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Recent advances in the discovery and development of antibody–drug conjugates have led to FDA approvals and a rich clinical pipeline of promising new cancer therapies.

Antibody–drug conjugates: current status and future directions

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Antibody–drug conjugates (ADCs) aim to take advantage of the specificity of monoclonal antibodies (mAbs) to deliver potent cytotoxic drugs selectively to antigen-expressing tumor cells. Despite the simple concept, various parameters must be considered when designing optimal ADCs, such as selection of the appropriate antigen target and conjugation method. Each component of the ADC (the antibody, linker and drug) must also be optimized to fully realize the goal of a targeted therapy with improved efficacy and tolerability. Advancements over the past several decades have led to a new generation of ADCs comprising non-immunogenic mAbs, linkers with balanced stability and highly potent cytotoxic agents. Although challenges remain, recent clinical success has generated intense interest in this therapeutic class.

Introduction

The past decade has seen significant advances in new cancer treatments through the development of highly selective small molecules that target a specific genetic abnormality responsible for the disease [1,2]. Although this approach has seen great success in application to malignancies with a single, well-defined oncolytic driver, resistance is commonly observed in more complex cancer settings [3,4]. Traditional cytotoxic agents are another approach to treating cancer; however, unlike target-specific approaches, they suffer from adverse effects stemming from nonspecific killing of both healthy and cancer cells. A strategy that combines the powerful cell-killing ability of potent cytotoxic agents with target specificity would represent a potentially new paradigm in cancer treatment. ADCs are such an approach, wherein the antibody component provides specificity for a tumor target antigen and the drug confers the cytotoxicity. Here, we present key considerations for the development of effective ADCs and discuss recent progress in ADC technology for application to the next wave of cancer therapeutics. Advances in other modalities of antibody-mediated targeting, such as immunotoxins, immunoliposomes and radionuclide conjugates, have been extensively reviewed elsewhere [5,6].

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Reviews • KEYNOTE REVIEW

Historical perspective

The origin of ADCs can be traced back over a century to the German physician and scientist Paul Ehrlich, who proposed the concept of selectively delivering a cytotoxic drug to a tumor via a targeting agent (Fig. 1) [7,8]. Ehrlich coined the term 'magic bullet' to describe his vision, similar to the descriptors 'warhead' or 'payload' commonly used for the drug component of current ADCs. Nearly 50 years later, Ehrlich's concept of targeted therapy was first exemplified when methotrexate (MTX) was linked to an antibody targeting leukemia cells [9]. Early research relied on available targeting agents, such as polyclonal antibodies, to enable preclinical efficacy studies in animal models with both noncovalent-linked ADCs and later covalently linked ADCs [10–12]. In 1975, the landmark development of mouse mAbs using hybridoma technology by Kohler and Milstein greatly advanced the field of ADCs [13]. The first human clinical trial followed less than a decade later, with the antimitotic vinca alkaloid vindesine as the cytotoxic payload [14]. Further advances in antibody engineering enabled the production of humanized mAbs with reduced immunogenicity in humans compared with the murine mAbs used for early ADCs [15].

First-generation ADCs typically used clinically approved drugs with well-established mechanisms of action (MOAs), such as antimetabolites (MTX and 5-fluorouracil), DNA crosslinkers (mitomycin) and antimicrotubule agents (vinblastine) [16]. In addition to the immunogenicity issues observed with murine mAbs, these early attempts were met with limited success for several reasons, including low drug potency, high antigen expression on normal cells and instability of the linker that attached the drug to the mAb [17]. Lessons learned from these initial failures led to a new generation of ADCs, several of which entered and later failed human clinical trials. For example, doxorubicin conjugate **1** (BR96-DOX) was designed using a bifunctional linker, wherein the drug was appended via a hydrazone, and a maleimide enabled conjugation to the BR96 antibody via cysteine residues (Fig. 2) [18]. Although curative efficacy was observed in human tumor xenograft models, the relatively low potency of doxorubicin necessitated high drug:antibody ratios (DARs, eight per antibody) and high doses of the ADC to achieve preclinical activity. In clinical trials, significant toxicity was observed due to nonspecific cleavage of the relatively labile hydrazone linker and expression of the antigen target in normal tissue [19].

Further advancements, including higher drug potency and carefully selected targets, ultimately led to the first ADC to gain US Food and Drug Administration (FDA) approval in 2000 (Mylotarg®, gemtuzumab ozogamicin, **2**) [20,21]. Despite initially encouraging clinical results, Mylotarg® was withdrawn from the market a decade later owing to a lack of improvement in overall survival. In 2011, following an accelerated approval process, a second ADC (Adcetris®, brentuximab vedotin, **3**) gained marketing approval
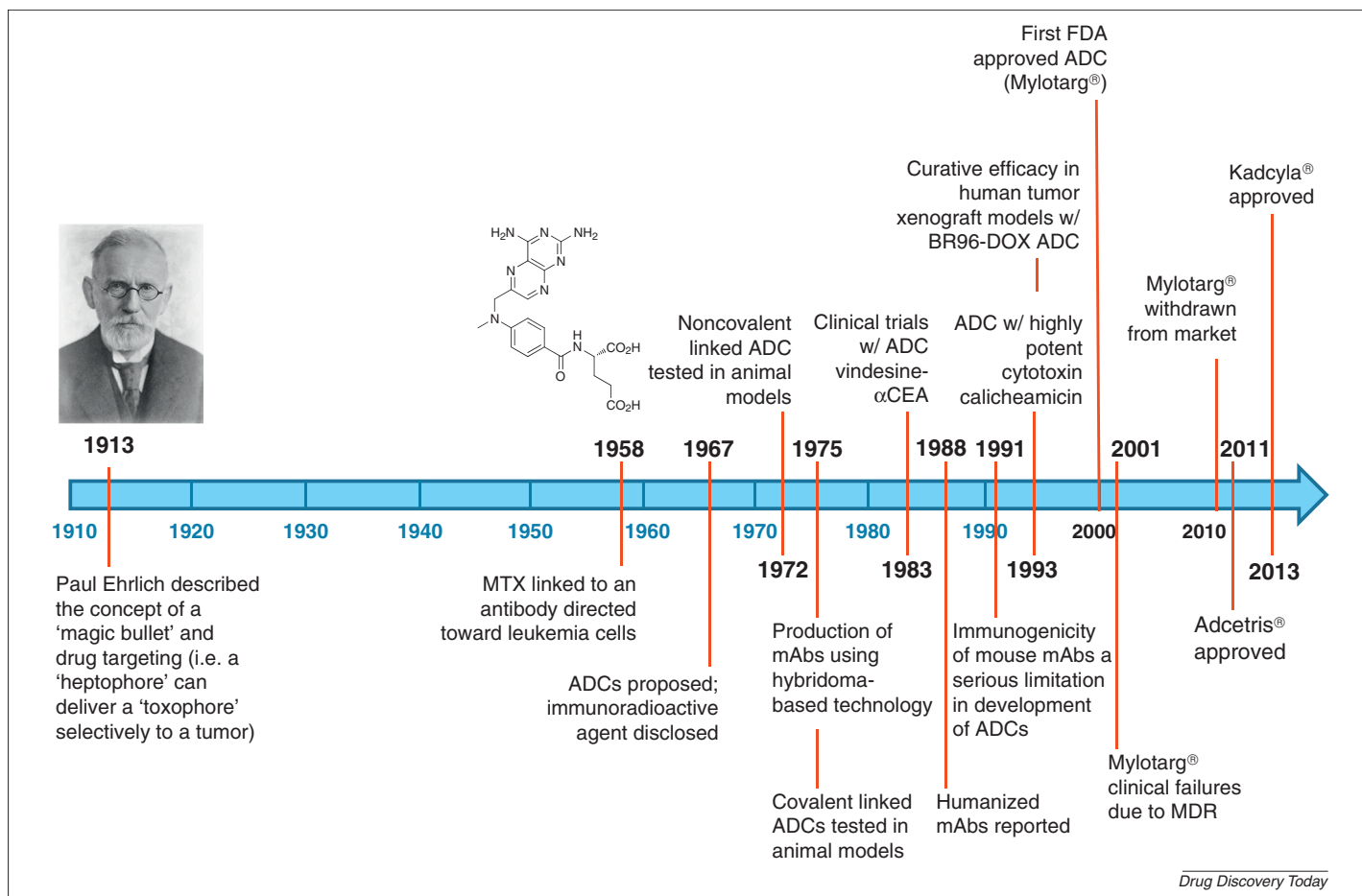

FIGURE 1

Antibody–drug conjugate (ADC) timeline. *Abbreviations*: mAbs, monoclonal antibodies; MDR, multidrug resistance; MTX, methotrexate.
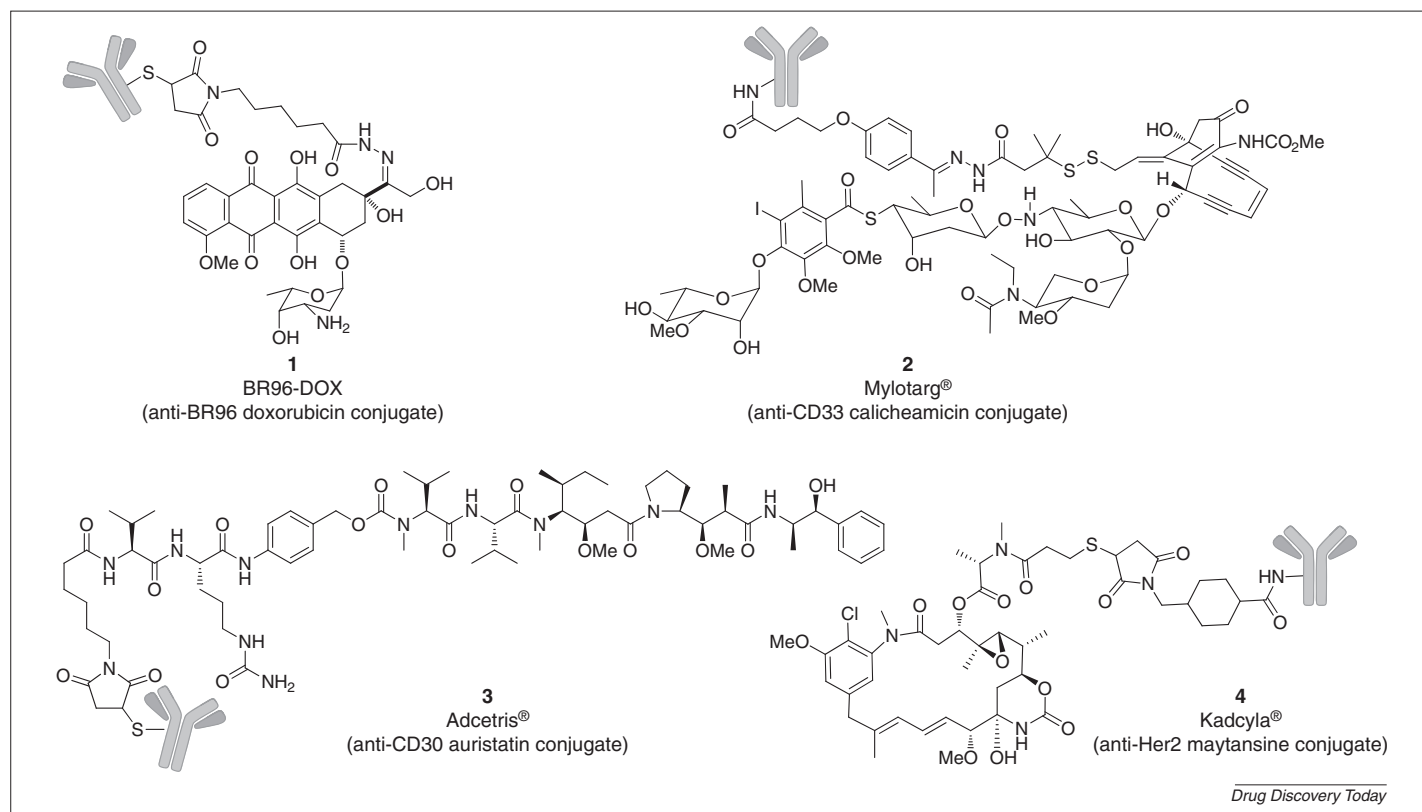
FIGURE 2

Structures of antibody–drug conjugates (ADCs).

from the FDA for the treatment of Hodgkin's (HL) and anaplastic large-cell lymphomas (ALCL) [22,23]. Most recently, Kadcyla® (ado-trastuzumab emtansine, T-DM1, **4**), which combines the humanized antibody trastuzumab with a potent antimicrotubule cytotoxic agent using a highly stable linker, was approved for the treatment of patients with Her2-positive breast cancer [24,25]. With nearly 30 additional ADCs currently in clinical development, the potential of this new therapeutic class might finally be coming to fruition [26].

ADC design

Although simple in concept, the success of a given ADC depends on careful optimization of each ADC building block: antibody, drug and linker (Fig. 3) [27]. The chosen antibody should target a well-characterized antigen with high expression at the tumor site and low expression on normal tissue to maximize the efficacy of the ADC while limiting toxicity. Bifunctional linkers with attachment sites for both the antibody and drug are used to join the two components. With respect to the mAb, existing linker attachment strategies typically rely on the modification of solvent-accessible cysteine or lysine residues on the antibody, resulting in heterogeneous ADC populations with variable DARs. Given that low drug loading reduces potency and high drug loading can negatively impact pharmacokinetics (PK), DARs can have a significant impact on ADC efficacy. In addition, the linker must remain stable in systemic circulation to minimize adverse effects, yet rapidly cleave after the ADC finds its intended target antigen. Upon antigen recognition and binding, the resulting ADC receptor complex is internalized through receptor-mediated endocytosis [28]. Once

inside the cell, the drug is released through one of several mechanisms, such as hydrolysis or enzymatic cleavage of the linker or via degradation of the antibody. Typically, the unconjugated drug should demonstrate high potency, ideally in the picomolar range, to enable efficient cell killing upon release from the ADC.

Target antigens and antibody selection

Although the basic premise that a successful ADC should target a well-internalized antigen with low normal tissue expression and high expression on tumors remains true, the field is evolving to refine these parameters. For example, antigen expression on normal tissues can be tolerated if expression on vital organs is minimal or absent. The FDA approval of Kadcyla® for Her2-positive breast cancer highlights this point since Her2/neu, a member of the epidermal growth factor receptor (EGFR) family, is not only expressed in breast tissue, but also in the skin, heart and on epithelial cells in the gastrointestinal, respiratory, reproductive and urinary tracts [29]. In addition, prostate-specific membrane antigen (PSMA) is an ADC target expressed both on prostate cancer cells as well as normal prostate and endothelial tissue [30]. Given that most patients with prostate cancer undergo surgery to remove their prostate, selective expression relative to normal prostate cells might not be crucial in this setting. Furthermore, apical expression of PSMA on the kidney and gastrointestinal tract might prevent the ADC from accessing these tissues. Other possible exceptions include hematological malignancies in which normal target tissues are able to regenerate, supported by the case of rituximab where depletion of normal B cells was not a major safety issue in patients [31]. Accessibility of the ADC to the target antigen is also
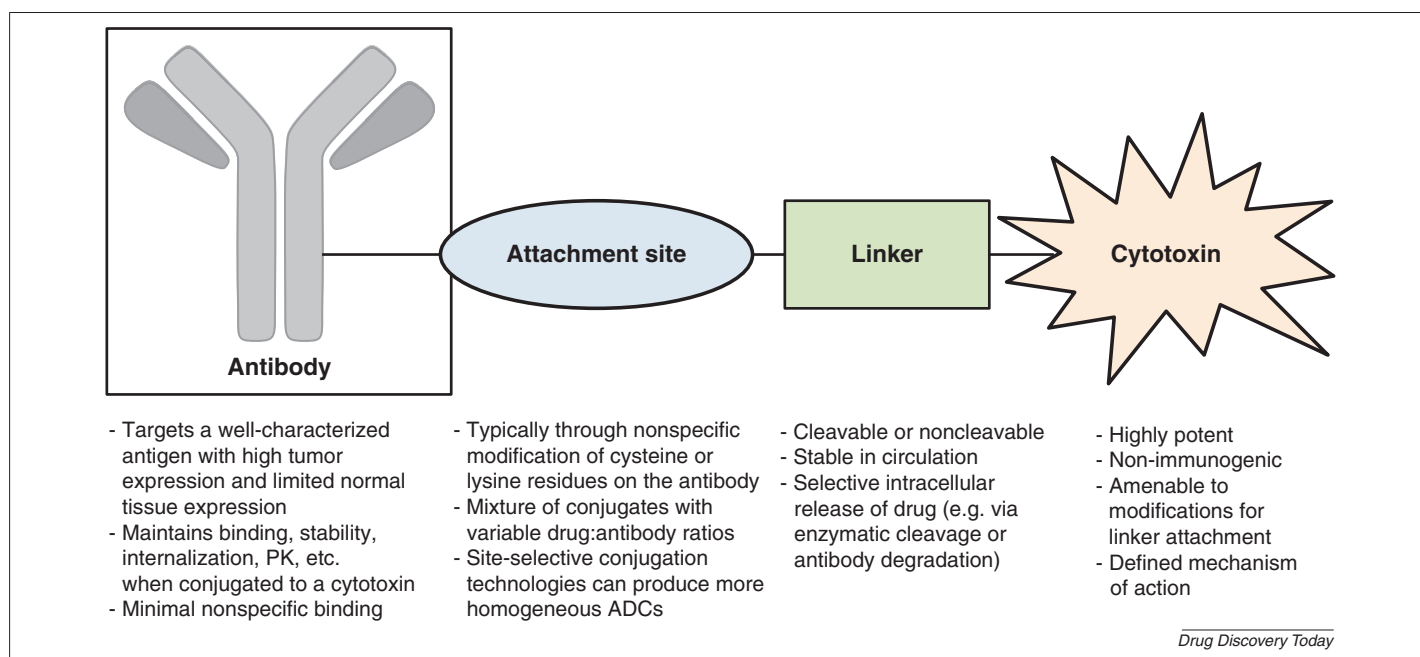
FIGURE 3

Key components of an antibody–drug conjugate (ADC). *Abbreviation*: PK, pharmacokinetics.

an important consideration. In addition to high interstitial pressure in the tumor, endothelial, stromal and epithelial barriers can limit ADC uptake, resulting in only a small percentage of the injected dose reaching the intended tumor target [32]. From a biology perspective, the design of an effective ADC relies on selection of an appropriate target antigen, taking into account tumor expression levels, rates of antigen internalization and antibody Fc format.

Tumor types

Table 1 highlights the broad range of hematologic and solid tumor indications targeted by ADCs currently in preclinical or clinical development. Several of these tumor-associated antigens exhibit remarkable specificity, such as CD30 for HL and MUC16 for ovarian cancer. Other antigens, such as CD74, are expressed in multiple tumor types.

Antigen expression

In general, optimal ADC targets are homogeneously and selectively expressed at high density on the surface of tumor cells. Homogenous tumor expression, although preferred, is likely not an absolute requirement owing to the ability of some ADCs to induce bystander killing. Under these circumstances, a membrane-permeable free drug liberated after intracellular cleavage of the linker can efflux from the cell and enter neighboring cells to facilitate cell death [33]. Most advanced ADCs in the clinic target hematological indications, in part due to the largely homogeneous expression of antigen in liquid tumors, despite frequently low receptor densities. Although the treatment of solid tumors with heterogeneous antigen expression might benefit from bystander killing, the potential to harm normal cells could contribute to systemic toxicity.

Current experimental evidence generally suggests that tumor antigen density (expression level) does not directly correlate with ADC efficacy [34]. When patient samples are accessible, the number of receptors per cell can be quantified using flow

cytometry, immunohistochemistry (IHC) or radiolabeled saturation-binding studies to assess the relation between target expression and efficacy [35]. In non-Hodgkin's lymphoma (NHL) cell lines, high CD79b expression was found to be a prerequisite for *in vitro* response to an anti-CD79b auristatin conjugate (RG-7596, Roche-Genentech); however, a wide range of sensitivities were observed, indicating that a minimal expression threshold exists [36]. Likewise, melanoma cells lines with receptor densities vary-

TABLE 1

Target antigens for ADCs in preclinical and clinical development

Indication	Targets
NHL	CD19, CD20, CD21, CD22, CD37, CD70, CD72, CD79a/b and CD180
HL	CD30
AML	CD33
MM	CD56, CD74, CD138 and endothelin B receptor
Lung	CD56, CD326, CRIPTO, FAP, mesothelin, GD2, 5T4 and alpha v beta6
CRC	CD74, CD174, CD227 (MUC-1), CD326 (Epcam), CRIPTO, FAP and ED-B
Pancreatic	CD74, CD227 (MUC-1), nectin-4 (ASG-22ME) and alpha v beta6
Breast	CD174, GPNMB, CRIPTO, nectin-4 (ASG-22ME) and LIV1A
Ovarian	MUC16 (CA125), TIM-1 (CDX-014) and mesothelin
Melanoma	GD2, GPNMB, ED-B, PMEL 17 and endothelin B receptor
Prostate	PSMA, STEAP-1 and TENB2
Renal	CAIX and TIM-1 (CDX-014)
Mesothelioma	Mesothelin

Abbreviations: AML, acute myeloid leukemia; MM, multiple myeloma; CRC, colorectal cancer.

ing from 20,000 to 280,000 binding sites per cell were sensitive to an anti-p97-auristatin conjugate [37]. This threshold level varies among different targets based on the unique factors of the antigen, such as rate of internalization and binding affinity for the ADC. For example, approximately 5,000–10,000 copies of CD33, the antigen target for Mylotarg®, are expressed per cell [38]. As with Mylotarg®, no significant correlation was observed between the activity of a preclinical anti-CD33 pyrrolobenzodiazepine conjugate (SG-CD33A, Seattle Genetics) and CD33 levels in a panel of acute myeloid leukemia (AML) cell lines [39]. An anti-PSMA auristatin conjugate (PSMA ADC, Progenics/Seattle Genetics) demonstrated potent *in vitro* cytotoxicity versus cells expressing $>10^5$ PSMA molecules per cell, with 10^4 receptors per cell serving as a threshold level [40]. For some tumor antigens, however, a relatively proportional relation between efficacy and receptor expression level has been observed. In the case of an anti-endothelin B receptor (EDNBR) auristatin conjugate, improved efficacy against human melanoma cells lines and xenograft tumor models generally correlated with increasing EDNBR expression (1,500–30,000 copies per cell) [41].

Antigen internalization

Ideally, once an ADC binds to a tumor-associated target, the ADC–antigen complex is internalized in a rapid and efficient manner. Although poorly understood, various factors are likely to influence the rate of internalization, such as the epitope on the chosen target antigen bound by the ADC, the affinity of the ADC–antigen interaction and the intracellular trafficking pattern of the ADC complex [42–44]. For example, anti-Her2 antibodies that bind distinct epitopes on Her2 have been shown to impact downstream trafficking and lysosomal accumulation differentially, despite binding to the same cell surface receptor [45]. Several ADCs, including Adcetris®, have been shown to internalize with rates similar to or greater than the corresponding unconjugated antibodies [46–48,37]. Certain antigens mediate exceptionally rapid accumulation of ADCs inside cells. When bound to ligand-activated EGFR, Her2 monomer is internalized at a rate up to 100-fold greater than carcinoembryonic antigen (CEA) [49,50]. Likewise, the catabolic rate of antibodies targeting CD74 is approximately 100 times faster than other antibodies that are considered to rapidly internalize, such as anti-CD19 and anti-CD22 [51]. The preclinical data for milatuzumab-DOX (Immu-110), an anti-CD74 doxorubicin conjugate in early clinical trials, suggest this agent is equipotent to ADCs comprising more potent drug payloads that target slower internalizing antigens [52].

Alternative approaches have been explored in which antigen internalization is not required for efficient cell killing. The extra-domain B (ED-B) of fibronectin is a marker of angiogenesis undetectable in healthy tissue, but highly expressed around tumor blood vessels [53]. Anti-ED-B antibodies have been shown to localize to the subendothelial extracellular matrix of tumor vasculature. Conjugation of these antibodies with a photosensitizer has led to agents that selectively disrupt tumor blood vessels upon irradiation, resulting in curative efficacy in mouse models [54].

Impact of format

The biological activity of an antibody can depend on the interaction of its Fc portion with cells that express Fc receptors (FcRs). Therefore, selection of the appropriate antibody format for an ADC is an important consideration. Broad understanding of the relation between antibody Fc format and ADC function is lacking since species differences in immune systems complicate preclinical studies. In one study, McDonagh et al. conjugated anti-CD70 antibody immunoglobin G (IgG) variants (IgG1, IgG2 and IgG4) to an auristatin (ADC toxin monomethyl auristatin F; MMAF) to determine the effect of format on ADC function [55]. In addition, the Fc regions of IgG1 and IgG4 were mutated (IgG1v1 and IgG4v3) to examine the influence of IgG receptor (FcγR) binding. Although all the ADCs demonstrated potent *in vitro* cytotoxicity and were well tolerated in mice, the engineered IgGv1-MMAF conjugate displayed improved antitumor activity and increased exposure, which correlated with a superior therapeutic index compared to the parent IgG1 conjugate.

In the absence of definitive guidelines for selecting an optimal antibody format, all human IgG isotypes, except for IgG3, are currently used for ADCs in clinical trials. IgG1, the most commonly used format, can potentially engage secondary immune functions, such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). These inherent effector functions could prove beneficial by providing additional antitumor activity, as in the case of Kadcyla®, which was shown to activate ADCC in preclinical models [56]. Adcetris®, however, demonstrated minimal ADCC and no detectable CDC despite its IgG1 format [57]. The absence of effector functions is potentially advantageous as binding of an ADC to effector cells could reduce tumor localization, hinder internalization and lead to off-target toxicity [55]. Unlike IgG1, IgG2 and IgG4 typically lack Fc-mediated effector functions. Mylotarg® and inotuzumab ozogamicin (CMC-544) exhibited no ADCC or CDC in preclinical studies, consistent with their IgG4 format [58]. Overall, the contribution of IgG effector functions to the efficacy, selectivity and toxicity of ADCs is not yet well understood.

In addition to effector functions, ADCs often retain other biological properties associated with their parent mAbs, such as immunogenicity potential. Limited therapeutic efficacy of early ADCs comprising murine mAbs prompted the development of chimeric and humanized antibodies, which minimize human immune response. Conversion of murine mAbs to human IgGs also results in longer retention in systemic circulation due to recognition by the human neonatal Fc receptor (FcRn) and a greater ability to elicit ADCC [59]. Technologies for the generation of fully human mAbs include the use of either phage display or transgenic mouse platforms, in which a mouse strain is engineered to produce human rather than mouse antibodies [60].
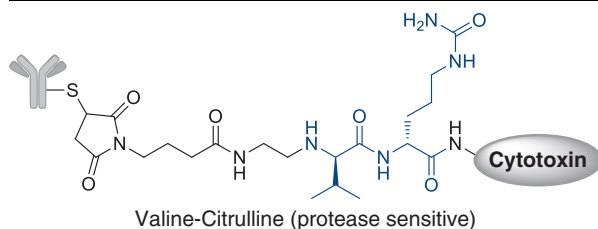
Linker technology and stability

The identity and stability of a linker that covalently tethers the antibody to the cytotoxic drug is crucial to the success of an ADC. Sufficient linker stability is necessary to enable the conjugate to circulate in the bloodstream for an extended period of time before reaching the tumor site without prematurely releasing the free drug and potentially damaging normal tissue. Once the ADC is internalized within the tumor, the linker should be labile enough to efficiently release the active free drug. Linker stability also influences overall toxicity, PK properties and the therapeutic index of an ADC. The lack of adequate therapeutic index for earlier
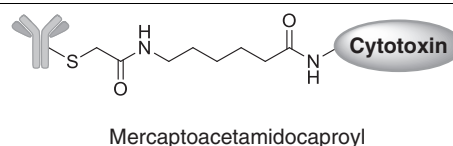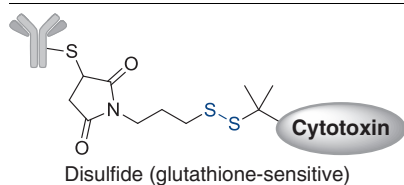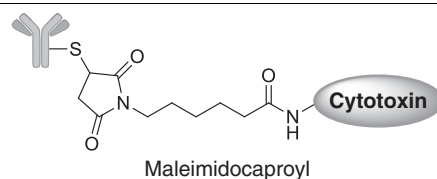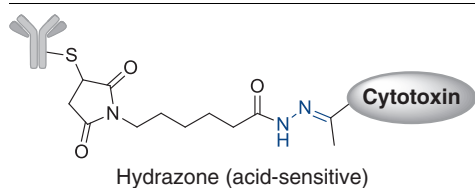
TABLE 2

Examples of ADC drug linkers

Cleavable linkers	Noncleavable linkers

Valine-Citrulline (protease sensitive)

N-Maleimidomethylcyclohexane-1-carboxylate (MCC)

Hydrazone (acid-sensitive)

Maleimidocaproyl

Disulfide (glutathione-sensitive)

Mercaptoacetamidocaproyl
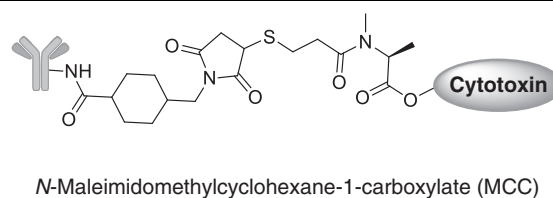
ADCs, such as BR96-DOX and Mylotarg®, has been attributed to poor linker stability (Fig. 1) [19,61].

The two main classes of ADC drug linkers currently being explored take advantage of different mechanisms for release of the drug payload from the antibody (Table 2). The first is a cleavable linker strategy, with three different types of release mechanism within this class.

(i) Lysosomal protease sensitive linkers. This strategy utilizes lysosomal proteases, such as cathepsin B (catB), that recognize and cleave a dipeptide bond to release the free drug from the conjugate [62]. Many ADCs in the clinic use a valine–citrulline dipeptide linker, which was designed to display an optimal balance between plasma stability and intracellular protease cleavage [63]. This linker strategy was successfully utilized by Seattle Genetics/Millennium in the case of Adcetris® [64].

(ii) Acid sensitive linkers. This class of linkers takes advantage of the low pH in the lysosomal compartment to trigger hydrolysis of an acid labile group within the linker, such as a hydrazone, and release the drug payload. In preclinical studies, hydrazone linker-based conjugates have shown stability ($t_{1/2}$) ranges from 2 to 3 days in mouse and human plasma, which may not be optimal for an ADC [65]. Hydrazone linkers were used in Mylotarg® (anti-CD33 calicheamicin conjugate) and recently in inotuzumab ozogamicin (anti-CD22 calicheamicin conjugate) [66,67]. The withdrawal of Mylotarg® from the market was attributed to toxicities related to hydrazone linker instability, which resulted in increased fatalities in patients treated with Mylotarg® plus chemotherapy as opposed to chemotherapy alone [65]. Similarly, inotuzumab ozogamicin was recently withdrawn from a phase III clinical trial owing to a lack of improvement in overall survival.

(iii) Glutathione sensitive linkers. This strategy exploits the higher concentration of thiols, such as glutathione, inside the cell relative to the bloodstream. Disulfide bonds within the linker are relatively stable in circulation yet are reduced by intracellular glutathione to release the free drug. To further increase plasma stability, the disulfide bond can be flanked with methyl groups that sterically hinder premature cleavage in the bloodstream [68]. This class of linker has been used in several clinical candidates, such as SAR3419 (anti-CD19 maytansine conjugate), IMGN901 (anti-CD56 maytansine conjugate) and AVE9633 (anti-CD33 maytansine conjugate) developed by ImmunoGen and its partners [67].

The second strategy is one that uses noncleavable linkers. This approach depends on complete degradation of the antibody after internalization of the ADC, resulting in release of the free drug with the linker attached to an amino acid residue from the mAb. As such, noncleavable linker strategies are best applied to payloads that are capable of exerting their antitumor effect despite being chemically modified. This type of strategy has been used successfully by Genentech/Immunogen with Kadcyla® (trastuzumab-MCC-DM1). The released modified payload (lysine-MCC-DM1) demonstrated similar potency compared with DM1 alone, although the charged lysine residue is likely to impair cell permeability and hence abate the bystander killing observed with the free drug [69]. One potential advantage of noncleavable linkers is their greater stability in circulation compared with cleavable linkers. However, no significant difference in terminal half-life ($t_{1/2}$) values was observed in the clinic between Kadcyla® [24], which contains a noncleavable linker, and Adcetris®, which employs a cleavable linker [22].

Preclinically, linker strategies continue to evolve [70,71]. Additional tumor-associated proteases, such as legumain, have been identified that release the ADC payload in nonlysosomal

compartments (i.e. the endosome) [72]. Other nonprotease enzymes have recently been exploited for the selective cleavage of β-glucuronidase and β-galactosidase sensitive linkers in the lysosome [73,74]. Demonstrating expanded utility, these approaches enable drug linkage via a phenol functional group in addition to a more traditional basic amine residue.

Cytotoxic agents
Payload classes and MOAs
There are two main classes of ADC payloads undergoing clinical evaluation. The first class comprises drugs that disrupt microtubule assembly and play an important role in mitosis. This class includes cytotoxics, such as dolastatin 10-based auristatin analogs (3, Adcetris®) [64] and maytansinoids (4, Kadcyla®) [75]. The second class of payloads consists of compounds that target DNA structure and includes calicheamicin analogs, such as Mylotarg® (2), that bind the minor groove of DNA causing DNA double-strand cleavage [76]. Duocarmycin analogs (MDX-1203, 5) [77] participate in a sequence-selective alkylation of adenine-N3 in the minor groove of DNA to induce apoptotic cell death (Fig. 4).

One common feature among these cytotoxic agents is that they demonstrate at least 100–1000-fold greater potency in *in vitro*

proliferation assays against a broad range of tumor cell lines compared with conventional chemotherapeutic agents, such as paclitaxel and doxorubicin [78,79]. The high potency of these alternative payloads is crucial since only an estimated 1–2% of the administered ADC dose will ultimately reach the tumor site, resulting in low intracellular drug concentrations [80]. Unlike earlier ADCs that failed to make a meaningful impact in the clinic owing to low drug potency and suboptimal delivery, newer, more potent cytotoxic compounds are now the focus of preclinical research. For example, pyrrolobenzodiazepine (PBD) dimers 6 covalently bind the minor groove of DNA, resulting in a lethal interaction due to cross-linking of opposing strands of DNA [81]. α-Amanitin 7, a cyclic octapeptide found in several species of the *Amanita* genus of mushrooms, strongly inhibits RNA polymerase II, leading to inhibition of DNA transcription and cell death [82]. Tubulysins 8, similar to auristatins and maytansine, inhibit tubulin polymerization to induce apoptosis [83–85].

Addressing drug resistance
In addition to potency, the sensitivity of cytotoxic agents to multidrug resistance (MDR) mechanisms is a factor to consider in selecting the optimal payload for an ADC. Cancer cells have the ability to become resistant to multiple drugs via increased efflux of
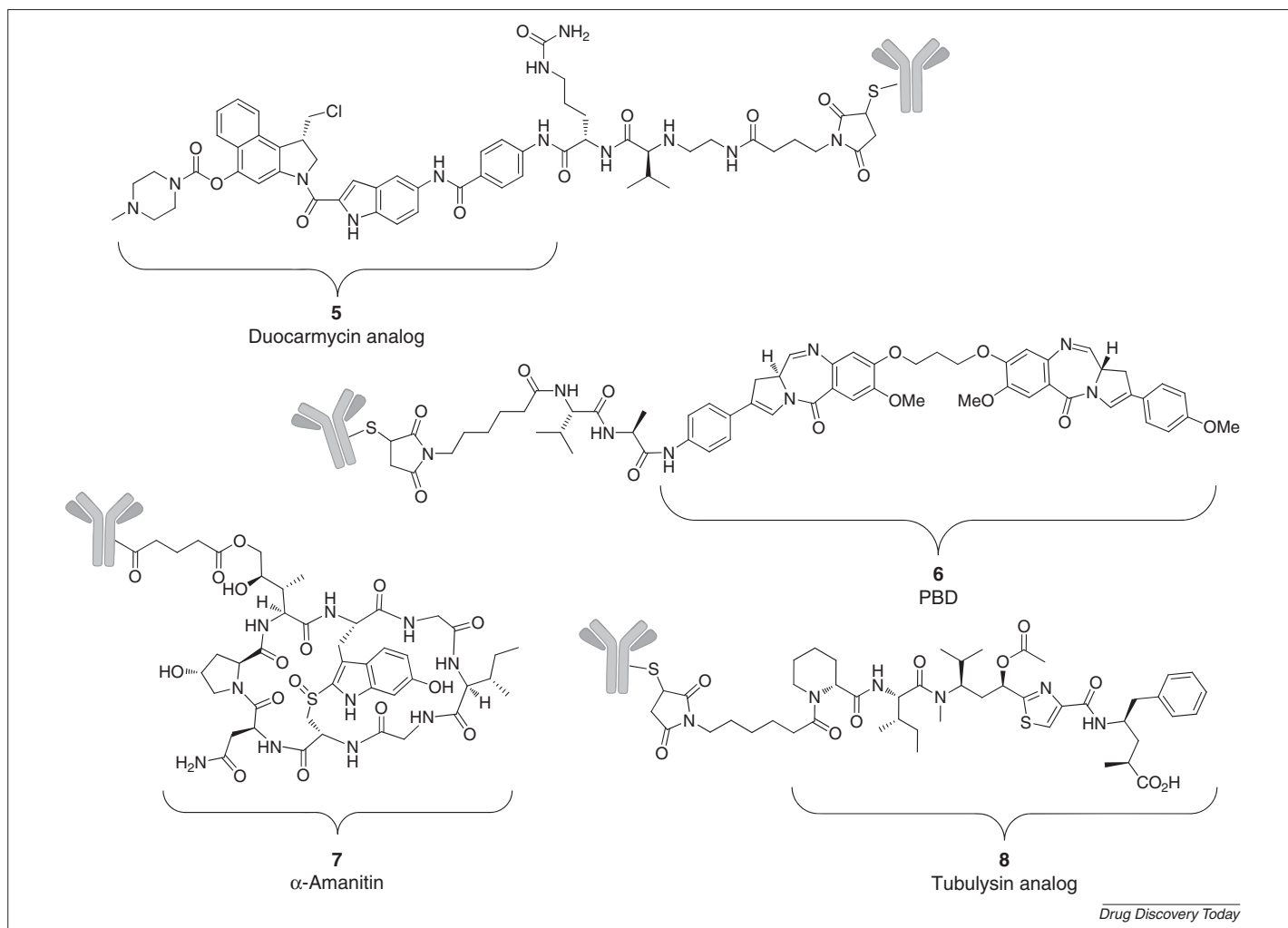

FIGURE 4

Representative antibody–drug conjugate (ADC) payload structures.
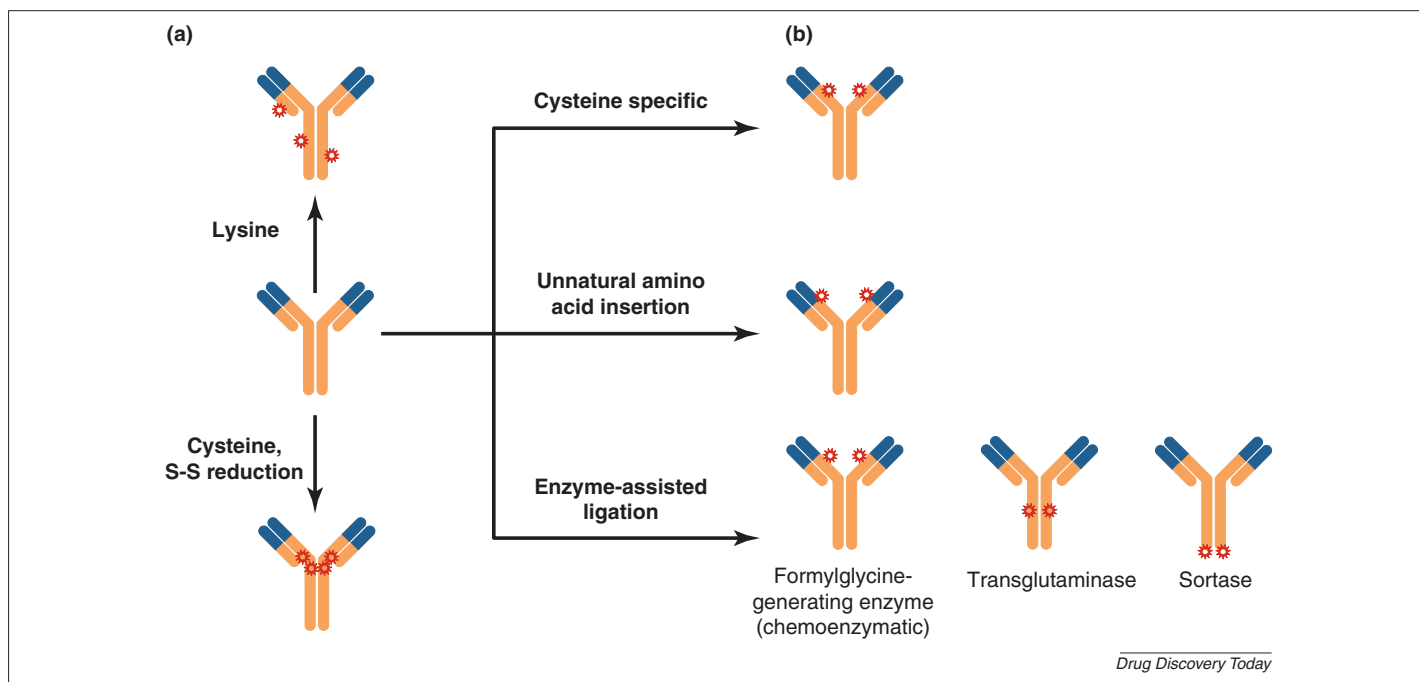
Reviews • KEYNOTE REVIEW

FIGURE 5

Random and site-specific conjugation strategies. Antibody–drug conjugate (ADC) products of random conjugation comprise chemically heterogeneous species **(a)**, whereas site-specific conjugation methods produce fairly homogeneous product profiles **(b)**.

the drug by either P-glycoprotein (Pgp) or other multidrug-resistance proteins (e.g. MRP1 and MRP3) [86]. The sensitivity of cytotoxic drugs to MDR mechanisms can be measured *in vitro*. In the case of Mylotarg®, *in vitro* cytotoxicity assays in AML cell lines indicated that Pgp expression altered the potency of the calicheamicin payload and that drug potency could be restored by adding known efflux transporter antagonists to inhibit Pgp and MRP-1 proteins [87]. These results were relevant for patients with AML as levels of Pgp expression in the clinic were found to correlate directly with responders and nonresponders [88,89].

Another interesting example related to MDR mechanisms involves AVE9633, which comprises an anti-CD33 antibody linked through a disulfide bond to the maytansine analog DM4. *In vitro* data clearly demonstrated that the cytotoxicity of AVE9633 and the DM4 free drug were highly dependent on the expression level of Pgp protein in myeloid cell lines [90]. As with the calicheamicin payload of Mylotarg®, the potency of DM4 could be restored in Pgp-overexpressing cell lines by adding known inhibitors of Pgp. However, Pgp activity was not found to be a major mechanism of resistance for the AVE9633 conjugate in cells from patients with AML. Reasons for the lack of correlation are unclear; other mechanisms such as microtubule alteration were proposed for chemoresistance to AVE9633.

Conjugation strategies

For most ADCs in clinical development, conjugation of the drug payload to the antibody involves a controlled chemical reaction with specific amino acid residues exposed on the surface of the mAb. Given that this process results in a mixture of ADC species with variable DARs and linkage sites, alternative conjugation strategies aimed at minimizing heterogeneity have been developed. In the overall design of an ADC, selection of the appropriate

drug-conjugation strategy significantly impacts efficacy, PK and tolerability. As such, careful consideration of the various conjugation technologies for ADC generation is warranted (Fig. 5).

Chemical conjugation

In one type of chemical conjugation, a reactive moiety pendant to the drug–linker is covalently joined to the antibody via an amino acid residue side chain, commonly the ε-amine of lysine. As demonstrated with Mylotarg®, direct conjugation of lysine residues on gemtuzumab was achieved using an *N*-hydroxysuccinimide (NHS) ester appended to the drug–linker to form stable amide bonds [91]. A two-step process can also be used in which surface lysines on the antibody are first modified to introduce a reactive group, such as a maleimide, and then conjugated to the drug–linker containing an appropriate reactive handle (e.g. a thiol) [92]. Such a strategy was utilized in the case of Kadcyla®. Alternatively, controlled reduction of existing disulfide bonds can liberate free cysteine residues on the antibody, which then react with a maleimide attached to the drug–linker. This approach, used in the preparation of Adcetris®, takes advantage of the reducible disulfide bonds of IgG antibodies in which controlled conditions enable reduction of only interchain disulfide bonds while intrachain disulfides remain unaffected, thus minimizing major structural disruptions to the antibody [19].

The random conjugation processes described above produce heterogeneous mixtures of conjugated species with variable DARs. Adding to the complexity, the site of conjugation could be different for each ADC species containing even only one drug. When lysines are used for conjugation, heterogeneity in overall charge can impact solubility, stability and PK [93]. Therefore, the clinical success of an ADC produced by random conjugation depends on robust manufacturing processes that provide the ability to monitor, control and purify the heterogeneous

products. Several organizations have developed expertise in this area to overcome the process development and manufacturing challenges associated with ADC commercialization [94].

Site-specific conjugation

Despite the success of Adcetris® and Kadcyla®, considerable enthusiasm for the next generation of ADCs has focused on the development of homogeneous products derived via site-specific conjugation. Currently, three strategies are at the forefront: insertion of cysteine residues in the antibody sequence by mutation or insertion, insertion of an unnatural amino acid with a bio-orthogonal reactive handle, and enzymatic conjugation.

Building on early studies that explored the introduction of surface cysteines on recombinant antibodies [95], several cysteine engineered antibodies have been produced and tested for use in site-specific attachment of cytotoxic drugs to yield homogeneous ADCs [96]. Junutula et al. reported a class of THIOMAB-drug conjugates (TDCs) prepared by taking advantage of: (i) phage display techniques to identify ideal sites for mutation and produce antibodies with minimal aggregation issues, and (ii) methods to reduce and re-oxidize the antibody under mild conditions to present only thiols of mutated cysteines for conjugation [92,97]. Compared with a conventional, randomly conjugated ADC, the analogous TDC displayed minimal heterogeneity with similar in vivo activity, improved PK and a superior therapeutic index. Moreover, McDonagh et al. engineered antibodies in which interchain cysteines were replaced with serines to reduce the number of potential conjugation sites, yielding ADCs with defined DARs (two or four drugs per antibody) and attachment sites [98]. Broad application of this approach to future ADCs will depend on further studies to evaluate the effect of these mutations on the overall stability and biological function of the engineered antibody.

Encouraged by studies with cysteine engineered antibodies, several investigators reasoned that the site and stoichiometry of conjugation could be controlled by inserting unnatural amino acids with orthogonal reactivity relative to the 20 natural amino acids. Axup et al. genetically engineered an orthogonal amber suppressor tRNA/aminoacyl-tRNA synthetase pair to insert site-specifically p-acetylphenylalanine (pAcPhe) in recombinantly expressed antibodies [99]. As a test case, pAcPhe was introduced at one of several positions in the constant region of trastuzumab (anti-Her2). These mutants were then conjugated to an alkoxy-amine auristatin derivative via formation of a stable oxime bond. The resulting chemically homogeneous ADCs demonstrated improved PK compared with nonspecifically conjugated ADCs and were highly efficacious in a Her2-positive human tumor xenograft model. In addition to pAcPhe, other unnatural amino acids are being explored through the use of appropriate tRNA–aminoacyl-tRNA synthetase pairs [100]. Recently, in vitro transcription and translation processes have also been developed and optimized to insert unnatural amino acids in antibodies for site-specific conjugation [101].

In addition to inserting unnatural amino acids into mAb sequences, chemoenzymatic approaches have been explored to generate bio-orthogonal reactive groups for selective conjugation. Bertozzi and co-workers utilized formylglycine-generating enzyme (FGE), which recognizes a CXPXR sequence and converts a cysteine residue to formylglycine to produce antibodies with aldehyde tags [102,103]. The reactive aldehyde functionality can then undergo conjugation to the drug–linker via oxime chemistry or a Pictet–Spengler reaction [104].

Harnessing enzymatic post-translational modification processes for site-specific labeling of proteins is a recently reviewed approach for the preparation of homogenous ADCs [105]. Bacterial trans-glutaminase (BTG) catalyzes the ligation of glutamine side chains with the primary ε-amine of lysine residues, resulting in a stable isopeptide bond. Jegar et al. exploited BTG to load four chelates on a deglycosylated antibody with an N297Q mutation in a site-specific manner [106]. Recently, Strop et al. conducted BTG-assisted conjugations by inserting LLQG sequences at different sites on an antibody [107]. These studies clearly demonstrated that the site of conjugation has a significant impact on the stability and PK of the ADC. Another enzyme, sortase A (SrtA), catalyzes hydrolysis of the threonine–glycine bond in a LPXTG motif to form a new peptide bond between the exposed C-terminus of threonine and an N-terminal glycine motif [108].

Next-generation ADCs

Key clinical assets

The nearly 30 ADCs currently in clinical development have been reviewed in detail elsewhere [109], and representative examples of the most advanced agents are summarized in Table 3. In addition to the FDA-approved ADCs discussed in preceding sections, several compounds are in late-stage clinical testing for both hematological and solid tumor indications. Despite the withdrawal of Mylotarg® from the market in 2010, promising results from ongoing clinical studies have shown that when combined with chemotherapy Mylotarg® increased overall survival in patients with newly diagnosed AML compared to those treated with chemotherapy alone [110]. Inotuzumab ozogamicin, which uses the same calicheamicin payload and cleavable hydrazone linker found in Mylotarg®, recently failed to demonstrate improved survival in a phase III study for patients with refractory aggressive NHL (Pfizer Inc. press release; May 20, 2013). No unexpected safety concerns were identified, however, and phase III studies continue for acute lymphoblastic leukemia (ALL) patients.

The vast majority of remaining ADCs in clinical development use either auristatin [monomethyl auristatin E (MMAE) or MMAF] or maytansinoid (DM1 or DM4) payloads, both potent inhibitors of tubulin polymerization. Several MMAE conjugates with cleavable linkers are currently under evaluation in phase II studies for various indications based on the target antigen. In general, these agents were well tolerated in phase I trials with toxicities consistent with the known mechanism of action for the auristatins (e.g. neutropenia or neuropathy) [22,111]. SAR3419, an anti-CD19 DM4 conjugate with a cleavable disulfide linker, demonstrated a dose-limiting toxicity (DLT) of reversible severely blurred vision in a phase I study for refractory B cell NHL, but was well tolerated on a modified dosing schedule [112]. Recently advanced to phase II studies for colorectal cancer (CRC), labetuzumab-SN-38 employs a cathepsin B-cleavable dipeptide linker and SN-38, the active metabolite of the clinically used anticancer agent irinotecan, as a payload. Initial phase I data indicated that labetuzumab-SN-38 was generally safe and well tolerated at effective clinical doses [113]. Lorvotuzumab mertansine utilizes a maytansinoid payload (DM1) and a disulfide linker to target CD56. No serious DLTs or

Reviews • KEYNOTE REVIEW

TABLE 3

Representative ADCs undergoing clinical evaluation[a]

Agent	Sponsor (licensee)	Status	Indication	Antigen	Cytotoxin	Linker
Adcetris® (brentuximab vedotin, SGN-35)	Seattle Genetics (Millennium)	Launched	HL, ALCL	CD30	MMAE	Cleavable, Val-Cit
Kadcyla® (ado-trastuzumab emtansine, T-DM1)	Roche-Genentech (ImmunoGen)	Launched	Her2+ metastatic breast cancer	HER2	DM1	Non-cleavable, thioether
Mylotarg® (gemtuzumab ozogamicin)	Pfizer (UCB)	Withdrawn	AML	CD33	Calicheamicin	Cleavable, hydrazone (Ac-But acid)
Inotuzumab ozogamicin (CMC-544)	Pfizer (UCB)	Ph III	ALL, NHL	CD22	Calicheamicin	Cleavable, hydrazone (Ac-But acid)
RG-7596	Roche-Genentech	Ph II	DLBCL, NHL	CD79b	MMAE	Cleavable, Val-Cit
Glembatumumab vedotin CDX-011)	Celldex (Seattle Genetics)	Ph II	Advanced breast cancer, melanoma	GPNMB	MMAE	Cleavable, Val-Cit
PSMA-ADC	Progenics (Seattle Genetics)	Ph II	HRPC	PSMA	MMAE	Cleavable, Val-Cit
SAR3419	Sanofi (ImmunoGen)	Ph II	Hematologic tumors	CD19	DM4	Cleavable, disulfide
Labetuzumab-SN-38 (IMUU-130)	Immunomedics	Ph II	Metastatic CRC	CEACAM5	SN-38	Cleavable, Phe-Lys
Lorvotuzumab mertansine (IMGN901)	ImmunoGen	Ph I/II	MM, solid tumors	CD56	DM1	Cleavable, disulfide
Milatuzumab-DOX (IMMU-110)	Immunomedics	Ph I/II	MM	CD74	Doxorubicin	Cleavable, hydrazone
BT-062	Biotest AG (ImmunoGen)	Ph I	MM	CD138	DM4	Cleavable, disulfide
BAY-94-9343	Bayer Schering (ImmunoGen)	Ph I	Solid tumors	Mesothelin	DM4	Cleavable, disulfide
ASG-5ME	Astellas (Seattle Genetics)	Ph I	Solid tumors	AGS-5	MMAE	Cleavable, Val-Cit
SGN-75	Seattle Genetics	Ph I	NHL, RCC	CD70	MMAF	Non-cleavable, MC
IMGN529	ImmunoGen	Ph I	Hematologic tumors	CD37	DM1	Non-cleavable, thioether
SAR-566658	Sanofi (ImmunoGen)	Ph I	Solid tumors	DS6	DM4	Cleavable, disulfide

[a] *Abbreviations*: CEACAM5, carcinoembryonic antigen cell adhesion molecule 5; HRPC: hormone refractory prostate cancer; MC: maleimidocaproyl; RCC: renal cell carcinoma; SN-38, 7-ethyl-10-hydroxycamptothecin.

drug-related adverse events were reported in early-phase multiple myeloma (MM) studies [114].

Although the modest potency of doxorubicin payloads limited the efficacy of early ADCs (BR96-DOX), milatuzumab-DOX targets CD74, an antigen with unique internalization and surface re-expression, and is currently in phase I/II trials based on encouraging preclinical efficacy in hematopoietic cancer xenograft models [52]. Select agents in phase I trials include ADCs containing DM1 or DM4 cytotoxic drugs under evaluation by ImmunoGen, and several ADCs with MMAE or MMAF developed by Seattle Genetics, each targeting a different antigen across a variety of tumor indications. Available data for these and other phase I agents generally provide initial evidence of efficacy and tolerability. Similar to SAR3419 (anti-CD19 DM4 conjugate), the DM4-based anti-mesothelin conjugate BAY-94-9343 has also been reported to induce Grade 2 and 4 ocular toxicity [115].

ADC PK

ADCs typically retain the PK properties of the antibody component, as opposed to the appended drug, and thus exhibit relatively low clearance and long half lives. Compared with the unconjugated antibody, ADCs can exhibit somewhat higher clearance due to introduction of an additional metabolic pathway (i.e. cleavage of the drug from the antibody). In addition, ADCs with higher DARs tend to clear faster than those with lower DARs [116]. Variable DARs and attachment sites, a consequence of current random conjugation methods, result in heterogeneous ADCs with PK parameters that can vary substantially compared to the unconjugated antibody [117]. Each ADC component, along with their respective metabolites, can potentially impact efficacy, safety and tolerability [118]. Both the type of linker used and the site of conjugation can influence the extent to which the drug is prematurely released from the antibody. Deconjugation of the payload from the antibody can result in ADCs with lower DARs, reduced efficacy and potentially increased toxicity owing to release of a highly potent cytotoxic drug in systemic circulation.

The PK parameters of Adcetris® and Kadcyla® were evaluated in mouse, rat and monkey in preclinical toxicity studies. Overall, these ADCs demonstrated similar PK properties, albeit with a few differences in mouse and monkey. The $t_{1/2}$ of Adcetris® in mouse, rat and monkey was 14, 10 and 2 days, respectively. The rapid clearance of Adcetris® in monkeys as compared with mouse or rat was hypothesized to result from nontherapeutic antibodies, target-mediated disposition and other factors [119]. In the case of Kadcyla®, the

$t_{1/2}$ in mouse, rat and monkey was 5.7, 8.3 and 11.6 days, respectively [120,121]. In humans, the PK characteristics of these two conjugates were similar, with a $t_{1/2}$ ranging from 3.5 to 5 days [22,24]. Of note, the $t_{1/2}$ of an ADC is often significantly shorter in humans compared with other species. The optimal $t_{1/2}$ for an ADC remains to be determined, but the clinical success of Adcetris® and Kadcyla® indicate that the range of 3–4 days is appropriate.

The long $t_{1/2}$ typical of ADCs and mAbs results from FcRn recycling [122]. In this process, antigen-independent internalization by endothelial cells is followed by FcRn binding and then FcRn-mediated return to the bloodstream. FcRn recycling essentially protects ADCs from catabolism; however, diversion of FcRn-bound ADCs to the lysosome can increase the risk of off-target toxicities. Although the factors that influence this process are poorly understood, the drug, linker, antibody and antigen can each affect FcRn-mediated ADC trafficking [123]. Another mechanism of off-target toxicity involves soluble cell-surface mannose receptors (MRs), which interact with agalactosylated glycans on the antibody Fc domain [124]. Cell-surface MRs can internalize, effectively delivering the ADC to the endosome and lysosome compartments where the potent cytotoxic drug is released. Importantly, locations of off-target ADC activities reportedly coincide with cell-surface MR locations. The shedding of antigen from the tumor cell surface into circulation may also increase the risk of toxicity. Binding of an ADC to shed antigen can, in some cases, lead to higher ADC clearance and impaired tumor localization as well as immune complex formation and accumulation in the kidney [125].

To determine the effect of linker stability on PK and efficacy, the noncleavable thioether linker of Kadcyla® was compared to the cleavable disulfide linker of a T-SPP-DM1 conjugate [121]. The nonreducible thioether-linked Kadcyla® demonstrated superior PK with greater plasma exposure (area under the curve) and increased maytansinoid tumor concentration. The disulfide-linked ADC demonstrated higher plasma clearance owing to the presence of the metabolically labile linker. Despite the difference in PK, both conjugates had similar in vivo efficacy. It was hypothesized that the drug released from the disulfide-linked T-SPP-DM1 conjugate would benefit from the bystander killing effect, whereas Kadcyla® ultimately liberates a maytansinoid appended to a charged lysine residue, which limits diffusion to neighboring tumor cells. Taken together, these results illustrate how minor structural changes can profoundly impact ADC PK and efficacy.

In addition to the type of linker used to join the drug and antibody, the conjugation site on the antibody has been shown to influence stability and, therefore, PK. A recent study examined the stability of MMAE conjugated to Her2 via a maleimide at various site-specifically engineered cysteines [126]. Highly solvent accessible conjugation sites were found to be labile, undergoing maleimide exchange with reactive thiols in the plasma, such as glutathione, albumin or free cysteine. At less accessible sites, the succinimide ring of the linker underwent hydrolysis, which served to protect the linker from maleimide exchange and resulted in enhanced stability and efficacy. In a separate study, the stability of monomethyl auristatin D (MMAD) conjugated to an anti-M1S1 antibody was examined using BTG to introduce the drug payload site specifically at either the heavy or light chain [107]. The conjugation site was found to influence stability and PK, with ADCs appended to the heavy chain demonstrating a higher rate of drug loss in rats via proteolysis of the valine–citrulline linker. Interestingly, these results were species specific since both conjugates demonstrated comparable stability in mice, which also serves to highlight the potential pitfall of performing safety and efficacy studies in different species.

Concluding remarks and future directions

Despite complexities in designing ADCs, the promise of this therapeutic class has generated intense interest for decades. A robust clinical pipeline and the recent FDA approvals of Adcetris® and Kadcyla® suggest that the potential benefit of ADCs may finally be realized. Evolving clinical data will continue to drive technological advancements in the field. Current methods for preclinical lead selection typically rely on systematic in vitro evaluation of a matrix of various mAbs, linkers and cytotoxic payloads. Whether in vitro models are sufficient to predict response remains to be seen; until further understanding of ADCs is realized, early in vivo studies might be crucial. Progress in site-specific conjugation modalities, optimization of linkers with balanced stability and identification of novel, potent cytotoxic agents should pave the way for greater insight into the contribution of these various factors to ADC efficacy, PK and safety. Challenges in target tumor selection will be addressed as the roles of antigen expression, heterogeneity and internalization rate are further elucidated. Guiding principles for the selection of an ideal antibody Fc format are, as of yet, lacking and prompt validation of current assumptions regarding antibody-dependent properties, such as specificity and immune effector functions. Ongoing efforts to address these issues will continue to broaden the impact of ADCs as targeted therapeutics for the treatment of cancer and potentially other diseases.

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