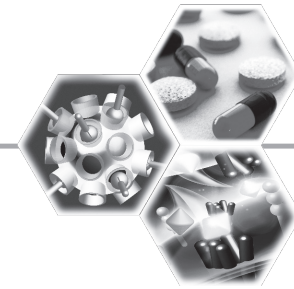

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Antibody–drug conjugates: using monoclonal antibodies for delivery of cytotoxic payloads to cancer cells

One approach to improving activity of anticancer drugs is to conjugate them to antibodies that recognize tumor-associated, cell-surface antigens. The antibody–drug conjugate concept evolved following major advances, first, in the development of humanized and fully human antibodies; second, in the discoveries of highly cytotoxic compounds ('drugs') linkable to antibodies; and finally, in the optimization of linkers that couple the drug to the antibody and provide sufficient stability of the antibody–drug conjugate in the circulation, optimal activation of the drug in the tumor, and the ability of the activated drug to overcome multidrug resistance. In this article, we will review the considerations for selecting a target antigen, the design of the conjugate, and the pre-clinical and clinical experiences with the current generation of antibody–drug conjugates.

Victor S Goldmacher[†1] & Yelena V Kovtun[1]

[1]ImmunoGen, Inc., 830 Winter Street, Waltham, MA 02451-1477, USA
[†]Author for correspondence:
Tel.: +1 781 895 0600
Fax: +1 781 895 0610
E-mail: victor.goldmacher@immunogen.com

Rationale for delivery of cytotoxic drugs with monoclonal antibodies

Cytotoxic chemotherapeutic drugs and therapeutic monoclonal antibodies (mAbs) represent two major classes of agents currently used for cancer treatment. The therapeutic activity of cytotoxic chemotherapeutic drugs is, in general, limited by their narrow therapeutic window, which may be attributed to their indiscriminate cytotoxicity, and/or poor pharmacokinetics and organ distribution [1]. Historically, most of the tested cytotoxic compounds have failed in clinical trials due to their excessive toxicity, and, accordingly, lack of sufficient therapeutic activity at the maximal tolerated dose (MTD).

Therapeutic mAbs display better pharmacokinetics than small-molecule drugs (longer circulation times: days vs minutes), various degrees of antigen-selective tumor accumulation, and often only moderate or no systemic toxicity [2]. Yet only a few of the large number of tested antibodies against various tumor-associated antigens, such as rituximab, trastuzumab, cetuximab and panitumumab, have made it into clinical practice [3], while the majority of the antibodies were found to lack sufficient therapeutic activity in clinical trials [4] or in preclinical studies.

The rationale for the development of **antibody–drug conjugates** (ADCs), as defined in a number of previous reviews [5–12], is to combine the selectivity, favorable pharmacokinetics, biodistribution and, when present, functional activity of antibodies with the high cytotoxic potency of a drug. An ideal ADC should: retain the favorable pharmacokinetic and functional properties of antibodies; remain intact and nontoxic in the compartment of its systemic delivery (typically blood); become active in the tumor compartment, with the active form of drug released in a sufficient amount to kill tumor cells; and combine the cytotoxic activity of the payload with the intrinsic anti-tumor activities of the antibody, including natural humoral immune responses such as the **antibody-dependent cellular cytotoxicity** (ADCC), **complement-dependent cytotoxicity** (CDC), antibody-dependent cellular phagocytosis and any direct functional activities of the antibody, such as blocking of a receptor signal transduction or engaging pro-apoptotic pathways.

Target antigen selection

The vast majority of antigens that are expressed on cancer cells have also been found to some degree on at least some normal tissues, and for this reason the term 'tumor-associated antigen' is more accurate than 'tumor-specific antigen'. The expression of an antigen of interest in some normal tissues does not necessarily preclude the development of an ADC targeting this antigen, if the normal tissue is either nonessential, or insensitive to the action of the drug (e.g., non-proliferating cells towards antimitotic agents such as **maytansinoids** or **auristatin**). Prostate-specific membrane antigen (PSMA) and HER2 are two examples of such target antigens. PSMA is expressed on normal prostate as well as on prostate cancer cells and has been the focus of

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Key Terms

Antibody–drug conjugate: A cytotoxic drug covalently linked to a monoclonal antibody that recognizes a tumor-associated antigen.

Antibody-dependent cellular cytotoxicity: Natural killer cells and neutrophils bind to the antibody Fc portion, thus making a contact with the antigen-expressing target cell. The effector cells then use the perforin-granzyme pathway and Fas ligand to kill the target cells. In addition to antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis may also contribute to the target cell eradication. Phagocytes such as monocytes/macrophages and dendritic cells take up and eliminate IgG-coated target cells.

Complement-dependent cytotoxicity: Some (but not all) human or humanized monoclonal antibodies of IgG1, IgG3, and (to a lesser degree) IgG2 isotypes trigger activation of complement pathways, which may lead to the destruction of the target cells.

Maytansinoids: Derivatives of the semi-synthetic microtubule-targeting agent maytansine.

Auristatin: Synthetic derivative of the microtubule-targeting agent dolastatin 10.

Human antimouse antibodies: Polyclonal antibodies that are produced by the human immune system against therapeutic murine monoclonal antibodies.

several ADC programs [13–16]. Targeting of normal prostate tissue may be of no safety concern and, in fact, most patients may have had their prostate surgically removed prior to ADC therapy. Trastuzumab emtansine (T–DM1) is an ADC consisting of the anti-HER2 mAb trastuzumab (Herceptin®) and the maytansinoid DM1, and has been administered safely at therapeutically effective doses, despite HER2 being expressed on some normal tissues [17–20]. Properties of a target antigen that are generally assumed to be favorable for an ADC approach include: strong expression on the surface of tumor cells; either good penetrance (percentage of tumor samples that are positive), or expression on tumors of cancer patients with high unmet medical need (a life-threatening disease with limited treatment choices); and, at the same time, little or no expression on essential normal tissues [8,11,21–23]. A requirement for the antigen of interest to be expressed homogeneously throughout a tumor tissue is probably not absolute, due to the ability of some ADCs to mediate bystander cell killing [24,25].

Accumulating, albeit still fragmentary, evidence suggests that a combination of several antigen-related factors determines the cytotoxic and anti-tumor activities of ADCs. These include, but are probably not limited to, the following parameters: the degree of the cell surface expression of the antigen; the rate of internalization of antigen–ADC complexes, and the rate and direction of their intracellular trafficking; the rate and/or degree of antigen–ADC complex processing leading to the release of the drug in its active form (proteolytic degradation of the antibody and/or cleavage of the linker). For some targets, the dynamics or efficiency of ADC internalization/trafficking may vary depending on the specific epitope on the antigen that is bound by a particular antibody. In addition, phosphorylation or other post-translational modification of the target protein or expression of cellular proteins that physically or functionally interact with the antigen may affect ADC internalization and/or its intracellular trafficking [10,11,26–31]. Available experimental evidence suggests that correlation between antigen expression and potency of an ADC is not a general phenomenon and is evident only for some antigens [12]. In one example, in an experiment evaluating the *in vitro* sensitivity of 18 non-Hodgkin lymphoma cell lines to an anti-CD22–DM1 conjugate, it was found that sensitivity to the maytansinoid component of the ADC was a more predictive indicator of sensitivity than was either the level of CD22 expression or the amount of internalized DM1 [32,33]. Therefore, selection of patients based solely on high tumor antigen expression may not guarantee clinical efficacy.

A potentially attractive property for a prospective ADC target antigen is a biological role in a pathway essential for tumor growth or survival. In principle, the intrinsic anti-tumor activity of the antibody that blocks such a pathway and the cytotoxic payload provided by the ADC format may produce a potent combined anti-tumor activity. T–DM1 [17–19] and SGN-35 [34] are examples where ADC clinical evaluation was preceded by clinical experience with the respective naked antibodies, trastuzumab and SGN-30. Trastuzumab (Herceptin) targets the HER2-receptor tyrosine kinase and suppresses the oncogenic function of this receptor, which is overexpressed on tumor cells. Trastuzumab was approved for the treatment of HER2-positive metastatic breast cancer [35]. Genentech/Roche are currently developing the ADC T–DM1 for patients with advanced HER2-positive breast cancer who have previously received multiple HER2-targeted medicines and chemotherapies [36]. Phase III studies are underway that could lead to approval of this agent in second-line and first-line therapy of HER2-positive metastatic breast cancer. Results of a Phase II study showed that T–DM1 has impressive anti-tumor activity in patients who have progressed on prior HER2-targeted therapies (including trastuzumab) [37]. The chimeric antibody SGN-30 targets CD30, a member of the TNF-R superfamily of immunoregulatory molecules, and was found to promote growth arrest and DNA fragmentation in cultured CD30-positive cells, and to have activity against Hodgkin lymphoma xenografts in immunodeficient mice [38]. Evidence of clinical activity has been reported for both SGN-30 and its conjugate with auristatin, brentuximab vedotin (SGN-35), and advanced trials are underway [34,39,40].

Apart from the biological role of the target antigen, there are two additional key considerations when developing an ADC that potentially combines anti-tumor activities provided by the cytotoxic payload with the intrinsic anti-tumor activity of its antibody component. First, the modification, conjugation, linker and payload chemistries employed must yield an ADC that retains the full biological properties of the functional antibody. T–DM1, an ADC created by conjugation of maytansinoids to trastuzumab through lysine residues, retains

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all of trastuzumab's anti-tumor mechanisms of action, including HER2 binding affinity, inhibition of HER2 signaling and Fc-mediated immune-effector activities, such as ADCC [41]. Similar combination of the antibody functional activity and the effector drug cytotoxicity may be expected for the anti-tumor activity of the SGN-30–auristatin conjugate SGN-35.

Second, to achieve the combined effect, such ADCs may have to be administered at high enough doses, since optimal anti-tumor activity of functional antibodies in cancer patients requires relatively high doses, typically several mg/kg or greater every 3 weeks (e.g., rastuzumab, cetuximab, rituximab or panitumumab). ADCs that employ calicheamicin as a payload, a highly cytoxic DNA minor groove-binding agent, achieve an MTD of only 9 mg/m^2 or lower [42–44], doses wherein the antibody component (<0.3 mg/kg) would be unlikely to contribute to the efficacy of the ADC. By contrast, maytansinoid conjugates incorporating multiple linker formats (both cleavable and noncleavable) can achieve MTDs greater than 3 mg/kg in clinical testing [5]. In Phase I studies, the reported MTD for T–DM1 is 3.6 mg/kg when given every 3 weeks, and 2.4 mg/kg when administered weekly [19,45], which is similar to the trastuzumab dose adminstered to patients with metastatic breast cancer (e.g., 2 mg/kg weekly, after the first dose of 4 mg/kg). Therefore, maytansinoid ADC formats can produce compounds that have the prospect of retaining anti-tumor activities of the antibody component, with the added cytotoxic potency provided by the maytansinoid payload.

A wide variety of tumor-associated antigens have been explored to date as potential targets for ADCs, including various cell-surface proteins and glycoproteins, gangliosides and extracellular proteins [12,46]. In addition, in recent years, there has been increasing interest in antibody-based targeting of the tumor vasculature and stromal tissue that often play a supporting role in tumor growth [3,47–51]. ADCs that target neovasculature may act by destroying the architecture of the tumor blood supply, leading to hypoxia and nutrient deprivation [47,52], while ADCs that target the tumor stroma may reduce the level of tumor growth-supporting factors [53,54]. IMGN388 is an ADC that consists of the maytansinoid DM4 attached to an antibody against αV integrin, which is expressed on the cells of many solid tumors and also on endothelial cells during angiogenesis [55]. IMGN388 is currently in a Phase I clinical trial, and has demonstrated favorable tolerability at the dose levels evaluated to date, and evidence of activity [56]. Since it targets both tumor cells and cells that form new blood vessels that support tumor growth, its activity may exceed the clinical benefit achievable by targeting either component separately, especially with well-vascularized tumors. FAP5–DM1 is an ADC that targets fibroblast activation protein-α, an antigen found on the surface of tumor stromal fibroblasts, and it was shown to have anti-tumor activity in tumor xenografts and models of lung, pancreas, head and neck cancers in immunodeficient mice [57]. Another potential ADC stromal target is Ptk7, a pseudokinase that is highly expressed on many cancer types either in the cancer cells themselves or in the tumor stroma [58].

Targeting cancer stem cell (CSC)-specific antigens with ADCs has also been explored recently [59,60]. If the CSC hypothesis is correct then, following the elimination of the majority of cancer cells, the tumor may regrow if the CSCs are not also eradicated [61–64]. In addition to being implicated in initiating and propagating tumors, CSCs may also contribute to angiogenesis and drug resistance [65–68]. Several CSC-associated antigens are potential targets of ADC therapeutics [29,69,70]. Targeting stromal cells surrounding tumors may eliminate a source of inhibitors of bone morphogenic proteins, allowing bone morphogenic proteins to induce differentiation and inhibition of CSC proliferation [71–74].

Antibody selection

The first mAbs to be examined in the clinic were murine. In most instances, even a single injection of a murine mAbs elicits the generation of **human antimouse antibodies** within 2 weeks of antibody administration, limiting the therapeutic efficacy of murine mAbs to the first and at most, the second dose administration [2]. To date, most antibodies for use in ADCs were generated as mouse mAbs that were subsequently converted to human IgGs [3,75,76]. The initial goal for antibody chimerization (substitution of human sequences into the Fc regions) and humanization (retention of the murine complementarity-determining regions only) was to minimize the immunogenicity of antibodies when administered to human patients [76–80], and both approaches have achieved this goal [80]. Beyond reduced immunogenicity, human IgGs have two other

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advantages over murine IgGs, first, they are retained longer in human circulation (because of their recognition by the human Fc neonatal receptor [FcRn], which regulates IgG circulation half-life [81]); and second, they are more effective than murine antibodies in eliciting ADCC with human effector cells [2]. Other approaches to generating human IgGs are to either directly raise human mAbs in transgenic mice in which the murine antibody-producing genes have been inactivated and replaced with human antibody-producing genes [82], or to select antibodies from large recombinant antibody libraries, such as those generated by phage display [76,83–87].

In general, desirable properties of antibodies for use in ADCs include: specificity; high binding affinity; long-circulation times; immune effector functions such as CDC, ADCC and antibody-dependent cellular phagocytosis; tumor-suppressing modulation of antigen's biological activity when possible; and minimal loss of the above-listed properties upon its conjugation to the drug of choice. To date, there are no clinical data available that would compare the anti-tumor activities of ADCs differing in one or more of these antibody-dependent properties (but made with the same drug, targeting the same antigen), and it remains unknown how much any of these properties contribute to (or interfere with) the anti-tumor efficacy of ADCs. Thus, the above-mentioned criteria are assumptions based largely on the available clinical and preclinical results obtained with naked therapeutic antibodies, and on preclinical results with ADCs in animal models, mostly immunodeficient mice. Significant differences exist between the human and murine immune systems [88,89], and between human patients' tumors and xenografts of human tumor cell lines in immunodeficient mice, and thus these criteria need further validation.

One of these factors, affinity, deserves a more detailed consideration. Typically, antibodies with the highest affinity are selected for use in ADCs on the tacit assumption that the higher affinity of an antibody will lead to the higher anti-tumor activity of its ADC. Apart from well documented *in vitro* correlation between ADC affinity and its cytotoxicity, there is no conclusive evidence of any *in vivo* correlation between affinity of an ADC and its efficacy in either preclinical models [32] or in the clinic, and it may well be that there is no such correlation because of the following considerations:

- ADCs are administered intravenously, at doses that can translate into an initial blood concentration of 0.1 µM or higher, which is much higher than the typical K_d values for antibodies used in ADCs (nM range or lower). If the ADC concentration at the tumor sites is as high as that in the blood, the ADC would be expected to saturate the antigen irrespective of its K_d;
- An exceedingly high affinity of an ADC may lead to its fast binding to the perivascular regions of the tumor, thus limiting its diffusion inside the tumor [90,91];
- A recently developed computational model of tumor targeting predicts that similar amounts of antibodies (and, by extention, ADCs) with affinities of 10^{-8} M or higher would be retained at the antigen-expressing tumor site [92].

Similarly, there are varying views on the desirability of the immune-effector functions in mAbs used in ADCs. The ADCC and CDC activities of antibodies are dependent on the antibody isotype. Human IgG1 and IgG3 isotypes elicit strong ADCC and CDC immune-effector responses, while IgG4 and IgG2 antibodies typically induce weak immune-effector responses [89,93,94]. It is generally assumed that CDC and ADCC may contribute to the anti-tumor activity of ADCs [93,94]. On the other hand, effector functions may interfere with target-cell localization or internalization of the ADC, and therefore, antibody isotypes with reduced effector functions, IgG2 and IgG4, were chosen for some ADCs [94–98].

Humanized IgG1 has been the most common isotype chosen for ADC antibodies in clinical development, with few instances of humanized IgG4 and IgG2 being selected to date **(Table 1)**. The circulation half-life of human IgG3 antibodies is shorter than that of IgG1, IgG2 and IgG4 isotypes, making them less desirable for therapeutic use [89,93]. One potential disadvantage of the IgG4 subclass is the propensity of IgG4 antibodies to exchange half-molecules with other IgG4 molecules *in vivo* and hence to become bi-specific, but this can be overcome by replacing the third constant domain of the IgG4 molecule with that of human IgG1 [99]. Only one ADC that underwent clinical testing, CRO11–valine–citrilline monomethylauristatin E (vcMMAE), was of an IgG2 type [100]. IgG2 antibodies are capable of forming covalent dimers [101], which conceivably may enhance solid-phase homotypic antibody–antibody

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Table 1. Antibody–cytotoxic compound conjugates in clinical development.

Antibody–cytotoxic compound	Target antigen	Linker–cytotoxic compound, class	Antibody	Tumor type(s)	Developer	Status
Gemtuzumab ozogamicin (Mylotarg®)	CD33 (Siglec-3)	Hydrazone, AcBu, *N*-acetyl-γ calicheamicin, calicheamicin	hP67.6 humanized IgG4	Acute myeloid leukaemia	Pfizer	US FDA conditionally approved, recently withdrawn
Inotuzumab ozogamicin (CMC–544)	CD22 (Siglec-2)	Hydrazone, AcBu, *N*-acetyl-γ calicheamicin, calicheamicin	G5/44 humanized IgG4	B-cell lymphomas	Pfizer	Phase I and I/II Phase III combination
Trastuzumab–DM1 (T–DM1)	HER2 (ErbB2)	Thioether, SMCC–DM1, maytansinoid	Transtuzumab humanized IgG1	Metastatic breast cancer	Roche	Phase II and III
Lorvotuzumab mertansine (IMGN901, huN901–DM1, BB10901)	CD56 (NCAM)	Hindered disulfide, SPP–DM1, maytansinoid	huN901 humanized IgG1	Solid tumors, multiple myeloma	ImmunoGen	Phase I and II
IMGN388 (CNTO365)	Integrin	Highly hindered disulfide, SPDB–DM4, maytansinoid	Anti-integrin human IgG1	Solid tumors	ImmunoGen	Phase I
SAR3419 (huB4–DM4)	CD19	Highly hindered disulfide, SPDB–DM4, maytansinoid	huB4 humanized IgG1	Non-Hodgkin lymphoma	Sanofi–Aventis	Phase I
BIIB015	Cripto	Highly hindered disulfide SPDB–DM4, maytansinoid	Anti-Cripto humanized IgG1	Cripto-positive solid tumors	Biogen Idec	Phase I
BT062	CD138	Highly hindered disulfide SPDB–DM4, maytansinoid	Anti-CD138 chimeric IgG4	Multiple myeloma	Biotest	Phase I
Brentuximab vedotin (SGN-35)	CD30	Dipeptide, vc-MMAE, auristatin	SGN-30 chimeric IgG1	Relapsed/refractory Hodgkin lymphoma, systemic anaplastic large-cell lymphoma	Seattle Genetics, Millenium	Phase II and III
Glembatumumab vedotin (CR011–vcMMAE, CDX-011)	Glycoprotein NMB (osteoactivin)	Dipeptide, vc-MMAE, auristatin	CRO11 fully human IgG2	Metastatic breast cancer and melanoma	Celldex Therapeutics	Phase I/II
SGN-75 (h1F6–mcMMAF)	CD70	Noncleavable, mc-MMAF, auristatin	SGN-70 humanized IgG1	Non-Hodgkin lymphoma and renal carcinoma	Seattle Genetics	Phase I
PSMA ADC	Prostate-specific membrane antigen	Dipeptide, vc-MMAE, auristatin	Anti-PSMA	Progressive, metastatic, hormone-refractory prostate cancer following prior taxane chemotherapy	Progenics Pharmaceuticals	Phase I
ASG–5ME	SLC44A4 (AGS-5)	Dipeptide, vc-MMAE, auristatin	Anti-AGS-5 human IgG2	Pancreatic cancer	Agensys, Seattle Genetics	Phase I
MDX–1203	CD70	Dipeptide, MGBA	Anti-CD-70 human	Non-Hodgkin lymphoma and advanced/recurrent clear-cell renal cell carcinoma	Bristol–Myers Squibb	Phase I

MMAE: Monomethylauristatin E; MMAF: Monomethylauristatin F; NCAM: Neural cell-adhesion molecule; PSMA: Prostate-specific membrane antigen; SPDB: N*-succinimidyl-3-(2-pyridyldithio)butyrate; SPP:* N*-succinimidyl 4-(2-pyridyldithio)pentanoate.*

associations to boost affinity and/or internalization, but may also contribute to aggregation issues. Antibody engineering can enhance the immune-effector functions and extend the circulation half-life of antibodies [85,102], with current technologies focusing on modifying the antibody amino acid sequence or on its defucosylation to enhance affinity for IgG Fc-γ receptor IIIa (FcγRIIIa) [103–107]. The effect of isotype and FcγR binding on the properties of monomethylauristatin F (MMAF) conjugates of an anti-CD70 antibody have been recently reported [88]. Conjugates were prepared from variant antibodies of the parental anti-CD70 IgG1, which included an IgG2, an IgG4 and an IgG1v1, the latter engineered for impaired binding to huFcγ receptors, but having only modest impairment of binding to FcRn, which regulates retention of IgG in circulation. All the conjugates bound well to antigen, had good cytotoxicity activity *in vitro* and were well-tolerated *in vivo* in mice. The IgG1v1 conjugate was found to have an increased anti-tumor activity in xenograft models, and a longer circulation half-life than the parental IgG1 conjugate. Interpretation of these results and their implications for ADC clinical therapy is difficult, however, because of the above-mentioned differences between the human and murine systems, it remains to be seen how much ADCC and CDC contribute to the overall activity of ADCs.

Cytotoxic drugs used in ADCs

■ Considerations for ADC drugs

One important requirement for a drug to be considered as an ADC effector is that this drug should be sufficiently cytotoxic at concentrations that can be achieved by antibody delivery. Early immunoconjugates developed for oncology incorporated the standard anticancer drugs in use at the time, such as methotrexate, doxorubicin or the vinca alkaloids, and yielded disappointing results, with little or no anti-tumor activity observed in clinical trials [7,8,108,109]. Delivery of the cytotoxic agent to a tumor cell via an ADC is limited by the amount of the target antigen on the cell surface, and by the access of the ADC to the cell within the tumor. It was later proposed that one of the reasons these early ADCs failed, and had a narrow (if any) therapeutic window, was insufficient potency of the conjugated drugs [7,25,108,110,111]. Cytotoxic drugs used in the current generation of ADCs are at least 100-fold more potent than those used in the early conjugates [7]. On the other hand, more potent ADCs may be more systemically toxic, which may narrow their therapeutic window. Another common feature of effectors utilized in the current generation of ADCs is their selectivity towards killing proliferating cells. An effector that preferentially kills proliferating cells may, in principle, improve the selectivity of the ADC since the incidence of proliferating cancer cells in tumors is higher than that in most normal tissues. Other preferable characteristics for a drug include low molecular weight, and the related lack of immunogenicity, a reasonably low cost of production, availability in a linkable form and sufficient solubility and stability in water to facilitate preparation and storage of the antibody conjugates [112].

■ Types of ADC drugs

The drugs used in ADCs currently in the clinic or preclinical testing can be divided into two categories, those that target DNA, and those that target microtubules. In addition, several potent ADC drugs have also been reported that have not yet reached the clinic. These include the microtubule-targeting taxol derivatives (taxanes) [7,8,113–116], the DNA-targeting streptonigrin, the HSP90-targeting geldanamycin and the topoisomerase-inhibiting camptothecin analogs [117,118]. These preclinical-stage ADCs will not be discussed here further.

■ DNA-damaging drugs

N-acetyl-γ-calicheamicin dimethyl hydrazide, a derivative of calicheamicin, is the cytotoxic component of Mylotarg® gemtuzumab ozogamicin, which targets CD33 [96,119], and was conditionally approved in May 2000 under the US FDA's accelerated-approval program, but was recently withdrawn from the USA market by Pfizer due to its failing to show inproved efficacy in a Phase III combination trial. Calicheamicin is a highly cytotoxic enediyne antibiotic that binds to the minor groove of DNA and induces double-strand DNA breaks that result in cell death [98,120]. The ADC showed higher *in vitro* potency than unconjugated calicheamicin against CD33-positive cells, and eradicated CD33-positive HL-60 xenografts in nude mouse models [96]. Several other ADCs comprised of antibodies linked to calicheamicin are being evaluated, including inotuzumab ozogamicin (CMC-544), which targets CD22 and is in Phase III clinical testing against non-Hodgkin lymphoma [98,121,122]. Calicheamicin conjugates of several other antibodies, the anti-CD20 antibody rituximab [123], the H8 antibody directed against

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the oncofetal antigen 5T4 expressed on various carcinomas [124], and an anti-MUC1 antibody targeting various solid tumors [125], are currently in preclinical evaluation.

Analogs of CC-1065 and the duocarmycins are highly cytotoxic DNA minor-groove binding alkylating agents (MGBA) [126]. The first MGBA-based ADCs were made with the CC-1065 analog DC1 [127], which turned out to be nearly insoluble, making conjugation reactions inefficient, and with DC4, a water-soluble phosphate prodrug of DC1 [8]. Several other conjugates have been reported more recently [16,128–132]. MDX-1203, a conjugate of the fully human anti-CD70 antibody and a duocarmycin analog has been developed at Medarex (now Bristol–Myers Squibb). In this conjugate, the cytotoxic effector is attached to the antibody in prodrug form, linked to it via a protease-cleavable dipeptide linker, and a maleimide attachment site, requiring not only release from the antibody but also esterase/protease cleavage to release the active cytotoxic drug [131]. MDX-1203 showed efficacy in renal cancer xenograft models, was well tolerated in cynomolgus monkey safety studies [131,132] and is currently in a Phase I clinical study in patients with advanced/recurrent clear-cell renal-cell carcinoma or relapsed/refractory B-cell non-Hodgkin lymphoma. Two other MGBA-based ADCs are being explored as potential prostate cancer therapeutics, one targeting PSMA, which is found on the surface of the prostate tumor cells, and another directed against the extracellular protein mindin/RG-1 which is thought to be retained in the extracellular matrix of human prostate tumors [16].

■ Microtubule-targeting drugs

Among ADCs based on microtubule-targeting cytotoxic drugs, two categories have reached the stage of clinical development, those utilizing derivatives of maytansine (maytansinoids), and those utilizing auristatins [5,6,8,9,110,133]. Maytansine, with IC_{50} values in the range of 10 to 100 pM depending upon the cell type [110,133], is significantly more cytotoxic against cell lines *in vitro* than clinically used microtubule-targeting drugs such as vinca alkaloids or taxol. Maytansine and maytansinoids appear to act by interfering with microtubule dynamics [134–136], thereby inducing cell-cycle arrest at G2/M, which eventually leads to cell death. This mode of cytotoxic action may explain why proliferating cells are more sensitive to maytansine than are nonproliferating cells [137]. Two maytansinoids are being used in ADCs currently in clinical development, DM1 and DM4, which differ in the degree of hindrance of the disulfide bond they afford in the linker between the drug and the antibody. The first antibody–maytansinoid conjugate to undergo clinical testing was cantuzumab mertansine [25,110], which was comprised of the cytotoxic maytansinoid compound DM1 conjugated by a disulfide linkage to the huC242 antibody, a humanized IgG1 antibody that targets the extracellular domain of the tumor-associated carbohydrate antigen CanAg, expressed on most colorectal, biliary and pancreatic cancers, as well as on many non-small-cell lung, gastric, uterine and bladder cancers [138–140]. Cantuzumab mertansine was shown to have specific and highly efficacious anti-tumor activity in preclinical xenograft models [25]. It was well-tolerated in Phase I clinical studies of CanAg-expressing solid tumors and showed evidence of biological activity in terms of tumor shrinkage and stable disease [139,141]. The development of this conjugate was stopped because its activity was considered insufficient. The evaluation of this initial antibody–maytansinoid conjugate has been followed by the development of an expanding number of antibody–maytansinoid conjugates that incorporate a growing portfolio of maytansinoid compounds and linker technologies [8,133]. At this time, six of these conjugates are undergoing clinical evaluation in a variety of tumor indications (discussed below), and many more are undergoing preclinical evaluation, including an ADC that targets CD79 on non-Hodgkin lymphoma [142]; an ADC that targets CD22, another B-cell antigen [143]; and SAR566658 (huDS6-DM4), which targets a Muc1 sialoglycotope on solid tumors [144,145]. Antibody–maytansinoid conjugates are discussed in greater detail in a recent review [5].

The auristatins, another group of antimicrotubule agents with potent cell-killing activity, are fully synthetic analogs of the pentapeptide natural product dolastatin 10 [146,147]. Auristatin E and MMAE are 50- to 200-fold more potent than the vinca alkaloids against a diverse panel of tumor cell lines, and ADCs prepared with these compounds were highly effective in preclinical studies [147]. An auristatin derivative with a negatively charged C-terminal phenylalanine residue, MMAF, was shown to have much lower cytotoxic activity as an unconjugated compound than MMAE, probably due to its reduced plasma membrane permeability

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and intracellular access [148]. Nonetheless, antibody–MMAF conjugates were found to be comparable with antibody–MMAE conjugates in their cytotoxic potency towards target antigen-positive cell lines, and over three orders of magnitude more potent than free MMAF, presumably because of the different route of drug delivery by ADCs, traversing the plasma membrane [148]. It is still an open question if the low cytotoxicity of the unconjugated MMAF will result in lower toxic side-effects of its respective ADCs compared with similar MMAE conjugates. Currently, five antibody–auristatin conjugates are undergoing clinical evaluation **(Table 1)**. Pre-clinical studies have been reported for several other antibody–auristatin conjugates, such as ADCs that target the B-cell antigens CD22 [143], CD19 [149,150] and CD79 [151], as well as EphA2, an antigen that is overexpressed in many solid tumors [152,153].

Linkers used in ADCs

The third ADC component is the linker used to covalently attach the drug to the antibody. Several recent reviews have focused on emerging linker technologies [8,9,11,112,154,155]. There are several important considerations regarding the linker component, including: the site of attachment on the antibody; the average number of attachment sites per antibody molecule; the cleavability of the linker, that is, its ability to chemically disintegrate releasing the drug; and the polarity of the linker.

Most drugs have been linked to antibodies at the amino groups of lysine residues or thiol groups of cysteine residues [11,112,156]. New approaches to engineer specific sites of modification are also being evaluated [157]. The relative benefit of the different approaches to the function of the resulting ADC is still largely unknown.

The number of cytotoxic molecules attached to the antibody is an important consideration. A low drug per antibody ratio (DAR) may result in low potency of the ADC, while a too-high degree of antibody modification may adversely affect its affinity towards the target antigen, FcγR, or FcRn, and may also result in aggregation and precipitation of the antibody and/or a decrease in its stability. The optimal DAR is determined empirically for each antibody on the basis of a variety of tests such as the feasibility of conjugate synthesis and solubility of the resulting conjugate, impact on antigen-binding affinity, antigen-specific as well as nontargeted cytotoxicity of the ADC, and its behavior in animal models such as anti-tumor activity, pharmacokinetics and systemic toxicity. Most of the conjugates currently in clinical testing (regardless of which cytotoxic compound, antibody or linker was used) have three to four cytotoxic molecules per antibody molecule. Gemtuzumab ozogamicin, prepared with a strategy designed to minimize aggregation, consists of an approximate 1:1 mixture of unconjugated antibody and conjugated antibody having four to six calicheamicin molecules per antibody [96,119], and inotuzumab ozogamicin has an average calicheamicin:antibody loading of five:seven per antibody [98].

The anti-tumor activity of antibody–auristatin conjugates with different molar ratios of auristatin to antibody was evaluated by Hamblett *et al.*, who separated a sample of an anti-CD30 antibody–MMAE conjugate into fractions having approximately two, four or eight MMAE molecules per antibody [158]. Although the potency of the resulting conjugates was found to correlate with auristatin load when tested *in vitro*, the therapeutic index in xenograft tumor models in mice did not improve with increasing DAR. The discrepancy between the *in vitro* and *in vivo* results was consistent with the observations that higher MMAE load resulted in faster clearance of the conjugate from circulation and increased toxicity in mice [158]. Whether the enhanced clearance is a direct effect of the increased load of MMAE on the antibody, or an effect on the antibody of progressively decreasing the number of inter-chain disulfide bonds that are used in linking MMAE, is unknown. Subsequently, the anti-CD30 antibody was engineered to generate conjugates with defined stoichiometries of either two or four auristatin molecules per antibody [159].

Since a key advantage of antibody-based therapeutics over most chemotherapeutic drugs is their long retention in circulation, an optimal conjugate linkage must be stable during circulation in the patient yet allow for efficient release of the cytotoxic compound in active form upon internalization into the tumor cell. A variety of linkers have been designed to take advantage of differences between the extracellular and intracellular environments to allow release of an active cytotoxic molecule only after antigen-specific, antibody-mediated internalization of the ADC into tumor cells has occurred. Linkers containing disulfide bonds were used to take advantage of the different concentrations of free thiols (free thiols are the main physiological substances

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that can cleave these bonds) in the intracellular and extracellular space. The most abundant intracellular thiol, reduced glutathione, is present in cells in the concentration range of 0.5 to 10 mM [160] compared with a significantly lower concentration of the most abundant low-molecular-weight thiol in circulation, cysteine, approximately 5 µM [161]. In addition, the intracellular enzyme protein disulfide isomerase, or similar enzymes that are also capable of cleaving disulfide bonds [162], may also contribute to the preferential cleavage of disulfide bonds inside cells. Acid-labile linkers were used because of the low pH in the late endosomes and lysosomes, compared with near-neutral pH in circulation and other extracellular environments. Peptide-based linkers were used because such linkers could be cleaved by lysosomal and endosomal proteolytic enzymes. In principle, these linkers may also be cleaved by cytoplasmic proteases as well [163].

Most ADCs currently undergoing clinical evaluation contain linkers that fall into two broad categories: 'cleavable', that is, those that can disintegrate inside the cell, such as hydrazone, disulfide or protease-sensitive peptide linkers; and 'noncleavable', which require proteolytic cleavage of the antibody portion of the ADC for release of the cytotoxic molecule, still attached to the linker and the single remaining amino acid derived from the antibody. For more details the reader can refer to the recent review by Singh and Erickson [112].

Calicheamicin-containing ADCs utilize a hydrazide functionality introduced into calicheamicin γ1 to enable its linkage to an antibody via an acid-hydrolizable bond [164]. In gemtuzumab ozogamicin, *N*-acetyl-γ-calicheamicin dimethyl hydrazide is conjugated to lysines in the antibody using a 4-(4'-acetylphenoxy) butanoic acid linker, which is stable in physiological buffers (pH 7.4), but hydrolyses at the pH of lysosomes (pH ~4) [164]. Gemtuzumab ozogamicin was found to be reasonably stable in blood in animal studies [95,165] and in acute myeloid leukaemia patients [166]. In mouse circulation, the amount of calicheamicin conjugated to the hP67.6 decreased twofold in approximately a 2-day period, and the presence of a CD33-expressing xenograft did not influence the rate of this apparent decomposition, although the method used in the study could not distinguish between cleavage of calicheamicin on all the conjugate molecules and the generation of a fraction of unconjugated antibody, and between conjugate decomposition and preferential clearance of a fraction of the conjugate with a higher DAR [165].

Antibody–maytansinoid conjugates currently in clinical testing utilize either cleavable disulfide or noncleavable thioether linkages. Both are formed from a heterobifunctional crosslinking reagent that reacts with a lysine residue on an antibody, and then maytansine derivatives containing thiol groups are reacted with the other end of the heterobifunctional reagent [110,133,167]. This method of conjugation results in a random distribution of the maytansinoid molecules randomly linked to a small subset of approximately 40 different sites [156]. Newer thiol-containing maytansinoids have been synthesized with one or more methyl groups (substituting hydrogen) on the carbon atoms adjacent to the disulfide linkage, to create linkers having varying degrees of steric hindrance around the disulfide bond [8,133]. These hindering modifications were designed to minimize cleavage of the conjugates during circulation in the blood, without interfering with efficient release of the cytotoxic agents once internalized into tumor cells. Preclinical studies comparing huC242–DM4 (IMGN242), a conjugate utilizing a more-hindered maytansinoid, with huC242–DM1 (cantuzumab mertansine), a conjugate having a less-hindered maytansinoid, revealed decreased plasma clearance for huC242–DM4 and also greater anti-tumor activity in xenograft models [133,168]. Several maytansinoid conjugates currently undergoing clinical testing, IMGN388, SAR3419, BIIB015 and BT062, utilize this more-hindered disulfide linkage. One ADC in clinical testing, T–DM1, utilizes a noncleavable thioether linkage to attach the maytansinoid molecule to the antibody [18].

The intracellular processing of disulfide- or thioether-linked maytansinoid conjugates has been examined [169,170]. Both types of maytansinoid conjugates were found to degrade in lysosomes to yield metabolites consisting of a lysine adduct of the maytansinoid and linker, and this lysosomal processing was required for their cytotoxic activity. The disulfide-containing metabolites were processed further via reduction of the disulfide bond and subsequent *S*-methylation, to produce *S*-methyl-maytansinoids. These lipophilic *S*-methyl-maytansinoid metabolites were highly cytotoxic towards cell lines when added exogenously (comparable to maytansine), whereas the more hydrophilic lysine-containing

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Key Term

P-glycoprotein: A transmembrane ATP-dependent drug transporter. Overexpression of P-glycoprotein often contributes to the emergence of multidrug resistance in tumors following chemotherapy.

maytansinoid metabolites were much less active. These observations may help explain both the phenomenon of target-cell-activated killing of bystander cells and the superior efficacy of disulfide-linked conjugates over noncleavable conjugates seen in some xenograft models [10,24,169,170]. Antigen-positive cells treated with a disulfide-linked conjugate, but not those treated with a thioether-linked conjugate, release the highly cytotoxic *S*-methyl-maytansinoid capable of killing nearby antigen-negative cells [24]. This additional indirect killing of adjacent tumor cells expressing little or no surface antigen may contribute to the activity of disulfide-linked antibody–maytansinoid conjugates against tumors having heterogeneous antigen expression. In addition, such bystander killing could potentially impact tumor-supporting neovasculature and proliferating stromal cells while causing minimal collateral toxicity to adjacent normal cells, as proliferating cells are much more sensitive to the cytotoxic effects of maytansine and its derivatives than are nonproliferating cells [10,24].

To prepare the auristatin conjugates, antibody disulfide bonds are reduced to generate two to eight cysteine residues that are subsequently linked to an auristatin derivative, either maleimide–MMAE or maleimide–MMAF, via a dipeptide linker [147,148,158,171]. One of the dipeptide linkers used, valine–citrulline, was designed to be cleaved by cathepsin B upon intracellular delivery of the conjugates into the lysosomal compartment [147,148]. Intracellular processing of brentuximab vedotin (anti-CD30–MMAE containing this dipeptide linker) was examined in detail [172]. Exposure of CD30-expressing cells to this conjugate resulted in its lysosomal degradation and intracellular release of chemically unmodified MMAE. Efflux of MMAE led to killing of cocultivated CD30-negative cells, a bystander killing effect similar to that observed with disulfide-linked maytansinoid ADCs.

In pre-clinical studies, the valine–citrulline peptide linkage appeared to be quite stable in circulation, with a half-life of auristatin release of 6 days in mice and 9.6 days in cynomolgus macaques [173], similar to that of *N*-succinimidyl 3-[2-pyridyldithio]-propionate (SPDB)-linked maytansinoid conjugates. New auristatin ADC linkers have been generated by replacing the maleimide with a halo-acetamide and have demonstrated improved *in vivo* stability in preclinical studies [154]. In another recent development, anti-CD70–auristatin conjugates have been synthesized utilizing novel dipeptide linkers and new auristatins linked through the C-terminus, and some of these new conjugates showed improved therapeutic windows over the original 1F6-Val-Cit-PABC–MMAF conjugate [155].

A method for site-specific antibody conjugation has been developed to reduce the heterogeneity of ADCs [157]. The 'ThiomAb' technology involves the engineering of reactive cysteine residues into specific sites of the antibody, to allow drug conjugation with defined stoichiometry and without disruption of interchain disulfide bonds [157]. A ThiomAb–drug conjugate comprised of MMAE conjugated to an anti-MUC16 antibody was found to be as efficacious as a conventional MMAE conjugate in mouse xenograft models and was better tolerated in rat and cynomolgus monkeys, possibly due to the absence of species carrying overly high MMAE loads [157].

Multidrug resistance (MDR) is a major obstacle to successful cancer chemotherapy and potentially to ADC therapy as well [27,57,133,174,175]. MDR1 (also known as **P-glycoprotein** or ABCB1), the best-characterized member of the ABC family of transporters, is expressed in a variety of cancers with evidence of increased incidence following chemotherapy, and its expression has been found to correlate with poor responses to chemotherapeutic compounds [176–178]. The majority of drugs used in ADCs are MDR1 substrates [167], and it is expected that improved intracellular retention of ADC's active metabolites would result in increased anti-tumor efficacy in cancers expressing MDR1. Efforts to evaluate cytotoxic compounds as substrates of the various MDR efflux pumps and to develop strategies to circumvent resistance mechanisms have become a priority in ADC research [125,167,179–181]. We have recently developed a new generation of hydrophilic linkers that enable antibody–maytansinoid conjugates to overcome MDR1-mediated resistance. Maytansinoid conjugates prepared with one of these linkers, maleimidyl-based PEG_4Mal, were processed by target cells to a yield a polar cytotoxic metabolite that appears to be a poor MDR substrate. The increased retention of this metabolite correlated with greater anti-mitotic and cytotoxic potency of the conjugates toward MDR1-expressing cells *in vitro* and with increased efficacy against MDR1-positive xenograft tumors [167,181].

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Clinical experience with ADCs

■ ADCs in clinical development

A list of ADCs in clinical development at the time of preparation of this review is presented in **Table 1**.

Gemtuzumab ozogamicin (Mylotarg) was conditionally approved by the FDA (accelerated approval) for treatment of acute myeloid leukemia, but was recently withdrawn from the US market. A confirmatory, postapproval trial was stopped early when no improvement in clinical benefit was observed, combined with serious drug-related toxicities. A second calicheamicin conjugate, inotuzumab ozogamicin (CMC-544), targets CD22 and is undergoing clinical testing against B-cell lymphomas [98,121,182].

Maytansinoid-containing conjugates are undergoing clinical testing in a variety of tumor indications. T–DM1, comprised of Roche's anti-HER2 antibody trastuzumab linked by a noncleavable thioether linkage to the maytansinoid DM1, is in broad clinical development for HER2-positive breast cancer. Results of a Phase II study, showed that T–DM1 has impressive anti-tumor activity in patients who have progressed on prior HER2-targeted therapies (including trastuzumab) [37]. One could speculate that residual intrinsic activity of trastuzumab, insufficient to suppress tumor growth on its own in these patients, may be contributing to the efficacy observed with T–DM1. Alternatively, the efficacy of T–DM1 observed in clinical trials could be a result of the high expression of HER2 and the ability of Herceptin to stimulate endocytosis of the T–DM1 complex with HER2 and deliver the maytansinoid into the cancer cells [183]. The FDA denied accelerated approval of T–DM1 Biologics License Application (BLA) for metastatic breast cancer. The BLA was submitted in July 2010 on the basis of the results of a single-arm, Phase II study, which showed that T–DM1 shrank tumors in one-third of women with advanced HER2-positive breast cancer who had received, on average, seven prior medicines, including two HER2-targeted medicines. In its review of the BLA, the FDA stated the T–DM1 trials did not meet the standard for accelerated approval because all available treatment choices approved for metastatic breast cancer, regardless of HER2 status, had not been exhausted in the study population. Roche stated that it will submit data from the amended Phase III randomized study to the FDA to support a new T–DM1 BLA in mid 2012. This study compares T–DM1 to lapatinib in combination with capecitabine in people with advanced HER2-positive breast cancer whose disease has worsened after receiving initial treatment.

Lorvotuzumab mertansine (IMGN901, BB-10901 or huN901-DM1) [184], which targets CD56 (neural cell adhesion molecule 1) comprises a humanized version of the N901 antibody [78] conjugated to DM1 via a hindered disulfide linker. CD56 is found on a variety of tumor types of hematopoietic and neuroendocrine origin, including multiple myeloma, certain types of leukemia, neuroblastoma, astrocytoma, glioma, most cases of small-cell lung cancer and many cases of ovarian cancer [184–186]. Its normal tissue expression is restricted to natural killer cells and a subset of T cells, as well as in nerve tissue and heart muscle [187]. Encouraging anti-tumor activity of lorvotuzumab mertansine was reported in clinical studies in multiple myeloma and Merkel-cell carcinoma [188,189] (see [5] for more detail). Its clinical evaluation in patients with solid tumors, in particular small-cell lung cancer and Merkel-cell carcinoma, and multiple myeloma is continuing.

IMGN388 is a maytansinoid ADC comprised of a humanized anti-integrin antibody conjugated to DM4 [55]. The target antigen is strongly expressed on a wide variety of solid tumors, including melanomas, sarcomas and a large number of carcinomas, as well as on endothelial cells in the process of forming new blood vessels. The targeting of neovasculature and/or stromal components supporting tumor growth, in addition to the tumor cells themselves, may afford greater efficacy than targeting the tumor cells alone. The initial clinical testing of IMGN388 showed favorable tolerability and evidence of activity [190]. Several other antibody–maytansinoid conjugates (utilizing conjugate technology from ImmunoGen) are in clinical development. SAR3419 (sanofi-aventis) targets the CD19 antigen and is in clinical testing in patients with relapsed or refractory B-cell non-Hodgkin lymphoma [191]. BIIB015 (Biogen Idec), a humanized IgG1 anti-Cripto antibody conjugated to DM4, is in clinical testing for treatment of relapsed or refractory Cripto-positive solid tumors. BT062 (Biotest AG), comprised of a chimerized anti-CD138 antibody and DM4 [192], is in Phase I clinical testing for multiple myeloma [193].

A total of five auristatin conjugates are in clinical evaluation; promising Phase I/II results have been reported for two

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of these conjugates (Table 1). SGN-35, which consists of a CD30-targeting antibody conjugated to MMAE [171,194] is undergoing clinical evaluation in Phase II and Phase III trials in patients with Hodgkin lymphoma and systemic anaplastic large-cell lymphoma. CD30 is a member of the tumor necrosis factor receptor superfamily, a transmembrane glycoprotein receptor that is highly expressed on Hodgkin lymphoma, anaplastic large cell lymphoma, and some other T-cell lymphoproliferative disorders, but is restricted on normal tissues primarily to expression on activated leukocytes [39,158]. Seattle Genetics expects to file a BLA submission to the FDA in the first half of 2011. CR011–vcMMAE is being developed by Celldex Therapeutics for the treatment of metastatic breast cancer and advanced melanoma [100,195,196]. The targeted antigen is the glycoprotein NMB (GPNMB), which is highly expressed in most metastatic melanoma samples [197], and is also expressed in liver carcinomas, squamous-cell lung cancers, breast cancers, soft-tissue tumors and gliomas [11,195,197]. CR011–vcMMAE is comprised of a fully human IgG2 antibody conjugated to MMAE via a valine–citrulline dipeptide linker, and is currently being evaluated in two Phase I/II clinical trials, for unresectable stage III or IV melanoma and for locally advanced or metastatic breast cancer, and in Phase IIb for GPNMB-expressing breast cancers.

Several auristatin conjugates have recently entered clinics. SGN-75, a conjugate of a humanized anti-CD70 mAb with MMAF, is being developed by Seattle Genetics and is currently in a Phase I clinical trial for relapsed or refractory non-Hodgkin lymphoma or metastatic renal cell carcinoma. CD70 is expressed on several types of non-Hodgkin lymphoma, multiple myeloma, renal-cell carcinoma, pancreatic, ovarian and lung cancer, while having limited expression in normal tissues. In preclinical studies, SGN-75 demonstrated potent antitumor activity in models of CD70-positive renal-cell carcinoma and hematologic malignancies. A conjugate of a fully human anti-PSMA mAb with MMAE [14] is being developed by Progenics Pharmaceuticals, and is in a Phase I trial in patients with progressive, metastatic and hormone-refractory prostate cancer [198]. PSMA is expressed on the surface of prostate cancer cells and on the neovasculature of many types of solid tumors, but has limited expression in normal tissues. PSMA expression is increased in high-grade, metastatic and hormone-refractory prostate cancer. Recently ASG–5ME, which consists of an anti-ASG-5 antibody attached via a valine–citrulline dipeptide linker to the auristatin derivative MMAE, has entered Phase I trials for pancreatic cancer patients.

■ Toxicity of ADCs

While ADCs retain antibody-like pharmacokinetics and biodistribution, these molecules ultimately are metabolized and eliminated *in vivo* by the same organ systems that normally metabolize circulating antibodies and/or chemotherapeutic drugs. Unlike 'naked' antibodies, ADCs will have inherent dose-limiting toxicities related to the systemic metabolism of the conjugate and its cytotoxic payload. The nature and degree of such 'nontargeted toxicity' of ADCs vary significantly depending on the cytotoxic payload and its metabolites; generally, one might expect that cytotoxic payloads with increased potency would yield ADCs with decreased MTDs. Similar to naked antibody therapeutics, ADCs also have the potential to cause toxicity in patients if they target a tumor antigen that is also expressed on normal tissues ('targeted toxicity').

Many ADCs tested to date, even those with some normal tissue crossreactivity, have been well-tolerated in patients, causing only minimal toxicities that were manageable and reversible. Examples of maytansinoid ADCs with favorable tolerability reported in clinical testing include lorvotuzumab mertansine [199–201], T–DM1 [17–19], cantuzumab mertansine (huC242–DM1) [139,141], MLN2704 [15] and AVE9633 [202]. However, sometimes significant ADC-related toxicities may only become apparent in the clinic. For example, treatment with gemtuzumab ozogamicin, which received conditional approval (accelerated approval) in 2000 based on single-arm Phase II trials [119,203] was associated with the serious, potentially fatal liver condition veno-occlusive disease [204], and the incidence of this disease increased in the confirmatory Phase III trial [301].

The clinical experience with bivatuzumab mertansine (Boehringer Ingelheim), an anti-CD44v6-directed maytansinoid ADC, provides an example of 'targeted toxicity' of an ADC. This compound showed evidence of efficacy in multiple early-stage clinical trials, but was discontinued due to skin-related adverse effects [205–208]. Since the observed toxicity was most likely due to abundant expression of the CD44v6 antigen in dividing epithelial cell layers of normal skin, as well as its high expression

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on head and neck cancer and other epithelial cancers [209], these clinical results testify to the specificity and potency of antibody-delivered cytotoxic agents.

Soluble antigen in circulation

The presence of target antigen in patient plasma, due to cleavage of the antigen from the surface of tumor cells ('shed' antigen), or from secretion of a soluble form of the antigen, can sometimes influence the pharmacokinetics and the efficacy of an ADC. Soluble antigen can bind the circulating ADC, potentially enhancing clearance of the latter and/or interfering with targeting of the antigen on tumor cells. A correlation between high peripheral blood antigen load and decreased efficacy has been suggested for gemtuzumab ozogamicin in acute myeloid leukaemia [210] and for an anti-MUC1 (hCTM01)–calicheamicin conjugate in ovarian cancer [125,211]. For other antibodies and ADCs, such as trastuzumab and IMGN242, high peripheral blood antigen levels have been correlated with altered pharmacokinetics, but have not been predictive of clinical outcome or associated with decreased efficacy (or did not reach statistical significance with the number of patients treated) [212–214]. The half-life of trastuzumab was reported to be 2 ± 1 days for patients with circulating levels of HER2 extracellular domain ≥500 ng/ml, as compared with a half-life of 9 ± 5 days for patients with <500 ng/ml serum HER2 [212]. However, the potential for soluble antigen to influence the pharmacokinetics of trastuzumab appears not to have precluded delivery of clinical benefit in early testing of T–DM1 for metastatic breast cancer patients, where the average terminal half-life of T–DM1 was reported to be 3.5 days at the MTD of 3.6 mg/kg given every 3 weeks [17–19].

Future perspective

The field of antibody-targeted drug delivery has been fueled by the development of mAb technology in 1975 and subsequent success in identification of many tumor-associated antigens, first with the help of mAbs and later also with a variety of other methods, most notably mRNA-expression profiles. It took many years to gradually develop the current (yet

Executive summary

- Antibody–drug conjugates (ADCs) combine the selectivity, favorable pharmacokinetics, biodistribution and, when present, functional activity of antibodies with the high cytotoxic potency of a small drug.
- Factors that determine the anti-tumor activity of ADCs include antigen expression, the rate of internalization and direction of intracellular trafficking of antigen–ADC complexes, the rate and/or degree of the intracellular release of the active drug and the ability of the antibody to interfere with tumor growth or tumor cell survival.
- A wide variety of tumor-associated cell-surface antigens have been explored as targets for ADCs. Other currently explored targets for ADCs include neovasculature, tumor stroma and cancer stem cells.
- Chimerization and humanization of murine IgGs or use of fully human antibodies minimized or eliminated their immunogenicity in humans, led to their longer retention in human circulation and increased their antibody-dependent cellular cytotoxicity activity.
- At present, there is no conclusive evidence of any *in vivo* correlation between the affinity of an ADC for its target and its efficacy in either preclinical models or in the clinic. A recently developed computational model of tumor targeting predicts that antigen-selective tumor uptake of ADCs depends on the antibody affinity in the range of K_d values of 10^{-8} M to 10^{-7} M or worse, but may not improve with the higher affinity antibodies.
- The desirability of the immune-effector functions in monoclonal antibodies used in ADCs is, at present, unclear.
- The drugs currently used in ADCs in the clinic or in preclinical testing are at least 100-fold more potent than those used in the early conjugates, and can be divided into two categories, those that target DNA and those that target microtubules. Two categories of ADCs based on microtubule-targeting cytotoxic drugs have reached the clinical development stage, those utilizing derivatives of maytansine and those utilizing auristatins.
- Most ADCs currently in clinical development contain linkers that fall into two categories: 'cleavable', that is, those that can disintegrate inside the cell; and 'noncleavable', which require proteolytic cleavage of the antibody portion of the ADC for release of the cytotoxic molecule. Antibody–maytansinoid conjugates utilize either cleavable disulfide or noncleavable thioether linkages. New linkers that enable maytansinoid ADCs to overcome P-glycoprotein-mediated resistance have been developed.
- Many ADCs tested to date, even those with some normal tissue crossreactivity, have been well-tolerated in patients, causing only minimal toxicities that were manageable and reversible.
- The chances for clinical success of ADCs have increased due to the continued improvements in their design: improved understanding of target-selection criteria, advances in antibody engineering, development of more potent cytotoxic drugs, and optimization of antibody–cytotoxic agent links.

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incomplete) knowledge of how to make ADCs that are potent, well-tolerated, nonimmunogenic in the clinic and feasible to develop from both an economic and logistical perspective. The chances for clinical success have increased due to the continued improvements in the design of ADCs: improved understanding of target-selection criteria, advances in antibody engineering, development of more potent cytotoxic drugs, and optimization of antibody–cytotoxic agent linkers. Future clinical studies with ADCs and therapeutic antibodies will give us further insight into the contribution of various factors, both in ADC design (the potency of the drug, the affinity of the antibody, the cleavability, polarity and solubility of the linker, and the roles of antibody-dependent humoral responses) and in the nature of the target tumors (antigen density and heterogeneity of expression, accessibility of the tumor to the ADC, MDR status, soluble antigen levels, FcγR genotype and tumor vascularization). ADCs are poised to provide a new generation of effective oncology treatment options in the near future.

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The authors are employees of ImmunoGen, Inc. and have stock options of ImmunoGen, Inc. (Victor Goldmacher currently does not own stock of ImmunoGen). Both authors are inventors in several patents and patent applications related to their employment at ImmunoGen. The authors have no other relevant affiliations or financial involvement with any other organization or entity with a financial interest in or financial conflict with the subject matter or material discussed in the manuscript. This includes employment consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royaltes.

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▪ of interest
▪▪ of considerable interest

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