab, cetuximab, and bevacizumab, are among the most commercially successful anticancer drugs [3]. However, many more antibodies to a variety of target antigens have been tested, both in preclinical studies and in clinical trials, and have proven to have insufficient anticancer activity to be developed as therapeutic agents. In general, the immunologic mechanisms for killing malignant cells induced upon binding of antibodies to cell surface antigens present in cancers appear to be insufficient to affect significant reduction in tumor cell burden in most instances. Thus, providing an additional killing mechanism to such anticancer antibodies via conjugation to cytotoxic agents was thought to be a solution to their lack of potency. From the perspective of an immunologist, enhancing antibody activity by creating ADCs was one approach to be able to fully exploit the full potential of their exquisite specificity toward tumor cells [4–6].

1.1.3 What is a Typical ADC and How Does it Act?

A typical ADC consists of several molecules of a potent cytotoxic agent (generally in the range of two to six molecules per antibody molecule on average), which are linked covalently to side chains of particular amino acid residues of a monoclonal antibody (Figure 1.2). The chosen linker chemistry should be
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sufficiently stable during in vivo circulation in the bloodstream so that the payload stays linked to the antibody during the time it takes for the antibody to distribute into tissues, yet must allow release of an active cytotoxic compound once the ADC is taken up by cancer cells within tumor tissue. Once at the tumor, the antibody component of the ADC binds specifically to its target antigen on cancer cells; in the case of a typical ADC, the cytotoxic payload is liberated after internalization of the antibody–antigen complex and routing to the relevant intracellular compartment for release of an active cytotoxic compound from the ADC (Figure 1.2).

1.1.4 Simple Concept, but Not So Simple to Execute

The earliest notion in the field of ADC research was that conjugation to specific monoclonal antibodies was a way to widen the therapeutic window of existing chemotherapeutic drugs, such as the vinca alkaloids [7], and doxorubicin [8], following on from the early attempts to provide specificity to cytotoxic drugs by conjugation to serum immunoglobulins [9]. However, despite the early optimism generated by some of the preclinical results [8], the results of clinical trials of such conjugates were disappointing [10–12]. During the 1980s, increased knowledge of the biodistribution properties of monoclonal antibodies based on clinical dosimetry measurements with radiolabeled antibodies pointed to one explanation for such disappointing results.

Figure 1.2 The components of an ADC and its mechanism of action. (See color plate section for the color representation of this figure.)

- ADC components:
  An integrated system
  - Targeting antibody
  - Cytotoxic agent
  - Linker

- ADC mechanism:
  - Binds to target on surface of cancer cell
  - Internalized into the cell
  - Cytotoxic agent is released inside the cell
  - Cytotoxic agent affects its target (eg., DNA; tubulin)
  - Cell death
It was found that the amount of antibody that could be localized to a solid tumor 24 h after administration, a time corresponding approximately to the peak delivered concentration, was only about 0.01% of the injected dose of antibody per gram of tumor tissue for a range of different antibodies, to a variety of targets in patients with a variety of tumor types [13]. Thus, it was reasoned that the lack of clinical benefit from ADCs made with conventional chemotherapeutic drugs was that not enough of these agents could be localized at the tumor via antibody-mediated delivery to have an antitumor effect. The use of these only moderately cytotoxic compounds as payloads for ADCs was at least one of the barriers to the successful execution of the ADC concept. The idea that conventional chemotherapeutic drugs were not potent enough to serve as payloads for ADCs has guided much of the subsequent research in the field [4–6].

1.2 The Building Blocks of a Typical ADC

All three parts of an ADC, the antibody, the cytotoxic payload, and the linker chemistry that joins them together, are important in designing an ideal ADC. The design goal is to add the potent tumor cell-killing mechanism afforded by the payload, while retaining all the favorable properties of the antibody in terms of in vivo pharmacokinetics and biodistribution, together with any intrinsic biologic activity and immunologic properties. It is beyond the scope of this chapter to discuss the properties of the cell surface target molecule, but suffice to say that selecting the right target, and matching the design of the ADC to the properties of the target, is vital to the creation of an effective therapeutic agent.

1.2.1 The Antibody

The first monoclonal antibodies used in ADCs and also in immunotoxins – antibodies conjugated to potent protein toxins such as derivatives of ricin, or diphtheria toxin [14] – were murine antibodies. However, apart from other limitations, such conjugates proved to be immunogenic in humans [10]. The advent of chimerization and a variety of humanization techniques (CDR grafting, resurfacing) for rendering murine antibodies less immunogenic or nonimmunogenic in humans [15], and the methods for cloning of human immunoglobulin genes into a variety of organisms, such as transgenic animals, bacteriophage, or yeast, for the generation of fully human antibodies [16–18], have largely addressed this problem (Figure 1.3), as has been generally borne out by the recent clinical experience with ADCs [19]. Of the 51 ADCs currently in clinical trials, at least two utilize chimeric antibodies, including the approved ADC, brentuximab vedotin, while for the other ADCs, antibody usage is, where known, fairly evenly split between humanized antibodies and fully human antibodies. Several of the humanizations were done by the method of variable domain resurfacing [15], for
example, the anti-CanAg antibody, cantuzumab, utilized in the first maytansinoid ADC (cantuzumab mertansine) to enter into clinical trials [20]. Recently, however, the World Health Organization decided to alter criteria for providing generic names to antibodies, resulting in the confusing situation of many humanized antibodies being given names bearing the suffix of a chimeric antibody (“-ximab”), for example, the anti-CD19 antibody, coltuximab [15, 21], and the antifolate receptor alpha (anti-FRα) antibody, mirvetuximab [22], both of which were humanized by the resurfacing method [15, 23].

1.2.1.1 Antibody Isotype in ADCs
Most of the antibodies utilized in ADCs evaluated in clinical trials to date, including those (about 20) now discontinued, have been of the human IgG₁ isotype (60 of 67 ADCs, with an additional four not disclosed, upon this author’s
review of source information). In general, the Fc regions of these IgG_1 antibodies are unmodified with respect to Fc receptor binding properties so that all could be capable of inducing immune effector cell killing or complement-mediated cytotoxicity (Figure 1.4). However, at least one ADC was designed with an IgG_1 antibody having enhanced FcγR (FcγRIIIa) binding for enhanced antibody-dependent cellular cytotoxicity (ADCC) activity by virtue of being produced in an afucosylated form [24]. Thus far, no ADC with a human IgG_1 isotype in clinical development has employed an antibody with amino acid mutations known to abrogate FcγR binding, despite some speculation that such modifications may reduce certain toxicities observed in clinical trials with some ADCs [25]. Indeed, where abrogation of FcγR binding was part of the stated design goal of the resulting ADC, the IgG_4 format has been the preferred option to date, known to be used in three ADCs currently in clinical trials (gemtuzumab ozogamicin, inotuzumab ozogamicin, and indatuximab ravidansine). At least three ADCs have employed a human IgG_2 antibody, all of which were fully human antibodies generated in transgenic mice engineered to express human immunoglobulin genes in place of the corresponding mouse genes [17].

1.2.1.2 Functional Activity of the Antibody Moiety in ADCs
Antibodies for ADCs may be developed to targets where the antibody may have functional activity beyond intrinsic immunological functions of ADCC, ADCP, or CDC. The primary exemplar of this would be the approved ADC, ado-trastuzumab emtansine, wherein the antibody component, trastuzumab, inhibits HER2-driven cell growth in HER2-positive (overexpressing) breast cancer [26]. In this case, arming the antibody with a payload provides an additional mechanism for cancer cell killing over and above its intrinsic biologic and immunologic activities (Figure 1.4). In another example, antibody selection for an ADC that targets CD37 (IMGN529) was based on screening for those antibodies that could directly induce apoptotic cell death in CD37-positive tumor B cell lines. The antitumor activity of the antibody was then further augmented by arming it with a payload to create the ADC compound that was taken into clinical development [27]. For targets that have no signaling function, one would not anticipate finding antibodies that can induce any biologic function upon binding to the target, saving perhaps for immunologic effector functions triggered by antibody binding to the cell surface. In general, antibodies whose only function upon binding to tumor cells is to induce ADCC and/or ADCP often exhibit very little antitumor activity in clinical trials, sparking efforts to enhance effector functions [28]. Most ADCs in development are to such targets, where arming the antibodies with a payload to exploit their specific binding to cells is one way to provide them with a direct cell-killing function. For these targets, the antibodies should be selected for the property of efficient payload delivery, as in the example of an ADC designed to target FRα, IMGN853, recently named mirvetuximab soravtansine [22].
Apart from specificity for their target, antibodies should bind with sufficient affinity for good retention at the tumor in vivo. Typically, the apparent binding affinities of the antibody component of most ADCs currently in clinical evaluation are in the range of about 0.1 to 1.0 nM. However, there is little published data regarding what the optimal binding affinity should be for an ADC. Some studies with antibodies suggest that very high affinity may compromise delivery of antibodies throughout solid tumors [29], although such findings may depend on target biology and tumor type. Since typical ADCs are designed to require intracellular release of an active payload, the antibody should be internalized upon binding to its target [30–33].

1.2.2 The Payload

For an ADC to exhibit potent antitumor activity, the cytotoxic agent that serves as the payload must be active at killing cells at the intracellular concentrations achievable within tumor cells by antibody-mediated distribution into tumor tissue followed by target-mediated uptake into tumor cells. As the constraints on payload delivery via antibody-mediated distribution and cellular uptake became better understood [13], it was reasoned that the cytotoxic compounds suitable for ADC approaches should have potency in the
picomolar range [4–6]. The structures of several highly potent cytotoxic compounds that are currently being used as payloads for ADCs are shown in Figure 1.5. All but calicheamicin, of those shown in Figure 1.5, were (or, in the case of SJG-136, are still being) evaluated in clinical trials, and all proved too toxic, with limited antitumor activity at the achievable maximum tolerated doses [5].

![Chemical structures of ADC payloads](image)

**Figure 1.5** Structures of highly cytotoxic compounds developed as payloads for ADCs. Calicheamicin antibiotics cause DNA double-stranded breaks via a radical mechanism, SJG-136, a pyrrolobenzodiazepine dimer, alkylates and cross-links DNA, and the duocarmycin, adozelesin, alkylates DNA [5]. Dolastatin 10 and maytansine are potent tubulin-interacting compounds that disrupt microtubule dynamics [5, 34].
1.2.2.1 DNA-Targeting Payloads

The first ADC to receive marketing approval by FDA, gemtuzumab ozogamicin [35], used calicheamicin as the payload, a potent DNA-targeting agent that causes double-stranded breaks in the DNA resulting in cell death [5]. However, in 2010, it was withdrawn from the US market by the sponsor, 10 years after its initial approval for treating acute myeloid leukemia (AML), following an unsuccessful confirmatory phase III trial [36] and unacceptable safety profile. Subsequently, results from other trials utilizing dose fractionation have suggested patient benefit and have revived interest in this compound [37], and also in CD33 as an ADC target for AML [38]. Calicheamicin is known to be used as the payload in at least two other ADCs in current clinical testing, inotuzumab ozogamicin that targets CD22 on malignant B cells and that is in a phase III trial for treating acute B-cell leukemia [39], and an ADC that targets EphA4, a marker expressed on the cell surface of tumor stem cells in certain solid tumors [40], that is being evaluated in a phase I trial.

Another potent class of DNA-targeting agent are derivatives of the anticancer agent, SJG-136 (Figure 1.5), a pyrrolobenzodiazepine (PBD) dimer [41] that cross-links DNA, which are being assessed as payloads for three ADCs in ongoing clinical trials (e.g., see references [38] and [42]). Others include the camptothecin analog SN38 that is the payload for two ADCs [43], and a duocarmycin, a member of a family of DNA-alkylating antibiotics which includes adozelesin (Figure 1.5), that is, the payload of an ADC targeting HER2 [44]. Recently, a potent DNA-alkylating indolinobenzodiazepine dimer has been developed as a payload for ADCs, the first of which, IMGN779, entered into clinical testing in early 2016 [45].

1.2.2.2 Payloads Targeting Tubulin

Although these DNA-acting cytotoxins have the desired attribute of extraordinary high potency to be effective as an ADC payload, such compounds do have drawbacks. In general, DNA-interacting compounds are hydrophobic and may lack sufficient solubility in aqueous conditions for facile conjugation to antibodies, and some (e.g., duocarmycins) may not be stable in aqueous environments, thus requiring the use of prodrug approaches to protect the DNA-alkylating function [44]. These factors may explain why, even though the first ADC to receive approval utilized calicheamicin as the payload [35], only 11 of the 51 ADCs in clinical development at the time of writing utilize DNA-targeting compounds as payloads. Currently, the most important classes of ADC payload are potent tubulin-acting agents, which are used in 37 of the 51 ADCs in development (the payloads for three of the 51 ADCs have not yet been publicly disclosed). There are two main classes of these potent tubulin-acting agents in widespread use in ADCs undergoing clinical testing. Where the payload structures are disclosed \( (n = 37) \), 60% use auristatins, analogs of dolastatin 10, while 35% utilize derivatives of maytansine (Figure 1.5).
The binding of auristatins or maytansinoids to tubulin interferes with microtubule dynamicity, causing cells to arrest in the G2/M phase of the cell cycle, which ultimately results in apoptotic cell death [31, 34, 46]. Since these agents act as antimitotic agents because of their effect at disrupting the mitotic spindle, they have a natural selectivity for rapidly dividing cells. In the context of an ADC, this attribute of a payload may bring an additional level of selectivity beyond that provided by the specific binding of the antibody moiety. Target antigens are rarely completely tumor specific, their selectivity being based on differential expression on tumor versus normal cells rather than the complete absence of expression on normal cells. In any case, in most circumstances, most of the administered antibody is eventually removed from circulation for catabolism via cells of the reticuloendothelial system with only a small portion of the injected material passing through and being retained in tumor tissue [13]. Thus, the lack of cytotoxicity of these potent microtubule-acting compounds toward nondividing, or only slowly dividing, normal cells may contribute to the tolerability of ADCs made using them as payloads.

1.2.3 Linker Chemistries

An optimal linker should be sufficiently stable in circulation in the bloodstream to take advantage of the pharmacokinetic properties of the antibody moiety (the long half-life), yet should allow efficient release of an active cytotoxic compound within the tumor cell. Linkers used in typical ADCs can be characterized as either cleavable or noncleavable. The only mechanism of release of an active metabolite from an ADC utilizing noncleavable linker chemistry is by the complete proteolysis of the antibody moiety down to its constituent amino acids, which requires that following antigen-mediated internalization of the ADC, it is trafficked to lysosomes for proteolytic degradation. The active cytotoxic metabolite is thus appended with an amino acid residue, a lysine or a cysteine residue in a typical ADC – the site of attachment of the payload to the antibody via the linker. The necessity for sufficient lysosomal trafficking of the ADCs designed with noncleavable linkers means that lysosomal trafficking becomes a key selection criterion for the antibody and its target for ADCs of this design [33].

Cleavable linkers are those whose structure includes a mechanism of cleavage of chemical bonds between the amino acid attachment site on the antibody and the payload, thus freeing the active cytotoxic metabolite from any residual amino acid residue derived from the antibody attachment site. The cleavage mechanisms used in typical ADCs with cleavable linkers include the hydrolysis of acid-labile bonds in acidic intracellular compartments, proteolytic cleavage of amide bonds by intracellular proteases, and reductive cleavage of disulfide bonds by the reducing environment inside cells (see Section 1.3). It is possible that these mechanisms can operate in the pre-endosomal and endosomal compartments.
of cells without a strict requirement for lysosomal trafficking, although in the case of proteolytic cleavage, one must design peptide linkers susceptible to the proteases present in such nonlysosomal compartments. When the chemical structure of the linker–payload results in the release of an unmodified payload, such linkers may be referred to as “traceless linkers.” In other cases, the final active cytotoxic metabolite released intracellularly from the ADC is a derivative of the “parent” cytotoxic compound, which now includes structures and/or functional groups introduced as part of the linker chemistry. Indeed, varying the linker–payload chemistry to alter the properties of the final active metabolite is part of the design space of developing an effective, well-tolerated ADC [26, 30, 32, 47]. For example, increasing the hydrophobicity of the cytotoxic metabolite may increase the rate of transfer across cellular membranes for more efficient exit of the released payload moiety from lysosomes to enable access to its target within the cell. Alternatively, increasing its hydrophilic nature, for example, via charged groups, may decrease the rate of transmembrane transfer and thereby increase cellular retention [47, 48].

Linkers can be “stand-alone” bifunctional reagents that have one functional group designed to react with a functional group on an antibody, typically the amino group of a lysine residue or the sulfhydryl group of a cysteine residue (Figure 1.6), and a second functional group capable of reacting with an appropriate complementary functional group of the cytotoxic payload. This approach is the one taken in making ADCs using the maytansinoid platform, as exemplified by ado-trastuzumab emtansine [5, 26, 49]. Alternatively, the linker chemistry can be built into the payload as a single chemical entity, which then contains a single functional group for reaction with the antibody protein, again usually targeting either lysine amino groups or sulfhydryl groups of lysine or cysteine residues, respectively (Figure 1.6). This approach is exemplified by ADCs such as brentuximab vedotin using the auristatin platform [4, 5, 31].

1.3 Building an ADC Molecule

1.3.1 Conjugation of Payloads to Antibodies at Lysine Residues

The surface-accessible amino groups of lysine residues in an antibody make good attachment sites for a linker–payload since a sizable fraction of them can be modified without disturbing the integrity of the protein structure, thus preserving the native function and favorable pharmacokinetic properties of the antibody [5]. Most linkers/linker–payloads designed for attachment to lysine amino groups utilize $N$-hydroxysuccinimide esters, which react readily and preferentially with primary amines to form stable amide
bonds between the linker and the side-chain amino group of the lysine. Lysine attachment sites are used in the approved ADC, ado-trastuzumab emtansine (Figure 1.7), and in the other maytansinoid ADCs in clinical development, as well as in calicheamicin-containing ADCs, such as gentuzumab ozogamicin and inotuzumab ozogamicin [35, 39, 40]. The examples of typical ADC structures conjugated through lysine residues, shown in Figure 1.7, include ADCs with an acid-labile hydrazine linker (the calicheamicin conjugates), an uncleavable linker (ado-trastuzumab emtansine), and a hindered disulfide linker cleavable by the reduction of the disulfide bond (mirvetuximab soravtansine).

**Figure 1.6** Functional groups of antibodies typically used in conjugation reactions. The ribbon diagram shows the structure of an IgG1, with the backbone color coded according to the inset. Lysine residues (purple) and those cysteine residues involved in interchain disulfide bonds (green) are shown with space-filling atomic spheres. N-hydroxysuccinimide ester cross-linkers (NHS-linker) are typically used for a two-step conjugation of maytansinoids (red space-filling) to lysine residues [5, 47], for example, in the preparation of ado-trastuzumab emtansine [5, 26, 49, 50]. Maleimido-linker–auristatin compounds (magenta space-filling) are typically used to conjugate auristatin derivatives to antibodies at free sulfhydryl groups formed by partial reduction of interchain cysteine–cysteine disulfide bonds [4], for example, in the preparation of brentuximab vedotin [31, 51]. Similar conjugation chemistry can conjugate payloads to sulfhydryl groups of cysteine residues introduced into antibody structures by protein engineering [38, 52, 53]. (See color plate section for the color representation of this figure.)
A typical human(ized) IgG antibody contains between 80 and 90 unique lysine residues within its amino acid sequence \([50, 54]\). The conditions of the modification reaction between the antibody and the linker/linker–payload (e.g., reagent concentrations, reaction pH) must be carefully controlled to limit the average level of payload addition to a typical range of about three to four conjugated sites per antibody molecule. For example, the average maytansinoid-to-antibody molar ratio (also characterized as “drug”–to-antibody ratio, or DAR) for ado-trastuzumab emtansine is about 3.5 \([26, 49, 50]\). The ratio was selected for the defined ADC product based on (i) minimizing the amount of nonconjugated antibody and (ii) avoiding species in the mixture with very high DAR, which may be problematic in manufacturing and formulation due to higher hydrophobicity and lower solubility \([26, 50]\). Furthermore, higher DAR species may have altered pharmacokinetic properties, the increased hydrophobicity resulting in more rapid clearance \([21]\). The relative abundance of ADC species with different numbers of payloads attached per antibody molecule can

![Figure 1.7 Examples of typical ADCs conjugated at lysine residues. Gemtuzumab ozogamicin and inotuzumab ozogamicin are conjugates of a calicheamicin payload where the linker includes an acid-labile hydrazone moiety (shaded gray), and also contains a hindered disulfide bond cleavable by reduction (average DAR of these ADCs are in the range of 2 to 4 – only one linker-payload structure drawn for simplicity). The two maytansinoid ADCs show examples of conjugates with either a non-cleavable link created by reaction of the sulfhydryl group of the maytansinoid DM1 with the maleimido group of the linker (thioether bond so formed is shaded gray), as in ado-trastuzumab emtansine, or with a hindered disulfide-containing link (disulfide shaded gray) that is cleavable by reduction, as in mirvetuximab soravtansine (values for \(n\) and \(m\) are between 3 and 4 maytansinoids per antibody). The linker for mirvetuximab soravtansine also bears a hydrophilic charged sulfonate group.](image-url)
be estimated by mass spectrometry [50, 54–56]. In the case of maytansinoid ADCs, for an average DAR of about 3.5 for which three representative mass analyses are shown in Figure 1.8 (three different linker–maytansinoid species), about 70–80% of the antibody molecules have between two and five maytansinoids per antibody and > 90% of the antibody molecules have individual DAR values in the range of 1 to 6 [55]. At this average level of payload addition (DAR ~3.5), only about 3% of the antibody was nonconjugated antibody and only a similarly low proportion of antibody molecules had DAR values ≥7 [55, 56]. The distribution pattern of species with different DAR found experimentally is quite predictable for a given average DAR and can be described by statistical models, either by Poisson distribution [50] or by the binomial distribution [55]. One implication of these observations is that measurement and control of the

Figure 1.8 Deconvoluted mass spectra of three deglycosylated ADCs. Shown are examples of three different antibodies conjugated to three different linker–maytansinoid moieties with an average DAR value of 3.5 for each conjugate (determined spectrophotometrically). (a) mAb1-SMCC-DM1; (b) mAb2-SPP-DM1; (c) mAb3-sulfo-SPDB-DM4. Source: Adapted with permission from Goldmacher, V.S., et al., Molecular Pharmaceutics, 12, 1738–1744, copyright 2015, American Chemical Society [55].
DAR value itself during conjugation reactions could be sufficient to control the levels of nonmodified antibody in the defined ADC preparation [50].

Mass spectroscopy is also a useful tool to analyze chromatographic peptide maps in order to determine the actual conjugation sites in the antibody moiety of the ADC. Depending on the sensitivity of the technique, from 40 to 70 individual lysine residues (more than half of the total possible) are partially modified in the example of maytansinoid technology [50, 54, 56]. The range of levels of modification of individual lysine residues in ado-trastuzumab emtansine, for example, is from about 25% to <1%, with a median value of about 4% [50]. Such peptide mapping techniques provide a fingerprint that can be used to compare different lots of an ADC to ensure process consistency and robustness during scale-up to commercial manufacturing.

1.3.2 Conjugation of Payloads to Antibodies at Cysteine Residues

Linker–payload constructs designed for attachment to sulfhydryl groups of cysteine residues of the antibody have made use of the rapid reaction between maleimido groups and sulfhydryl groups to form thioether bonds [31, 42, 51]. The auristatin (MMAE) used as the payload of the approved ADC, brentuximab vedotin, and the auristatins used in the other ADCs in current clinical development, which use this payload class (MMAE and MMAF), are synthesized as maleimide-bearing linker–payload compounds for reaction with protein sulfhydryl groups. ADCs made with the DNA-crosslinking payload, PBD, can also be conjugated by this approach [42]. Figure 1.9 shows the structure of brentuximab vedotin and also shows a structure for an MMAF conjugate. The valine-citrulline-para-aminobenzyl-containing linker of brentuximab vedotin is an example of a protease-cleavable linker; the amide bond between the dipeptide and the para-aminobenzyl moiety is cleaved by the lysosomal protease, cathepsin B [5, 51]. It is also an example of a “traceless linker,” since cleavage of the amide bond is followed by self-immolation of the para-aminobenzyl moiety with loss of carbon dioxide to yield MMAE as the final metabolite [5, 51]. Other payloads exploit a similar mechanism of release [42]. The MMAF conjugate has an uncleavable link so that the final metabolite released inside the cell contains the linker plus a cysteine residue [5].

Antibodies generally do not contain free, solvent-accessible sulfhydryl groups, but rather they contain cysteine residues whose sulfhydryl groups are oxidized to form disulfide bonds between pairs of cysteine residues (see Figure 1.6). An IgG1 contains 16 disulfide bonds, four interchain disulfide bonds, two between the two heavy chains and one between each light chain and a heavy chain, and 12 intrachain disulfides. The four interchain bonds can be readily reduced and maintained as pairs of free sulfhydryl groups under nonoxidative conditions, and these can serve as sites of reaction with maleimido-linker–payload compounds [4, 5, 51].
The level of interchain disulfide bond reduction is carefully controlled so that in a typical auristatin conjugate, the DAR is limited to an average of about four [51]. As mentioned earlier for lysine conjugation, the ratio of about four auristatins per antibody molecule was selected to minimize the amount of non-conjugated antibody, and also to minimize the amount of ADC species with DAR values of eight, obtained by complete reduction of the intrachain disulfide bonds of an IgG antibody followed by complete reaction of all sulfhydryl groups thus produced with the maleimido-linker–payload linkage (shown shaded gray) that is cleavable by the cellular protease, cathepsin B. Also shown is a structure for a mAb-maleimido-caproyl-MMAF conjugate where the charged auristatin, MMAF, is linked to cysteine residues via a non-cleavable link (shown shaded gray). Values for $n$ are typically about 4 in each case.

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The loss of interchain disulfide bonds that accompanies conjugation of maleimido-linker–payloads to an antibody may come at a cost of some degree of stability in vivo, which may vary from antibody to antibody, and may become...
particularly apparent when such modification is pushed to completion (all four disulfides of an IgG1 reduced to yield an ADC bearing eight linker–payload moieties) [57]. Auristatin conjugates with DAR values of eight have a very short half-life in vivo, likely accounting for poor efficacy, and perhaps increased toxicity [52, 57]. One approach to address this issue is to express antibody genes bearing mutations, which result in the replacement of a solvent-accessible amino acid with a cysteine residue whose unpaired sulfhydryl group can serve as a payload attachment site [38, 52]. This approach also allows an ADC to be made with a DAR value of about two, with most conjugation occurring at a single site on each half-antibody (see Figure 1.6). Such site-specific conjugation minimizes the generation of any ADC species with DAR values ≥3, which may be an important consideration when conjugating very hydrophobic payloads (e.g., see reference [38]). However, selecting the correct site in the antibody molecule up front is not trivial [58], and furthermore, having 100% of the modification at a single site could potentially increase the possibility of an adverse immune response to the ADC. Several ADCs made using site-specific cysteine conjugation have recently entered clinical trials (structures are disclosed for four such “site-specific” ADCs at time of writing). There are a variety of other site-specific conjugation approaches that are being evaluated in the research laboratory [53, 59, 60]; however, a detailed description of these approaches is beyond the scope of this chapter and is about “typical” ADCs at this time.

1.4 Attributes of a Typical ADC

1.4.1 Structural Attributes of a Typical ADC

A typical ADC with a DAR value in the range of 3.5 to 4.0 has a molecular mass that is about 2% greater than that of the corresponding “naked” antibody moiety. Ideally, the biochemical parameters of an ADC should be broadly similar to those of its nonmodified antibody moiety, behaving mostly as intact monomeric molecules upon size-exclusion chromatography, with very little aggregate present [56, 61]. However, addition of payloads does alter surface hydrophobicity properties, with effects on solubility and the propensity to aggregate. The magnitude of such effects depends on DAR and relative hydrophobicity of the payload and must be carefully studied to ensure appropriate formulation designed to minimize any aggregation [56, 61]. In many cases, ADCs can be formulated in a similar manner to the parent “naked” antibody and can be provided to the clinical pharmacy as vials of either a liquid (e.g., coltuximab ravtansine) or a lyophilized (e.g., ado-trastuzumab emtansine) drug product [62, 63]. It is important to have an accurate assay to test for the presence of any nonconjugated (“free”) payload, including any small molecule linker–payload derivatives, which are typically at levels that are less than a few percent of the total conjugated
payload [56]. As mentioned earlier, size distribution analysis by mass spectrometry or by hydrophobic interaction chromatography are important tools for characterizing the ADC product, together with other biochemical and biophysical techniques such as imaged capillary isoelectric focusing for charge-based separation of species, and peptide mapping by liquid chromatography coupled to mass spectrometry [54, 56, 61]. Many of these biochemical and biophysical tools are the same tools that are already widely used to characterize “naked” antibody products, which are themselves heterogeneous molecules, for example, with respect to glycosylation patterns, deamidation of glutamine or asparagine, level of C-terminal lysine, and so on [54, 56, 61].

1.4.2 Functional Characteristics of a Typical ADC

1.4.2.1 In Vitro Properties
Ideally, conjugation of 3.0–4.0 payloads per antibody should have little or no effect on the binding specificity and apparent binding affinity of the antibody component of the ADC to its cell surface target [64, 65]. ADCs should also exhibit specificity and selectivity in their cytotoxicity upon evaluation of their in vitro potency. For example, a maytansinoid ADC targeting FRα made with the uncleavable SMCC-DM1 linker–payload format shows specific killing of cells expressing human FRα on their cell surface relative to an isotype-matched nonbinding control conjugate (Figure 1.10). Also shown in Figure 1.10 is the kill curve for a maytansinoid ADC targeting FRα made with a cleavable sulfo-SPDB-DM4 linker–payload format (IMGN853) that shows selectivity for antigen-mediated killing as demonstrated by the blockade of cytotoxic effects by the addition of a large excess of nonmodified “naked” antibody (Figure 1.10) [22]. Thus, for maytansinoid ADCs targeting FRα, in vitro cytotoxicity assays do not distinguish between a conjugate made with a cleavable linker (disulfide, in this case) and a noncleavable linker, a common observation in such in vitro assays [30, 32, 47]. However, differences in potency may be observed in vivo depending on the biology of the target [21, 22, 30, 32, 49].

It is worth noting that the magnitude of the difference between antigen-mediated killing and that of a nonbinding control ADC (about 100-fold as shown in Figure 1.10) is largely a function of antibody binding affinity to its target in these in vitro assays. An antibody with an apparent $K_D$ only in the 5–10 nM range will show only a small degree of specificity in vitro. However, it may be that the apparent binding affinity of an antibody is only one of the factors important for in vivo potency of an antibody/ADC, and high affinity may actually decrease overall uptake and retention by a tumor mass in vivo under some circumstances [29, 66, 67].

1.4.2.2 In Vivo Efficacy
Once an ADC has met the biochemical quality attributes (monomeric molecule, with only low levels of aggregates and of free payload species), and exhibits
Typical Antibody–Drug Conjugates

Appropriate in vitro activity, its antitumor activity should be evaluated in several in vivo models representing human tumors that are relevant indications for the particular ADC. These preclinical models can be xenograft models derived from human tumor cell lines or from patient-derived tumor xenograft models (PDx models). Clearly, it is a prerequisite that whatever in vivo models are to be used for the determination of antitumor activity, their levels of target antigen expression should be assessed. Immunohistochemical methods that are calibrated to have a dynamic range covering a relevant range of antigen expression levels appropriate for the cell-killing capability of the ADC are typically used for this purpose and should as closely as possible match the test method to be used on clinical samples for cancer patients.

Figure 1.10 In vitro cytotoxicity was measured after a 5-day exposure of FRα-positive KB cells to (i) a maytansinoid ADC targeting FRα made with a cleavable sulfo-SPDB-DM4 linker-payload format (IMGN853), with (dotted line, open circles) or without (solid line, closed circles) a blocking concentration (2 μM) of the “parent” non-conjugated anti-FRα antibody (M9346A), and (ii) M9346A-SMCC-DM1, a maytansinoid ADC targeting FRα made with a non-cleavable SMCC-DM1 linker-payload format (solid line, closed triangles), compared with the cytotoxicity curve of a non-targeting hulgG1-SMCC-DM1 (dotted line, open triangles). Source: Adapted from Ab, O., et al., Molecular Cancer Therapeutics, 14(7); 1605–1613, copyright 2015, American Association for Cancer Research [22].
Figure 1.11 shows an *in vivo* efficacy assessment for an example of an ADC, mirvetuximab soravtansine (IMGN853), in a xenograft model derived from the FRα-positive Ovcar-3 cell line (Figure 1.11a) and in an FRα-positive PDx model (Figure 1.11b). The conjugate was found to be highly active in both models,

![Image](image1.png)

**Figure 1.11** *In vivo* efficacy of mirvetuximab soravtansine (IMGN853) in three FRα-positive xenograft models. The dose–response curves for IMGN853-treatment of mice that bore xenograft tumors derived from the Ovcar-3 ovarian cancer cell line, and from the LXFA-737 xenograft derived from an ovarian cancer patient, are shown in panels (a) and (b), respectively. Mice bearing tumor xenografts (tumor volume about 130 mm³) received a single intravenous injection of IMGN853, vehicle control (solid diamonds, dotted line), or a nontargeting huIgG1-sulfo-SPDB-DM4 conjugate (latter only in the LXFA-737 model; open circles). The tumor-bearing mice were treated with IMGN853 at 1.2 ± 0.1 mg/kg (triangles), 2.4 ± 0.2 mg/kg (circles), or 5.0 ± 0.3 mg/kg (squares), which are equivalent to 24 ± 3, 49 ± 2, and 98 ± 4 µg/kg conjugated maytansinoid, respectively. Nontargeting huIgG1-sulfo-SPDB-DM4 conjugate was injected at 5.0 mg/kg (90 µg/kg conjugated maytansinoid). The effect of weekly administration of IMGN853 on the antitumor activity against KB xenograft tumors is shown in (c). Mice with established tumors of about 130 mm³ were intravenously treated with a single injection of vehicle control (solid diamonds), or IMGN853 at 50 µg of conjugated maytansinoid per kg (equivalent to 2.8 mg/kg antibody) on day 6 postinoculation (open triangles), or with multiple injections (qw × 3) on day 7, 14, and 21 postinoculation (solid circles). Source: Adapted from Ab, O., et al., *Molecular Cancer Therapeutics*, 14(7); 1605–1613, copyright 2015, American Association for Cancer Research [22].
with partial and complete regressions being observed when the tumor-bearing mice were given a single intravenous dose of 5 mg/kg (antibody dose, equivalent to about 90–100 µg/kg of conjugated maytansinoid) of the ADC [22]. The isotype-matched human IgG1 control conjugate (same linker–payload at the same DAR) showed no activity at this dose (Figure 1.11b). A lower dose of the FRα-targeting conjugate (about 2.4 mg/kg) caused a growth delay of 10–15 days in the PDx model, while partial and complete responses were still observed in the Ovcar-3 xenograft model. The effect of repeated dose administration was also studied: Figure 1.11c shows a xenograft model derived from the FRα-positive KB cell line where administration of a single dose of 2.8 mg/kg caused a partial response, and administration of the same dose weekly for 3 weeks resulted in complete regressions of the tumor xenograft.

Importantly, the antitumor activity of a typical ADC should be manifest at doses that achieve plasma concentrations in mice that are not markedly higher than the maximum plasma concentrations ultimately achievable in human clinical trials, and at doses that should not exhibit any signs of toxicity to the mice. Such dosing comparisons can be approximated in units of mg/kg, given that mouse and human each have approximately similar ratios of plasma volume to body weight, 40 to 43 mL/kg [68]. Although one also needs to take into account potential differences in pharmacokinetic properties of the ADC between mouse and human, as well as any differences in normal tissue antigen expression in the case of antibodies that do not cross-react with the mouse antigen which can result in differences in antigen-mediated clearance, assessment of anti-tumor activity in units of mg/kg may offer valuable initial insight into the potential of an ADC for development. For example, in the case of maytansinoid-ADCs, the maximum tolerated doses achieved in clinical trials are in the range of single-digit mg/kg doses [20, 26, 69], and such doses have shown promising, or confirmed, anti-tumor activity for some of them (e.g., [26, 69]). Even though the maximum tolerated doses in mice for maytansinoid-ADCs can be >80 mg/kg [22, 48], it is important that the doses found to be highly active in preclinical evaluation should be in a similar single-digit mg/kg dose range as the maximum doses likely to be achieved in humans for the best opportunity for successful development in clinical trials (see e.g., Figure 1.11).

1.4.2.3 Pharmacokinetics of ADCs
The pharmacokinetic characteristics of an ADC should also be characterized in preclinical models in order to properly model activity with exposure [70]. Typically, measurements are made of total antibody, irrespective of DAR (including any nonconjugated antibody), intact conjugate, and free payload species [71]. The pharmacokinetic parameters of a conjugate are somewhat variable – there is no “typical” set of parameters – since they will depend on the nature of the antibody, the presence of the target on normal tissue
(antigen-mediated clearance), the chemistry of the linker, and the type of payload and DAR value [32, 57]. Considering maytansinoid ADCs, an example of an ADC made with a cleavable hindered disulfide linker, coltuximab rivanosine, shows a half-life of about 7 days in a phase II clinical trial in patients with non-Hodgkin’s lymphoma [72], while an ADC with an uncleavable linker, ado-trastuzumab emtansine, shows a half-life of about 3–4 days in breast cancer patients showing the effect of antigen-mediated clearance [19].

1.5 Summary

In an attempt to increase antitumor activity of anticancer drugs, oncologists have evaluated several families of highly cytotoxic agents such as the potent tubulin-acting agents, maytansine and dolastatin 10, and highly active DNA-acting agents such as the duocarmycins (adozelesin, bizelesin). Unfortunately, clinical evaluation of such potent compounds failed to show any clinical benefit; such compounds lacked a sufficient therapeutic window to be useful in cancer treatment (see e.g., [73]). However, with the advent of nonimmunogenic monoclonal antibodies (engineered antibodies, or derived from transgenic organisms expressing human immunoglobulin genes), medicinal chemists had the possibility of being able to harness the great potency of these cytotoxic compounds by using the potential tumor selectivity of antibodies as vehicles to concentrate them at the tumor. The proof of concept for this approach has been achieved with the approval of two ADCs bearing highly potent tubulin-acting agents, brentuximab vedotin, which received accelerated approval by FDA in 2011 for treating Hodgkin’s lymphoma and acute large-cell lymphoma [51], and ado-trastuzumab emtansine that received full approval from FDA in 2013 for treating metastatic HER2-positive breast cancer [26, 74]. These approvals have re-invigorated research into ADCs within academic institutions as well as in the biopharmaceutical industry. With over 50 compounds in clinical testing at the time of writing, the prospects are exciting for ADCs to make a significant contribution to improving the therapeutic options for cancer patients, offering agents that have excellent antitumor activity while lacking the severe toxicities that are frequently associated with cytotoxic chemotherapy.

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Abbreviations

ADC  antibody–drug conjugate
ADCC  antibody-dependent cellular cytotoxicity
ADCP  antibody-dependent cellular phagocytosis
AML  acute myeloid leukemia
CDC  complement-dependent cytotoxicity
CDR  complementarity-determining region (of an antibody)
DAR  drug-to-antibody molar ratio
DM1  $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)-maytansine
DM4  $N^{2'}$-deacetyl-$N^{2'}$-(4-mercapto-4-methyl-1-oxopentyl)-maytansine
FRα  folate receptor alpha (FOLR1)
IMGN853  mirvetuximab soravtansine
MMAE/F  monomethyl auristatin E/monomethyl auristatin F
PDx  patient-derived tumor xenograft model
SMCC  succinimidyl-4-($N$-maleimidomethyl)cyclohexane-1-carboxylate
SPDB  $N$-succinimidyl-4-(2-pyridyldithio)butanoate
SPP  $N$-succinimidyl 4-(2-pyridyldithio)pentanoate
Sulfo-SPDB  $N$-succinimidyl 4-(2-pyridyldithio)-2-sulfobutanoate

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