Strategies and challenges for the next generation of antibody–drug conjugates

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Abstract | Antibody–drug conjugates (ADCs) are one of the fastest growing classes of oncology therapeutics. After half a century of research, the approvals of brentuximab vedotin (in 2011) and trastuzumab emtansine (in 2013) have paved the way for ongoing clinical trials that are evaluating more than 60 further ADC candidates. The limited success of first-generation ADCs (developed in the early 2000s) informed strategies to bring second-generation ADCs to the market, which have higher levels of cytotoxic drug conjugation, lower levels of naked antibodies and more-stable linkers between the drug and the antibody. Furthermore, lessons learned during the past decade are now being used in the development of third-generation ADCs. In this Review, we discuss strategies to select the best target antigens as well as suitable cytotoxic drugs; the design of optimized linkers; the discovery of bioorthogonal conjugation chemistries; and toxicity issues. The selection and engineering of antibodies for site-specific drug conjugation, which will result in higher homogeneity and increased stability, as well as the quest for new conjugation chemistries and mechanisms of action, are priorities in ADC research.

Antibody–drug conjugates (ADCs) consist of recombinant monoclonal antibodies (mAbs) that are covalently bound to cytotoxic chemicals (known as warheads) via synthetic linkers. Such immunoconjugates combine the antitumour potency of highly cytotoxic small-molecule drugs (300–1,000 Da, with subnanomolar half-maximal inhibitory concentration (IC_{50}) values) with the high selectivity, stability and favourable pharmacokinetic profile of mAbs.

Covalent conjugation of mAbs and drugs using chemical linkers is not a recent concept. In the 1960s, the use of ADCs in animal models was described in the literature, and in the 1980s clinical trials with ADCs based on mouse immunoglobulin G (IgG) molecules were conducted[1]. The first ADC to gain regulatory approval (in 2000) from the US Food and Drug Administration (FDA) was gemtuzumab ozogamicin (developed by Wyeth) (FIG. 1a) — an anti-CD33 mAb that is conjugated to calicheamicin — for the treatment of patients with acute myeloid leukaemia (AML) (BOX 1). However, in a required post-approval study, gemtuzumab ozogamicin in combination with chemotherapy did not demonstrate improved survival and showed a higher rate of fatal toxicity than chemotherapy alone, which led to the voluntary withdrawal of this ADC from the market by Pfizer (which acquired Wyeth) in 2010 (REF. 2). It was never approved in Europe.

This was followed by the approval of two second-generation ADCs: brentuximab vedotin (developed by Seattle Genetics) in 2011 (REFS 3,4) and trastuzumab emtansine (also known as T-DM1 and ado-trastuzumab emtansine; developed by Roche) in 2013 (REF. 5), which target the cancer antigens CD30 (also known TNFRSF8) and human epidermal growth factor receptor 2 (HER2; also known as ERBB2), respectively (FIG. 1b,c). These are currently the only ADCs approved by the FDA and the European Medicines Agency (EMA).

Since 2013, the field has become very dynamic. More than 30 additional ADCs have entered clinical development (all for oncological indications), and there are currently more than 60 ADCs in clinical trials[6]. The features of first-, second- and now third-generation ADCs, which are more homogeneous, stable and potent, as well as the iterative development process ('from bench-top to bedside and back to benchtop'), are illustrated in FIG. 2.

Interestingly, the cytotoxic warheads of two-thirds of the ADCs that are currently in clinical trials are based on only two families of antimitotic agents: auristatins and maytansinoids[7]. This is an indication of how difficult it is to identify cytotoxic molecules that fulfil the numerous — and sometimes contradictory — criteria to be suitable as ADC warheads. These criteria include a high level of

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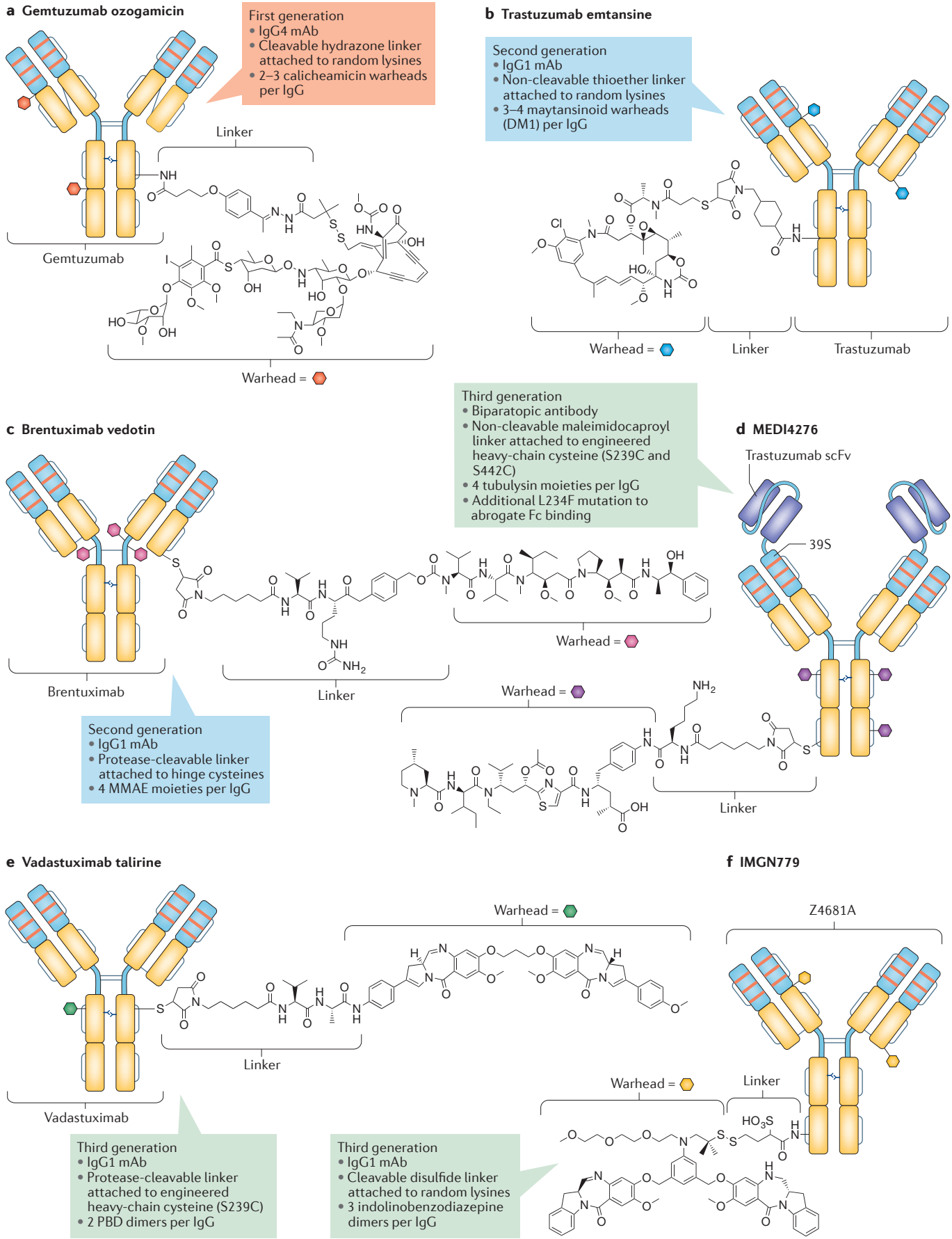


Figure 1 | **Structures of selected first-, second- and third-generation ADCs.** Schematic overview of selected antibody–drug conjugates (ADCs). Cytotoxic warheads are conjugated to human, humanized or chimeric monoclonal antibodies (mAbs) via a range of linker types. **a** | The first-generation ADC gemtuzumab ozogamicin is a conjugate of a humanized immunoglobulin G4 (IgG4) mAb specific for CD33 and 2–3 calicheamicin moieties per IgG, which are attached via cleavable hydrazone linkers to random lysine residues. **b** | The second-generation ADC trastuzumab emtansine consists of conjugate of a humanized IgG1 mAb specific for human epidermal growth factor receptor 2 (HER2) and 3–4 DM1 moieties per IgG, which are attached via non-cleavable thioether linkers to random lysine residues. **c** | The second-generation ADC brentuximab vedotin is a conjugate of a chimeric IgG1 mAb specific for CD30 and 4 monomethyl auristatin E (MMAE) moieties per IgG, which are attached to the hinge region through a protease-cleavable linker. **d** | The third-generation ADC MEDI4276 consists of a biparatopic antibody that targets two non-overlapping epitopes on HER2, conjugated to 4 tubulysin moieties per antibody through a maleimidocaproyl linker. **e** | The third-generation ADC vadastuximab talirine is a conjugate of a humanized IgG1 mAb specific for CD33 and 2 pyrrolobenzodiazepine (PBD) moieties per IgG, which are attached to engineered cysteines (S239C) in the heavy chain through a protease-cleavable linker. **f** | The third-generation ADC IMGN779 is is a conjugate of a humanized IgG1 mAb specific for CD33 and 3 indolinobenzodiazepine moieties per IgG, which are attached to random lysine residues by a cleavable disulfide linker. scFv, single-chain variable fragment.

potency, relative hydrophilicity, a lack of susceptibility to multidrug resistance protein 1 (MDR1; also known as permeability glycoprotein 1 or ABCB1)-mediated efflux, which is a common resistance mechanism for ADCs[8], and a suitable chemical 'handle' for attachment to the antibody moiety[9]. The difficulty of meeting these criteria is also illustrated by the clinical failures of methotrexate-based, doxorubicin-based and vinca alkaloid-based ADCs, which were not potent enough in patients with cancer[10]. To overcome resistance to current drugs, there is a need for new warheads that have different mechanisms of action.

Linker optimization is another key feature of ADC development: linkers must be stable while the ADC is circulating in the blood to limit off-target toxicity, but allow for release of the drug once it is inside the target cancer cells. Several recent studies of pharmacokinetics and metabolism show that the blood stability of second-generation ADCs is limited[11]. Most second-generation ADCs in clinical development have maleimide-type linkers and undergo a so-called deconjugation phenomenon in the serum, which results in off-target cytotoxicity. In particular, this phenomenon has been observed for conjugates to lysine and cysteine residues[11], and efforts have been made to address it in third-generation ADCs, as discussed below.

Another major trend in the field is the engineering of IgG molecules to allow for warhead linkage at defined positions that are suitable for drug conjugation and thus to obtain more homogeneous drug conjugates. This concept was first illustrated by mutating two or four of the eight native IgG1 hinge cysteines to serines[12]. Next, engineered cysteine substitutions at light- and heavy-chain positions that provide reactive thiol groups were reported to yield so-called THIOMAB drug conjugates (TDCs)[13], which have a near-uniform stoichiometry of two cytotoxic molecules per antibody molecule without disruption of interchain disulfide bonds (cysteine bridges). Unfortunately, the first TDCs also had a high deconjugation rate in the circulation, which was investigated in metabolic studies. The highly solvent-accessible site rapidly lost conjugated thiol-reactive linkers in plasma owing to maleimide exchange with reactive thiol groups in albumin, free cysteine or glutathione[14]. The high deconjugation rate was addressed in the next generation of TDCs, as it was found that accessible sites with a positively charged environment promote hydrolysis of the succinimide ring in the linker, thereby preventing this exchange reaction[14].

The instability of ADCs in the circulation may also be overcome by alternative bioconjugation chemistries in combination with optimal antibody engineering[15]. As a result, more than 40 site-specific drug conjugate technologies, which are often combined with alternative conjugation chemistries, have been developed, and at least 10 (publicly disclosed) ADCs that are based on these technologies have reached clinical development (see below). The main objectives of these technologies are to enhance homogeneity and to reduce the drug deconjugation rate in the circulation to limit off-target toxicity, thereby increasing the delivery of highly cytotoxic drugs to tumours while also improving tolerability (FIG. 3).

In this Review, we discuss antigen target selection, warheads used in clinical-stage ADCs, design and optimization of linkers, selection and optimization of antibodies, site-specific and alternative conjugation chemistries, and strategies to enhance potency, including for non-oncology ADCs.

Antigen target selection

A major issue in the development of ADCs for cancer is the identification and validation of adequate antigenic targets for the mAb component. Several factors need to be considered in antigen selection.

First, to reduce off-target toxicity and result in an acceptable therapeutic index for the ADC, target antigens should ideally have high expression levels in tumours and little or no expression in normal tissues, or at least expression limited to a given tissue type[16]. Of the diverse range of novel targets that are currently being (or have been) investigated in clinical trials (TABLES 1–3), some seem to be relatively specific for a given tumour type, but several are being explored in a variety of tumour types (for example, 5T4 (also known as trophoblast glycoprotein) in solid tumours[17], mesothelin in pancreatic and ovarian cancers[18], and CD138 (also known as SYND1) in multiple myeloma and solid tumours[19]). For haematological malignancies, potentially promising targets have been reported, such as surface antigen in leukaemia (SAIL)[20] and CD37 (REF. 21), which seem to be widely expressed in these types of cancer. ADCs can also be designed to target antigens in the tumour microenvironment, insofar as it contains preferentially expressed antigens. For example, an ADC that is directed against the tetraspanin-like protein transmembrane 4 L6 family member 1 (TM4SF1) was shown to bind to both tumour cells and tumour vasculature[22]. Moreover, a trifunctional antibody–cytokine–drug conjugate has

Permeability glycoprotein 1 (PGP; also known as multidrug resistance protein 1 (MDR1), ATP-binding cassette subfamily B member 1 (ABCB1) or CD243). It is an important protein of the cell membrane that pumps many foreign substances out of cells.

Box 1 | **Three generations of CD33 ADCs in acute myeloid leukaemia**

First generation: gemtuzumab ozogamicin

Gemtuzumab ozogamicin (developed by Wyeth) is an anti-CD33 monoclonal antibody (mAb) that is conjugated to calicheamicin, which is an enediyne DNA-binding antibiotic. It was approved by the US Food and Drug Administration (FDA) in 2000, but the required post-approval study showed no improvement in survival and a higher fatal toxicity rate in patients who were treated with gemtuzumab ozogamicin and chemotherapy compared with patients treated with chemotherapy alone, which led to the voluntary withdrawal of the drug in 2010 (REF. 2).

Reasons for failure may include the fact that the payload is susceptible to drug efflux. Moreover, two different gemtuzumab ozogamicin internalization mechanisms have been demonstrated: a CD33-specific mechanism that occurs at lower gemtuzumab ozogamicin concentrations and a CD33-independent mechanism that occurs in cells with endocytic capacity, which may explain some of the toxic side effects of gemtuzumab ozogamicin treatment. These data provide an important mechanistic insight into the clinical observation that gemtuzumab ozogamicin can show effectiveness in patients with $CD33^-$ leukaemias, and that lower concentrations of gemtuzumab ozogamicin may reduce off-target effects by preferentially targeting $CD33^+$ cells. It should also be noted that gemtuzumab ozogamicin is a highly heterogeneous mixture of 50% antibody–drug conjugates (ADCs), comprising 1–8 calicheamicin moieties per IgG molecule, and 50% unconjugated antibody[159,160], which competes with the ADC for cancer cell internalization. Also, the linker is labile towards hydrolysis and the release of free payload may explain the activity towards $CD33^-$ cells.

Nevertheless, gemtuzumab ozogamicin is still considered to be an interesting product, as the use of fractionated lower doses of gemtuzumab ozogamicin allows the safe delivery of higher cumulative doses and substantially improves outcome in patients with acute myeloid leukaemia (AML). The Acute Leukaemia French Association is currently reassessing gemtuzumab ozogamicin as front-line therapy for AML[161].

Second generation: AVE9633

The antibody–maytansinoid derivative AVE9633 (developed by Sanofi) is an example of a second-generation anti-CD33 ADC. It is composed of a thiol-containing maytansine derivative (DM4) that is conjugated via a hindered linker to a humanized IgG1 anti-CD33 mAb (huMy9-6) with a drug-to-antibody ratio (DAR) of about 3.5 (REF. 162).

Three phase I studies of AVE9633 as a single agent have been initiated in patients with relapsed or refractory AML. Unlike gemtuzumab ozogamicin, which can be eliminated from cells via the efflux pumps multidrug resistance protein 1 (MDR1) and multidrug resistance-associated protein 1 (MRP1; also known as ABCC1)[127], MDR1-mediated drug efflux does not seem to be involved in resistance to AVE9633 (REF. 163). In a completed phase I study with AVE9633, both saturation and down-modulation of the CD33 antigen were observed on peripheral blasts at doses of 75 mg per m^2 or higher, but discontinuation of its development was a result of the modest clinical activity of AVE9633 that was observed. Its poor activity was likely to be related to the low density of CD33 that was observed in the majority of patients, which may have been insufficient to deliver and sustain a sufficient intracellular concentration of active DM4. One might also hypothesize that the fraction of cells in G2/M transition in AML is low[164], thus allowing cells to escape the effect of the antimitotic agent. These data suggested that incorporating a more-active cytotoxic agent that is not a substrate for MDR1 and is not cell-cycle-dependent may be better for targeting the CD33 antigen in AML.

Third generation: vadastuximab talirine and IMGN779

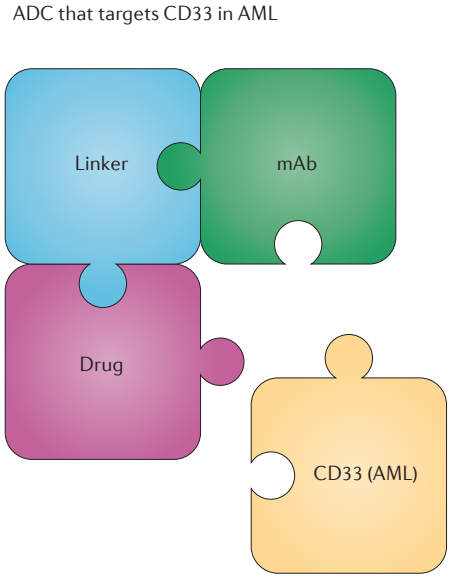
Vadastuximab talirine (also known as SGN-CD33A; developed by Seattle Genetics) contains a novel synthetic pyrrolobenzodiazepine (PBD) dimer (developed by Spirogen) that is structurally related to anthramycin and causes cell death by crosslinking DNA and blocking cell division. In vadastuximab talirine, PBD is coupled to a humanized anti-CD33 IgG1 antibody via a maleimidocaproyl valine-alanine dipeptide linker. To allow site-specific conjugation, the antibody was engineered to contain a cysteine at position 239 on both heavy chains. Vadastuximab talirine demonstrated robust activity in a series of AML animal models, including those in which gemtuzumab ozogamicin had minimal effect[43]. CD33-directed delivery of PBD dimers may overcome transporter-mediated multidrug resistance. In a phase I trial with 17 patients, single-agent treatment with vadastuximab talirine, administered every 3 weeks, was associated with a promising 29% complete response rate[165]. Vadastuximab talirine, in combination with azacitidine or decitabine, is now being investigated in a phase III trial in older patients with newly diagnosed AML.

IMGN779 is based on the indolinobenzodiazepine DGN462 and was selected to balance efficacy (DNA-alkylating properties) and tolerability (without DNA-crosslinking properties, which are associated with significant delayed toxicity)[32]. IMGN779 contains an optimized cleavable disulfide linker, which is designed to enhance bystander killing but without increasing systemic toxicity[48], and has recently entered phase I trials.

been developed that recognizes an alternatively spliced domain of fibronectin that is found in the tumour microenvironment[23].

Second, the target antigen should be present on the cell surface to be accessible to the circulating mAb. Third, it should be an internalizing antigen so that, after binding, the ADC is transported into the cell, where the cytotoxic agent can exert its effects. However, it has been reported that non-internalized ADCs can display significant toxicity in some cases and that ADCs often induce a strong 'bystander effect' (REF. 24), as discussed below.

The target antigen of an ADC does not necessarily need to be a target for which naked mAbs show activity. In the case of HER2 expressed by breast cancer cells, this antigen was first successfully targeted by the approved mAb trastuzumab (developed by Roche) in the late 1990s. The same mAb was then used to develop the approved ADC trastuzumab emtansine, with the maytansine derivative DM1 ($N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)-maytansine) as the cytotoxic drug component. By contrast, the approved ADC brentuximab vedotin is active in various lymphoproliferative



First generation

Gemtuzumab ozogamicin (marketed from 2000–2010 by Wyeth/Pfizer)
- Highly hydrophobic calicheamicin warhead that cleaves DNA
- 50% naked IgG
- Poor CMC characteristics
- High toxicity

Second generation

AVE9633 (developed by Sanofi up to phase I; stopped in 2011)
- Maytansinoid warhead (DM4; developed by Immunogen) that targets tubulin
- Better CMC characteristics

Third generation

Vadastuximab talirine (developed by Seattle Genetics; in phase III since 2016)
- Pyrrolobenzodiazepine dimer warhead (developed by Spirogen) that alkylates DNA
- Site-specific conjugation of warhead to an engineered cysteine through a cleavable dipeptide linker

IMGN779 (developed by Immunogen; in phase I since 2016)
- Indolinobenzodiazepine warhead that alkylates DNA
- Cleavable disulfide linker optimized to enhance bystander killing without increasing systemic toxicity

Figure 2 | **Example of first-, second- and third-generation ADC research and development.** As illustrated for the antigen CD33, which is one target for acute myeloid leukaemia (AML) (BOX 1), antibody–drug conjugate (ADC) research and development is an iterative process with fine-tuning of all of the pieces that must fit (antigen target and biology, antibody, linker, conjugation chemistry and cytotoxic warhead). CMC, chemistry, manufacturing and controls; IgG, immunoglobulin G; mAb, monoclonal antibody.

diseases, even though the anti-CD30 antibody that it is derived from has only shown modest clinical antitumour activity in anaplastic large-cell lymphoma (ALCL)[4]. Similar observations were made with anti-CD138 antibodies[25]. Therefore, the validation of activity of the naked mAb is not a requirement for the development of an active ADC.

For targets that have been validated with naked mAbs, another question is whether extracellular mechanisms of action, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent cell-mediated phagocytosis (ADCP), should be maintained. For example, trastuzumab emtansine has been shown to be ADCC-competent. The future design of ADCs will have to take into account the relative roles of the cytotoxic drug and the antibody in the antitumour activity and toxicity profiles of the overall ADC.

For some indications, there are well-established treatments with naked mAbs. For example, patients with B cell non-Hodgkin lymphoma (B-NHL) are routinely treated with rituximab or other anti-CD20 antibodies that lead to the complete deletion of all B cells. ADCs that are directed against several other targets, including CD19, CD22, CD79b (also known as B cell antigen receptor complex-associated protein β-chain) or others, are in clinical trials in patients with relapsed or refractory B-NHL[26,27].

ADCs are also being developed in indications for which there are no approved naked mAbs. An example of this is triple-negative breast cancer (TNBC), which has a very poor prognosis. An ADC (PF-06647263; developed by Pfizer), which comprises a humanized mAb that is directed against the breast cancer antigen ephrin A4 and is conjugated to calicheamicin, achieved sustained tumour regressions in preclinical experiments with both TNBC and ovarian cancer patient-derived xenografts and is now being investigated in a phase I clinical trial[28]. Glembatumumab vedotin, which is an ADC that targets glycoprotein non-metastatic melanoma protein B (GPNMB), was found to prolong progression-free survival in patients with advanced TNBC[28,29].

As the repertoire of validated target antigens becomes increasingly diversified, it is becoming clear that tumours will be classified not only according to their organ of origin and/or the existence of a targetable intracellular abnormality (such as a mutated kinase), but also according to the surface expression of targetable antigens[30].

Warheads used in clinical-stage ADCs

As shown in TABLES 1–3, ADCs that are currently in clinical trials only use a limited number of families of cytotoxic drugs as warheads. Most of these drugs target DNA (these are cytotoxic for proliferating and non-proliferating cells) or microtubules (these are cytotoxic for proliferating cells), and are optimized for high potency (with an IC_{50} range of approximately 10^{-10}–10^{-12} M). As there are often only a limited number of antigens on the tumour cell surface (ranging from approximately 5,000–10^6 antigens per cell) and the average drug-to-antibody ratio (DAR) of most current clinical-stage ADCs is limited to 3.5–4, the amount of the drug delivered by ADCs into tumour cells is low. This is thought to be the main reason for the clinical failure of ADCs incorporating conventional cytotoxic drugs such as methotrexate, taxoids or anthracyclines.

Many cytotoxic drugs that are used in ADCs are hydrophobic and tend to induce antibody aggregation, which must be avoided to ensure a long shelf life and to limit

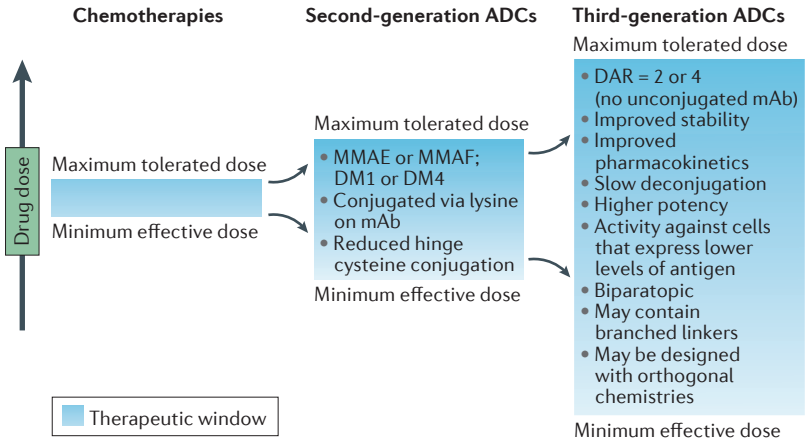


Figure 3 | **Third-generation ADCs are designed to expand the therapeutic window.** Antibody–drug conjugates (ADCs) can increase the efficacy and decrease the toxicity of their payloads in comparison with traditional cytotoxic drugs. The targeted delivery of cytotoxic drugs to cancer cells increases the percentage of drug molecules that reach the tumour, thus lowering the minimum effective dose and increasing the maximum tolerated dose[166]. Nevertheless, the therapeutic window remained narrow for most of the current second-generation ADCs. This is due to off-target toxicity[167], which is linked to retro-Michael deconjugation, competition with unconjugated antibody and aggregation or fast clearance of conjugates with a drug-to-antibody ratio (DAR) of 8. The toxicities that have been reported for active and discontinued drugs as well as the optimization of the antibody, the linker and the conjugation chemistry are important to drive the rational design and improve the therapeutic index of third-generation ADCs[168]. mAb, monoclonal antibody; MMAE, monomethyl auristatin E; MMAF, monomethyl auristatin F.

fast clearance rates and immunogenicity[31]. The drug must also retain its potency when modified for linkage (or made 'linkable'), show acceptable aqueous solubility and be stable in aqueous formulation as a conjugate[32]. Moreover, the drug must be synthetically accessible and obtainable under conditions of good manufacturing practice by a cost-effective process. The recent approval of brentuximab vedotin and trastuzumab emtansine demonstrates that their warheads, an auristatin (Seattle Genetics technology; TABLE 1) and a maytansinoid (ImmunoGen technology; TABLE 2), respectively, fulfil these criteria. The auristatins and maytansinoids, which act by inhibiting tubulin assembly, constitute the majority of the warheads in ADCs that are currently in clinical trials (TABLES 1,2). The remaining warheads are based on pyrrolobenzodiazepines (PBDs), indolinobenzodiazepines, calicheamicins, irinotecan derivatives, duocarmycins, tubulysins and doxorubicin[32] (TABLE 3). Indeed, because of the highly competitive nature of the field, increasing numbers of ADCs are being investigated in early clinical trials without the disclosure of the antigen targets and/or the chemical structures of warheads and linkers[7].

Auristatins. The largest group of ADCs in clinical trials are those based on monomethyl auristatin E (MMAE) and MMAF, with two different linkers, which are licensed by Seattle Genetics to AbbVie, Astellas/Agensys, Bayer, Celldex, Genmab, GlaxoSmithKline (GSK), Pfizer, Progenics, Roche/Genentech and Takeda/Millenium (TABLE 1). Both MMAE and MMAF are synthetic analogues of dolastatin 10, which is a natural antimitotic drug that is extracted from the sea hare *Dolabella auricularia* and is too toxic to be used in its unconjugated form[3]. MMAE and MMAF have been selected among hundreds of candidates for their high potency, water solubility, stability under physiological conditions and suitability for the attachment of stable linkers. Other auristatin analogues are also being investigated by several companies, including Ambrx, Bayer, Pfizer[33], Novartis, Pierre Fabre and Sanofi/Genzyme[34].

Maytansinoids. The second largest class of ADCs in clinical trials are those based on maytansinoids (DM1 and DM4) with four different linkers (TABLE 2), which are licensed by ImmunoGen to Amgen, Bayer, Biotest, CytomX, Novartis, Roche/Genentech, Sanofi and Takeda. DM1 and DM4 are derived from maytansine, which is a natural benzoansamacrolide product isolated from the bark of the African shrub *Maytenus ovatus*[5]. Maytansine binds to the same site on tubulin as the vinca alkaloids, with similar *in vitro* inhibition constants, but is a more-potent cytotoxin. It failed as an anticancer agent in clinical trials because of systemic toxicity[35]. However, it has excellent stability and acceptable solubility in aqueous solutions for use as an ADC building block.

Tubulysins. Tubulysins are antimitotic peptides originally isolated from myxobacteria. Tubulysins inhibit microtubule polymerization during mitosis to induce cell death and may bypass the efflux pumps for DM1 (REF. 36). The warhead AZ13599185 (developed by AstraZeneca/MedImmune) is a variant of tubulysin with low picomolar potency. AZ13599185 is conjugated to four engineered cysteines in the 'biparatopic' ADC MEDI4276 (AstraZeneca/MedImmune; FIG. 1d), which targets two non-overlapping epitopes on HER2. A phase I trial to assess the safety and preliminary efficacy of MEDI4276 is underway in patients who are refractory to or ineligible for current HER2-targeted therapies[36]. MEDI4276 targets two distinct epitopes in the HER2 extracellular domain. It can crosslink the target to form a large cluster on the cell surface, which results in rapid internalization, enhanced lysosomal trafficking and killing of cancer cells, even if they have low expression of HER2.

Calicheamicins. Calicheamicin is a highly potent enediyne antitumour antibiotic originally isolated from the actinomycete *Micromonospora echinospora*. It binds to the minor groove of DNA and cleaves double-stranded DNA in a site-specific manner[37]. *N*-Acetyl-γ-calicheamicin was used by Wyeth (now part of Pfizer) and Celltech (now part of UCB) for gemtuzumab ozogamicin (BOX 1; FIG. 1a) and inotuzumab ozogamicin. Inotuzumab ozogamicin is an anti-CD22 ADC, which obtained fast-track designation in 2015 from the FDA for patients with advanced acute lymphoblastic leukaemia (ALL) (TABLE 3). Calicheamicin is very hydrophobic, and only a few molecules per immunoglobulin can be conjugated before high levels of aggregated protein appear. As noted above, PF-06647263 (Pfizer),

Table 1 | **Clinical pipeline of auristatin-based ADCs (vedotin, mafodotin)**

Name	IgG isotype	Target	Linker–drug*	Developer	Indication (stage)	ClinicalTrials.gov identifier
Brentuximab vedotin (marketed as Adcetris)	IgG1	CD30	vc–MMAE	Seattle Genetics/ Takeda	ALCL and Hodgkin lymphoma (entered market in 2011)	–
Glembatumumab vedotin (also known as CDX-011)	IgG2	GPNMB	vc–MMAE	Celldex	Melanoma, osteosarcoma and TNBC (pivotal phase II)	NCT01997333
Depatuxizumab mafodotin (also known as ABT-414)	IgG1	EGFRvIII	mc–MMAF	AbbVie	Glioblastoma and solid tumours (phase II)	NCT02573324
PSMA ADC	IgG1	PSMA	vc–MMAE	Progenics/Seattle Genetics	Prostate cancer (phase II)	NCT01695044
Polatuzumab vedotin (also known as RG7596 or DCDS4501A)	IgG1	CD79b	vc–MMAE	Genentech/ Roche	NHL (phase II)	NCT02257567
Denintuzumab mafodotin (also known as SGN-CD19A)	IgG1	CD19	mc–MMAF	Seattle Genetics	B-NHL (phase II)	NCT01786096
AGS-16C3F	IgG2	ENPP3	mc–MMAF	Agensys/Astellas	RCC (phase II)	NCT01672775
CDX-014	IgG1	TIM1	vc–MMAE	Celldex	RCC (phase I/II)	NCT02837991
RG7841 (also known as DLYE5953A)	IgG1	LY6E	vc–MMAE	Genentech/ Roche	$HER2^-$ breast cancer and NSCLC (phase I)	NCT02092792
RG7882 (also known as DMUC406A)	IgG1	Undisclosed	vc–MMAE	Genentech/ Roche	Ovarian and pancreatic cancers (phase I)	NCT02146313
RG7986 (also known as DCDS0780A)	IgG1	Undisclosed	vc–MMAE	Genentech/ Roche	NHL (phase I)	NCT02453087
SGN-LIV1A	IgG1	LIV1	vc–MMAE	Seattle Genetics	Breast cancer (phase I)	NCT01969643
Enfortumab vedotin (also known as ASG-22ME)	IgG1	Nectin 4	vc–MMAE	Agensys/Astellas	Solid and urothelial tumours (phase I)	NCT02091999
ASG-15ME	IgG2	SLITRK6	vc–MMAE	Agensys/Astellas	Metastatic urothelial cancer (phase I)	NCT01963052
AGS67E	IgG2	CD37	vc–MMAE	Agensys/Astellas	NHL (phase I)	NCT02175433
Telisotuzumab vedotin (also known as ABBV-399)	Engineered IgG1	HGFR (also known as cMet)	vc–MMAE	AbbVie/Pierre Fabre	Advanced solid tumours (phase I)	NCT02099058
ABBV-838	IgG1	SLAMF7 (also known as CS1)	vc–MMAE	AbbVie	Multiple myeloma (phase I)	NCT02462525
ABBV-221	IgG1	EGFR	vc–MMAE	AbbVie	Solid tumours (phase I)	NCT02365662
ABBV-085	IgG1	Undisclosed	vc–MMAE	AbbVie	Solid tumours (phase I)	NCT02565758
GSK-2857916	Engineered afucosylated IgG1	BCMA	mc–MMAF	GSK	Multiple myeloma and haematological malignancies (phase I)	NCT02064387
Tisotumab vedotin (also known as HuMax-TF-ADC)	IgG1	Tissue factor (also known as CD142)	vc–MMAE	Genmab	Multiple solid tumours (phase I)	NCT02001623

Table 1 (cont.) | **Clinical pipeline of auristatin-based ADCs (vedotin, mafodotin)**

Name	IgG isotype	Target	Linker–drug*	Developer	Indication (stage)	ClinicalTrials.gov identifier
HuMax-Axl-ADC	IgG1	AXL	vc–MMAE	Genmab	Multiple solid tumours (phase I)	NCT02988817
Pinatuzumab vedotin (also known as RG7593 or DCDT2980S)	IgG1	CD22	vc–MMAE	Genentech/ Roche	NHL (phase II; stopped)	NCT01691898
Lifastuzumab vedotin (also known as RG7599 or DNIB0600A)	IgG1	NaPi2B	vc–MMAE	Genentech/ Roche	NSCLC and ovarian cancer (phase II; stopped)	NCT01991210
Indusatumab vedotin (also known as MLN-0264 or TAK-264)	IgG1	GCC	vc–MMAE	Millenium/Takeda	Gastrointestinal malignancies (phase II; stopped)	NCT02202785
Vandortuzumab vedotin (also known as RG7450 or DSTP3086S)	IgG1	STEAP1	vc–MMAE	Genentech/ Roche	Prostate cancer (phase I; stopped)	NCT01283373
Sofituzumab vedotin (also known as RG7458 or DMUC5754A)	IgG1	MUC16	vc–MMAE	Genentech/ Roche	Ovarian cancer (phase I; stopped)	NCT01335958
RG7600 (also known as DMOT4039A)	IgG1	Mesothelin	vc–MMAE	Genentech/ Roche	Ovarian and pancreatic cancers (phase I; stopped)	NCT01469793
RG7636 (also known as DEDN6526A)	IgG1	ETBR	vc–MMAE	Genentech/ Roche	Melanoma (phase I; stopped)	NCT01522664
Vorsetuzumab mafodotin (also known as SGN-75)	IgG1	CD70	mc–MMAF	Seattle Genetics	NHL and RCC (phase I; stopped)	NCT01677390
MEDI547	IgG1	EphA2	mc–MMAF	MedImmune	Solid tumours (phase I; stopped)	NCT00796055
PF-06263507 (also known as ADC 5T4)	IgG1	5T4	mc–MMAF	Oxford Biotech/ Pfizer	Solid tumours (phase I; stopped)	NCT01891669

ALCL, anaplastic large-cell lymphoma; BCMA, B cell maturation antigen; B-NHL, B cell non-Hodgkin lymphoma; EGFRvIII, epidermal growth factor receptor variant III; ENPP3, ectonucleotide pyrophosphatase/phosphodiesterase family member 3; EphA2, ephrin type A receptor 2; ETBR, endothelin B receptor; GCC, guanylyl cyclase C; GPNMB, glycoprotein NMB; GSK, GlaxoSmithKline; HER2, human epidermal growth factor receptor 2; HGFR, hepatocyte growth factor receptor; Ig, immunoglobulin; LY6E, lymphocyte antigen 6E; MUC16, mucin 16; NaPi2B, sodium-dependent phosphate transport protein 2B; NHL, non-Hodgkin lymphoma; NSCLC, non-small-cell lung cancer; PSMA, prostate-specific membrane antigen; RCC, renal cell carcinoma; SLAMF7, signalling lymphocytic activation molecule family member 7; SLITRK6, SLIT and NTRK-like protein 6; STEAP1, six-transmembrane epithelial antigen of the prostate 1; TIM1, T cell immunoglobulin mucin receptor 1; TNBC, triple-negative breast cancer. *The conjugates vc–MMAE and mc–MMAF denote valine-citrulline linked to monomethyl auristatin E (MMAE) and maleimidocaproic acid linked to monomethyl auristatin F (MMAF), respectively; MMAE and MMAF are licensed by Seattle Genetics to companies that include AbbVie, Astellas/Agensys, Bayer, Celldex, Genmab, GSK, Pfizer, Progenics, Roche/Genentech and Takeda/Millenium.

a calicheamicin-containing ADC that is directed against ephrin A4, has recently entered phase I trials in TNBC. It has optimized chemistry, manufacturing and controls (CMC) properties, with an average DAR of 4 and does not contain naked (unconjugated) antibodies[28].

Duocarmycins. Duocarmycins are DNA minor groove-alkylating agents. BMS-936561 (also known as MDX-1203; developed by Medarex, now Bristol-Myers Squibb (BMS)), which is a conjugate of a human anti-CD70 antibody (MDX-1115) and a duocarmycin analogue (MED-2460), was investigated in a phase I clinical trial in patients with advanced clear-cell renal cell carcinomas (RCCs) and B-NHL and was shown to be well tolerated at doses of up to 8 mg per kg (REF. 38) (TABLE 3).

Other duocarmycin analogues have been developed by Syntarga (now Synthon); these were conjugated to trastuzumab, and the resultant ADCs are being investigated as 'biobetter' versions of trastuzumab emtansine. Synthon has initiated treatment of the first patients in a phase I trial of trastuzumab duocarmazine (also known as SYD985), which is a HER2-targeting ADC that is based on trastuzumab attached to the duocarmycin prodrug seco-DUBA with a new cleavable linker[39]. Trastuzumab duocarmazine has demonstrated antitumour activity in preclinical breast and gastric cancer models that have low expression levels of HER2 (REF. 40).

Benzodiazepines. PBDs are based on naturally occurring antitumour antibiotics that bind to the DNA minor groove in a sequence-specific manner. PBDs are being

developed by Spirogen (now part of AstraZeneca) and have been licensed to several companies, such as Seattle Genetics, Roche/Genentech, Stemcentrx (now part of AbbVie), ADC Therapeutics, Kolltan Pharmaceuticals and Mitsubishi Tanabe Pharma. The dimerization of two PBD units that use different tethers to yield symmetrical and non-symmetrical dimers allows the resulting compound to crosslink DNA by binding to the N2 position of guanine on opposing strands of DNA[41]. PBD dimers have picomolar activity against many human tumour cell lines. They are generally not substrates for MDR1 and thus retain activity in MDR1$^+$ tumours and in tumours that are refractory to gemtuzumab ozogamicin treatment[42]. Since 2013, at least ten ADCs that are based on Spirogen's PBD dimer warheads have entered clinical trials, which makes them the third most prominent class of payloads after auristatins and maytansinoids (TABLE 3).

Five PBD-based ADCs — namely, vadastuximab talirine (also known as SGN-CD33A; FIG. 1e), SGN-CD70A, SGN-CD19B, SGN-CD123A and SGN-352A — are currently being investigated by Seattle Genetics in AML (phase III)[43,44], RCC (phase I)[44], B-NHL (phase I), AML (phase I) and multiple myeloma (phase I), respectively. All of these, which are the first publicly disclosed site-specific ADCs that have reached clinical trials, are homogeneous third-generation ADCs that are based on engineered cysteine mAbs (EC-mAbs) and are directed against CD33, CD70, CD19, CD123 (also known as IL-3Rα) and CD352, respectively. They are conjugated via a protease-cleavable valine-alanine linker that is connected to an aniline on the SGD1882 PBD (with an average DAR of 2).

Several other PBD-based ADCs are currently being investigated in clinical trials by AbbVie (Stemcentrx). These include rovalpituzumab tesirine (Rova-T; also known as SC16LD6.5), which is a biomarker-specific ADC that targets the cancer stem cell surface antigen Delta-like protein 3 (DLL3). It contains the SG3199 PBD, which is randomly conjugated to a native interchain cysteine (with an average DAR of 2) via a cleavable (valine-alanine) maleimide-type linker that contains a polyethylene glycol spacer (PEG8)[45]. Rova-T is currently being investigated in a phase III trial in patients with small-cell lung cancer (SCLC)[46]. SC-002 and SC-003, two other PBD-based ADCs being developed by AbbVie (Stemcentrx), are in phase I trials (in patients with SCLC and ovarian cancer, respectively) and are directed against undisclosed targets.

In addition, ADC Therapeutics are currently testing the PBD-conjugated ADCs ADCT-301 (REF. 47), which is based on an anti-CD25 antibody (HuMax-TAC; developed by Genmab) and ADCT-402, which is directed against CD19, in phase I trials in patients with NHL and B-ALL, respectively. Both are based on the PBD SG3249.

ImmunoGen is developing its own family of benzodiazepines that are based on an indolinobenzodiazepine pseudodimer backbone[48]. These compounds (termed IGNs) have been reported to be more potent than SJG-136 (developed by Spirogen), which is a PBD that has been evaluated alone as a cytotoxic drug in phase II trials for solid tumours and haematological malignancies[32]. Studies to optimize IGNs found that modifying the diimine form of the IGNs to a monoimine form resulted in compounds that alkylate only one strand of the target DNA rather than both (which leads to DNA crosslinking), and that retain the potency of the diimine IGNs without their off-target toxicity[48]. In addition, potency and bystander killing can be enhanced by using cleavable linkers. IMGN779 (FIG. 1f), which is based on the indolinobenzodiazepine DGN462 conjugated to a CD33-targeting mAb, was developed to balance efficacy (DNA-alkylating properties) and tolerability — that is, without the DNA-crosslinking properties that are associated with delayed systemic toxicity[32] — and is currently in phase I trials in patients with AML.

Camptothecin analogues. SN-38 and DX-8951f (also known as exatecan mesylate) are two camptothecin analogues that are used as warheads in clinical-stage ADCs being developed by Immunomedics and Daiichi Sankyo, respectively.

SN-38, the active metabolite of the anticancer prodrug irinotecan, acts via inhibition of DNA topoisomerase 1 (TOP1). SN-38 is approximately three orders of magnitude more potent than irinotecan and cannot be given directly to patients because of its toxicity and poor solubility. The antibody–SN-38 conjugates labetuzumab govitecan (also known as IMMU-130) and sacituzumab govitecan (also known as IMMU-132; formerly known as isactuzumab govitecan), which have both been developed by Immunomedics, are soluble in water and are designed with a near-homogeneous DAR of 8 (REF. 49). Labetuzumab govitecan targets carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) and sacituzumab govitecan targets TROP2 (also known as TACSTD2), which is a cell-surface glycoprotein that is reported to be overexpressed in various types of adenocarcinomas[50,51]. Labetuzumab govitecan is in phase II trials, and sacituzumab govitecan has recently entered phase III trials. Sacituzumab govitecan has received FDA breakthrough therapy designation for the treatment of patients with TNBC who have failed at least two previous therapies for metastatic disease.

DX-8951f is a water-soluble camptothecin analogue that exhibits stronger TOP1 inhibitory activity and antitumour activity than the other camptothecin analogues and is effective against MDR1-mediated multidrug-resistant cells[52]. DS-8201a (developed by Daiichi Sankyo) is a HER2-targeting ADC that is composed of an anti-HER2 mAb coupled to a derivative of DX-8951f by a maleimide–Gly-Gly-Phe-Gly peptide linker[53]. It is currently being investigated in a phase I study in patients with advanced solid malignant tumours.

Doxorubicin. Doxorubicin is an actinomycete-derived antimitotic anticancer agent that is routinely used in the clinic. BMS-182248 (also known as SGN-15; developed by BMS and Seattle Genetics), an ADC in which doxorubicin is conjugated to the mAb BR96, is targeted at Lewis Y antigen, which is expressed on a range of tumours, including squamous cell lung carcinoma, lung adenocarcinoma, ovarian carcinoma and colorectal adenocarcinoma. BMS-182248 was the first doxorubicin-based ADC to

Table 2 | **Clinical pipeline of maytansinoid-based ADCs (emtansine, mertansine, ravtansine, soravtansine)**

Name	IgG isotype	Target	Linker–drug*	Developer	Indication (stage)	ClinicalTrials.gov identifier
Trastuzumab emtansine (marketed as Kadcyla; also known as T-DM1)	IgG1	HER2	SMCC–DM1	Genentech/ Roche	HER2+ metastatic breast cancer (entered market in 2013)	–
Mirvetuximab soravtansine (also known as IMGN853)	IgG1	FOLR1	sulfo-SPDB–DM4	ImmunoGen	Advanced epithelial ovarian cancer (phase III)	NCT02631876
Coltuximab ravtansine (also known as SAR3419)	IgG1	CD19	SPDB–DM4	ImmunoGen	DLBCL (phase II)	NCT01472887
Naratuximab emtansine (also known as IMGN529)	IgG1	CD37	SMCC–DM1	ImmunoGen	NHL (phase II)	NCT01534715
Indatuximab ravtansine (also known as BT-062)	IgG4	CD138	SPDB–DM4	Biotest	Multiple myeloma (phase II)	NCT01638936
Anetumab ravtansine (also known as BAY 94–9343)	IgG1	Mesothelin	SPDB–DM4	Bayer HealthCare	Mesothelin-expressing tumours (phase II)	NCT01439152
SAR408701	IgG1	CEACAM5 (also known as CD66e)	SPDB–DM4	Sanofi	Solid tumours (phase II)	NCT02187848
SAR428926	IgG1	LAMP1	SPDB–DM4	Sanofi	Solid tumours (phase II)	NCT02575781
AMG 224	IgG1	Undisclosed	Undisclosed	Amgen	Relapsed or refractory multiple myeloma (phase I)	NCT02561962
PCA062	IgG1	Cadherin 3 (also known as P-cadherin)	Undisclosed	Novartis	Head and neck cancer, oesophageal cancer and TNBC (phase I)	NCT02375958
HKT288	IgG1	Cadherin 6	SPDB–DM4	Novartis	EOC and RCC (phase I)	NCT02947152
LY3076226	IgG1	FGFR3	Undisclosed	Eli Lilly	Advanced metastatic cancers (phase I)	NCT02529553
SAR566658	IgG1	CA6	SPDB–DM4	Sanofi	Breast, cervical, lung and ovarian cancers (phase I)	NCT01156870
Lorvotuzumab mertansine (also known as IMGN901)	IgG1	CD56	SPP–DM1	ImmunoGen	MCC, multiple myeloma and ovarian cancer (phase II; stopped)	NCT01237678
Cantuzumab mertansine (also known as SB-408075)	IgG1	CanAg (a novel glycoform of MUC1	SPP–DM1	ImmunoGen	Solid tumours (phase I; stopped)	–
Cantuzumab ravtansine (also known as IMGN242)	IgG1	CanAg	SPDB–DM4	ImmunoGen	Pancreatic cancer (phase I; stopped)	NCT00352131
Laprituximab emtansine (also known as IMGN289)	IgG1	EGFR	SMCC–DM1	ImmunoGen	NSCLC and SCCHN (phase I; stopped)	NCT01963715
IMGN388	IgG1	Integrin αV	SPDB–DM4	ImmunoGen	Solid tumours (phase I; stopped)	NCT00721669
Bivatuzumab mertansine	IgG1	CD44v6	SPP–DM1	Boehringer Ingelheim	SCCHN (phase I; stopped)	–
AVE9633	IgG1	CD33	SPDB–DM4	Sanofi	AML (phase I; stopped)	NCT00543972
BIIB015	IgG1	Cripto 1 growth factor (also known as TDGF1)	SPDB–DM4	Biogen	Solid tumours (phase I; stopped)	NCT00674947
MLN2704	IgG1	PSMA	SPP–DM1	Millenium/ Takeda	Prostatic carcinoma (phase I; stopped)	NCT00070837
AMG 172	IgG1	CD70	SMCC–DM1	Amgen	Renal cancer (phase I; stopped)	NCT01497821
AMG 595	IgG1	EGFRvIII	SMCC–DM1	Amgen	Recurrent gliomas (phase I; stopped)	NCT01475006
LOP 628	IgG1	KIT (also known as CD117)	SMCC–DM1	Novartis	AML and solid tumours (phase I; stopped)	NCT02221505

AML, acute myeloid leukaemia; B-ALL, B cell acute lymphocytic leukaemia; CA6, carbonic anhydrase 6; CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; DLBCL, diffuse large B cell lymphoma; EGFRvIII, epidermal growth factor receptor variant III; EOC, epithelial ovarian cancer; FGFR3, fibroblast growth factor receptor 3; FOLR1, folate receptor 1; HER2, human epidermal growth factor receptor 2; LAMP1, lysosomal-associated membrane glycoprotein 1; MCC, Merkel cell carcinoma; NHL, non-Hodgkin lymphoma; NSCLC, non-small-cell lung cancer; PSMA, prostate-specific membrane antigen; RCC, renal cell carcinoma; SCCHN, squamous cell carcinoma of the head and neck; SMCC, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SPDB, *N*-hydroxysuccinimidyl 4-(2-pyridydithio)butanoate; SPP, *N*-succinimidyl 4-(2-pyridyldithio)pentanoate; sulfo-SPDB, *N*-hydroxysuccinimidyl 4-(2-pyridyldithio)-2-sulfobutanoate; TDGF1, teratocarcinoma-derived growth factor 1; TNBC, triple-negative breast cancer. *DM1 and DM4 are licensed by Immunogen to companies that include Amgen, Bayer, Biotest, CytomX, Novartis, Roche/Genentech, Sanofi and Takeda.

enter clinical trials, reaching phase II trials in patients with non-small-cell lung cancer[54], but although the data were encouraging, Seattle Genetics decided to discontinue its development to focus on advancing its other programmes.

Milatuzumab doxorubicin (also known as IMMU-115; developed by Immunomedics) is an ADC that is directed against CD74, which is an antigen that is associated with haematological tumours, and is currently being investigated in phase II trials in chronic lymphocytic leukaemia (CLL) and NHL[55]. The ADC incorporates a pH-sensitive linker, which allows rapid detachment of the drug once the ADC enters the acidic environment of the tumour cell, and it delivers a high concentration of the intact drug after intracellular release of the drug from the mAb. After internalization, CD74 is shuttled back to the surface, which enables loading of the target cell with further drug molecules.

Non-clinical-stage cytotoxic warheads. In addition to the warheads discussed above, several promising new compounds are in preclinical development. Among these are α-amanitin (an RNA polymerase II inhibitor)[56], cryptophycins (tubulin inhibitors that are an order of magnitude more potent than MMAE and DM1 (REF. 9)), tubulysins[57], new highly potent anthracyclines (including PNU-159682, which is three orders of magnitude more cytotoxic than doxorubicin)[58], rhizoxin[59] (a microtubule inhibitor), and spliceostatins[60] and thailanstatins[61] (both of which are RNA spliceosome inhibitors).

Design and optimization of linkers

Premature release of drugs in the circulation can lead to systemic toxicity and a lower therapeutic index. Effective linker design has to balance the need for good stability during several days in the circulation and efficient cleavage upon delivery into the target cell. Several strategies are being investigated to enhance the solubility and the DAR of ADCs, and to overcome resistance induced by proteins that can transport the chemotherapeutic agent out of the cells, such as MDR1. These strategies include the conditional release (based on cleavable linkers) of the drug in the cytoplasm of the target cell; the enhancement of the bystander effect, which is achieved through nonpolar linker–drug metabolites that are able to cross biomembranes; and the limitation of the bystander effect, which is achieved through charged linker–drug metabolites that do not cross biomembranes.

Cleavable and non-cleavable linkers. Both cleavable and non-cleavable linkers have been used in approved second-generation ADCs and in third-generation ADCs that are currently being investigated in clinical trials[32].

Cleavable linkers include motifs that are either sensitive to lysosomal proteases (such as cathepsin B, which cleaves the valine-citrulline bond in brentuximab vedotin; TABLE 1) or sensitive to an acidic pH (such as hydrazone, which is hydrolysed to cleave the linker in gemtuzumab ozogamicin and inotuzumab ozogamicin; TABLE 2), or they can contain disulfide bridges that can be reduced by glutathione (TABLE 3). The steric hindrance of disulfide bridges can be optimized to limit premature cleavage inside the cell. For example, this was achieved for the linker–drug pair *N*-succinimidyl-4-(2-pyridyldithio) pentanoate linked to DM1 (SPP–DM1), as used in the ADC lorvotuzumab mertansine, and for the linker–drug pair *N*-hydroxysuccinimidyl-4-(2-pyridyldithio) butanoate linked to DM4 (SPDB–DM4), which is used in the ADCs coltuximab ravtansine and anetumab ravtansine. The disulfide linker is initially cleaved to release the thiol compound DM4, which is subsequently *S*-methylated by cellular methyltransferase activity[62].

Acid-cleavable linkers, such as hydrazone, are designed to remain stable at the neutral pH in the blood circulation, but in acidic cellular compartments they undergo hydrolysis and release the cytotoxic drug. However, these linkers have been associated with nonspecific release of the drug in clinical studies[63].

Examples of non-cleavable linkers include the thioether linker succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate linked to DM1 (SMCC–DM1), which is catabolized to lysine-SMCC–DM1, as used in the ADC trastuzumab emtansine (TABLE 2), or maleimidocaproic acid linked to MMAF (mc–MMAF), as used in the ADC depatuxizumab mafodotin, which is catabolized to cysteine-mc–MMAF (TABLE 1). This is consistent with proteolytic degradation of the ADC to release the linker–drug moiety attached to a lysine or cysteine residue of the degraded mAb, respectively[64].

Reducing or enhancing the bystander effect. Solid tumours often express the target antigen in a heterogeneous manner. As a result, ADCs that selectively kill only antigen-positive cells and spare neighbouring antigen-negative cancer cells may be ineffective in eradicating such tumours. Therefore, ADCs may be designed to kill not only antigen-positive cells but also other cells in the vicinity, irrespective of the expression of the target antigen on their surface[24]; this is known as the bystander effect. The charge of the linker–drug derivatives that are released from ADCs determines the bystander potency[65].

For ADCs that incorporate MMAE, such as brentuximab vedotin, cleavage of the ADC releases MMAE, which is neutral and able to cross biomembranes and kill neighbouring epithelial cells[66–68]. By contrast, for ADCs that incorporate MMAF, such as denintuzumab mafodotin, cleavage results in a metabolite with a charged carboxy-terminal phenylalanine residue, which does not cross biomembranes and so is less toxic to bystander cells than MMAE.

Conjugates that are, for example, linked via a reducible disulfide bond also have bystander cytotoxicity, whereas conjugates that are linked via a non-reducible thioether link, such as in trastuzumab emtansine, exhibit no bystander effect. For example, trastuzumab duocarmazine efficiently induced bystander killing *in vitro* in $HER2^-$ cells that were mixed with HER2-expressing cell lines, whereas trastuzumab emtansine did not[40]. In *in vitro* studies under pH conditions that mimic those in tumours, cathepsin B-mediated cleavage showed efficient release of the warhead from trastuzumab duocarmazine but not from trastuzumab emtansine[40]. The bystander killing effect was also recently confirmed for both coltuximab ravtansine,

Table 3 | **ADCs based on other novel warheads that have reached clinical trials**

Name	IgG isotype	Target	Linker–drug	Developer	Indication (stage)	ClinicalTrials.gov identifier
PBDs (talirine, tesirine)						
Vadastuximab talirine (also known as SGN-CD33A)*‡	Engineered IgG1	CD33	va–SGD1882	Seattle Genetics	AML (phase III)	NCT02785900
SGN-CD70A*‡	Engineered IgG1	CD70	va–SGD1882	Seattle Genetics	RCC (phase I)	NCT02216890
SGN-CD19B*‡	Engineered IgG1	CD19	va–SGD1882	Seattle Genetics	Relapsed NHL (phase I)	NCT02702141
SGN-CD123A*‡	Engineered IgG1	CD123	va–SGD1882	Seattle Genetics	AML (phase I)	–
SGN-CD352A*‡	Engineered IgG1	CD352	va–SGD1882	Seattle Genetics	Multiple myeloma (phase I)	NCT02954796
Rovalpituzumab tesirine (Rova-T; also known as SC16LD6.5) ‡	IgG1	DLL3	PEG8-va–SG3199	AbbVie (Stemcentrx)	SCLC (phase III)	NCT03061812
SC-002	Undisclosed	Undisclosed	Undisclosed	AbbVie (Stemcentrx)	SCLC and LCNEC (phase I)	NCT02500914
SC-003	Undisclosed	Undisclosed	PBD	AbbVie (Stemcentrx)	Ovarian cancer (phase I)	NCT02539719
ADCT-301 (also known as HuMax-TAC-PBD)	IgG1	CD25	PEG8-va–SG3199	ADC Therapeutics/ Genmab	Hodgkin lymphoma and NHL (phase I)	NCT02432235
ADCT-402	IgG1	CD19	PEG8-va–SG3199	ADC Therapeutics	B-ALL (phase I)	NCT02669264
MEDI3726 (also known as ADC-401)	IgG1	PSMA	PEG8-va–SG3199	MedImmune	Prostate cancer (phase I)	NCT02991911
Indolinobenzodiazepines						
IMGN779	IgG1	CD33	sulfo-SPDB–DGN462	ImmunoGen	AML (phase I)	NCT02614560
IMGN632*‡	Engineered IgG1	CD123	DGN549 (linker undisclosed)	ImmunoGen	–	–
Calicheamicin (ozogamicin)						
Gemtuzumab ozogamicin (marketed as Mylotarg in Japan)	Engineered IgG4	CD33	Hydrazone–CM1	Pfizer	AML (approved in Japan)	–
Inotuzumab ozogamicin (also known as CMC-544)	Engineered IgG4	CD22	Hydrazone–CM1	Pfizer	ALL and CLL (pre-registration)	–
PF-06647263	IgG1	Ephrin A4	Hydrazone–CM1	Pfizer/AbbVie (Stemcentrx)	TNBC and ovarian cancer (phase I)	NCT02078752
CMD-193	Engineered IgG4	Lewis Y antigen (also known as CD174)	Hydrazone–CM1	Pfizer	Neoplasms (phase I; stopped)	NCT00257881
CMB-401	Engineered IgG4	MUC1 (also known as CD227)	Hydrazone–CM1	Pfizer	Ovarian carcinoma (phase I; stopped)	–
Duocarmycin						
Trastuzumab duocarmazine (also known as SYD985)	IgG1	HER2^{++}	vc–seco-DUBA	Synthon	Breast and gastric cancers (phase I)	NCT02277717
BMS-936561 (also known as MDX-1203)	IgG1	CD70	mb-vc–MGBA	BMS	RCC and NHL (phase I; stopped)	NCT00944905
SN38 (irinotecan prodrug)						
Sacituzumab govitecan (also known as IMMU-132)‖	IgG1	TROP2	CL2A–SN38	Immunomedics (licensed to Seattle Genetics)	TNBC (phase III)	NCT02574455
Labetuzumab govitecan (also known as IMMU-130)‖	IgG1	CEACAM5	CL2A–SN38	Immunomedics	Metastatic CRC (phase II)	NCT01915472
DXd (exatecan derivative)						
DS-8201a‖	IgG1	HER2^{++}	Peptide linker with DX-8951 derivative	Daiichi Sankyo	Solid tumours (phase II)	NCT02564900

Table 3 (cont.) | **ADCs based on other novel warheads that have reached clinical trials**

Name	IgG isotype	Target	Linker–drug	Developer	Indication (stage)	ClinicalTrials.gov identifier
DXd (exatecan derivative) (cont.)						
U3-1402[‖]	IgG1	HER3[+]	Peptide linker with DX-8951 derivative	Daiichi Sankyo	Solid tumours (phase I)	NCT02980341
Doxorubicin						
Milatuzumab doxorubicin (also known as IMMU-110 or hLL1-DOX)	IgG1	CD74	Hydrazone–doxorubicin	Immunomedics	NHL, CLL (phase II; stopped)	NCT01585688
Undisclosed warhead						
BMS-986148	IgG1	Mesothelin	Undisclosed	BMS	Solid tumours (phase I/IIa)	NCT02341625
Auristatin (Remegen)						
RC48-ADC (also known as hertuzumab-vc–MMAE)	IgG1	HER2	vc–MMAE	Remegen	Breast cancer (phase I)	NCT02881190
Novel auristatins (Pfizer technology)						
PF-06647020	IgG1	PTK7	Cleavable vc-based linker with Aur0101	Pfizer/AbbVie (Stemcentrx)	NSCLC, TNBC and ovarian cancers (phase I)	NCT02222922
PF-06650808	IgG1	NOTCH3	Auristatin derivative (linker undisclosed)	Pfizer	Breast cancer (phase I; stopped)	NCT02129205
PF-06664178* (RN927C)	Engineered IgG1	TROP2	vc–PF06380101	Pfizer	NSCLC, breast and ovarian cancers (phase I; stopped)	NCT02122146
Novel auristatins (Bayer technology)						
Lupartumab amadotin (also known as BAY1129980)	IgG1	C4.4A	Auristatin W	Bayer	Lung squamous cell carcinoma (phase I)	NCT02134197
Aprutumab ixadotin (also known as BAY1187982)	IgG1	FGFR2	Auristatin W	Bayer	Solid tumours (phase I; stopped)	NCT02368951
Amberstatin269 (AmbrX technology)						
ARX788*[‡]	Engineered IgG1	HER2	Auroxime	Zhejiang Medicine Co./Ambrx	Breast and gastric cancers (phase I)	NCT02512237
AGS62P1*	Engineered IgG1	FLT3	Auroxime	Agensys/Astellas	AML (phase I)	NCT02864290
Auristatin (Mersana technology)						
XMT-1522[§]	IgG1	HER2	Fleximer polymer linker with auristatin F	Mersana	NSCLC, breast and gastric cancers (phase I)	NCT02952729
Tubulin inhibitor (AbGenomics technology)						
AbGn-107	IgG1	Transferrin receptor protein 1 (also known as CD71) glycotope	Cleavable linker–tubulin inhibitor	AbGenomics	Colorectal, pancreatic and stomach cancers (phase I)	NCT02908451
Tubulysin analogue (MedImmune technology)						
MEDI4276*	Engineered IgG1	HER2	AZ13599185	MedImmune	Solid tumours (phase I)	NCT02576548
Antibody–antibiotic conjugate (AAC)						
DSTA4637S* (also known as RG7861)	Engineered IgG1	*S. aureus*	vc–rifalogue	Genentech/Symphogen	*S. aureus* infection (phase I)	NCT02596399

ADC, antibody–drug conjugate; ALL, acute lymphocytic leukaemia; AML, acute myeloid leukaemia; B-ALL, B cell acute lymphocytic leukaemia; BMS, Bristol-Myers Squibb; BTG, bacterial transglutaminase; CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; CLL, chronic lymphocytic leukaemia; CRC, colorectal cancer; DLL3, Delta-like protein 3; FGFR2, fibroblast growth factor receptor 2; FLT3, Fms-like tyrosine kinase 3; HER, human epidermal growth factor receptor; LCNEC, large-cell neuroendocrine carcinoma; MGBA, minor groove-binding alkylating agent; MMAE, monomethyl auristatin E; MUC1, mucin 1; NHL, non-Hodgkin lymphoma; NSCLC, non-small-cell lung cancer; PBD, pyrrolobenzodiazepine; PEG8, polyethylene glycol 8; PSMA, prostate-specific membrane antigen; PTK7, protein tyrosine kinase 7; RCC, renal cell carcinoma; *S. aureus*, *Staphylococcus aureus*; SCLC, small-cell lung cancer; seco-DUBA, seco-duocarmycin-hydroxy-benzamide-azaindole; sulfo-SPDB, *N*-hydroxysuccinimidyl-4-(2-pyridyldithio)-2-sulfobutanoate; TNBC, triple-negative breast cancer; va, valine-alanine; vc, valine-citrulline. *Site-specific ADCs. [‡]ADCs with a drug–antibody ratio of 2. [§]ADC with a drug–antibody ratio of 15. [‖]ADC with a drug–antibody ratio of 7–8.

which targets CD19 and has been studied in a phase II trial for the treatment of diffuse large B cell lymphoma (DLBCL)[27], and anetumab ravtansine[69], which is directed against mesothelin and is currently in phase I trials. Both of these ADCs have intracellular cleavable disulfide linkers that are catabolized to release *S*-methylated maytansinoids, which are able to cross biomembranes.

Polar linkers to improve solubility and reduce MDR. Cancer cells frequently become resistant to drugs by upregulating the expression of MDR1. Maytansinoid-based ADCs with non-charged or nonpolar linkers have been shown to have lower *in vitro* potency against $MDR1^+$ cells than against $MDR1^-$ cells. MDR1 is known to transport hydrophobic compounds more efficiently than hydrophilic compounds. As a consequence, charged or hydrophilic linkers were developed and the resulting ADCs were shown to produce highly charged or polar metabolites, which led to improved potency against $MDR1^+$ cells. *N*-Hydroxysuccinimidyl-4-(2-pyridyldithio)-2-sulfobutanoate (sulfo-SPDB) and mal-PEG4-*N*-hydroxysuccinimide are examples of polar linkers[32]. Mirvetuximab soravtansine (also known as IMGN853; developed by ImmunoGen), the lead ADC based on sulfo-SPDB, demonstrated notable single-agent activity in patients with folate receptor-α (FRα)-positive platinum-resistant ovarian cancer in a recent phase I trial[70].

Reducing the hydrophobicity of homogeneous ADCs improves pharmacokinetics and the therapeutic index[71]. The *in vitro* potency of ADCs increases as the DAR increases. However, ADC plasma clearance can increase as the DAR increases, which reduces exposure and *in vivo* efficacy[72]. It has been shown that increased ADC hydrophobicity correlates with accelerated ADC clearance, which can be modulated through linker–drug design. This was demonstrated using auristatin-based hydrophilic linker–drug constructs and pegylated ADCs, which resulted in uniform high-DAR ADCs with superior *in vivo* performance[71].

Selection and optimization of antibodies

Improving antibody homogeneity and developability is mandatory for both naked antibodies and ADCs to reduce the attrition rate of drug candidates[73]. In the past decade, several hundred papers have been published on the analytical and structural characterization of mAbs, and the trend has accelerated in the past 2 years[74]. Liquid chromatography, electrophoresis and mass spectrometry are used at all stages of mAb discovery and preclinical and clinical development. These analytical techniques are helpful for the selection of the best antibody-producing clones with suitable glycosylation profiles for full structural characterization of research leads and potential clinical candidates. They are also used for the identification of 'hotspots' on the antibody that may be deleterious for stability as well as for pharmacokinetic and pharmacological properties. Importantly, the early use of mass spectrometry methods in the research and development process also helps to optimize the structure of next-generation mAbs from a pharmaceutical standpoint, allowing the development of candidates with reduced CMC liabilities and better drug-like properties (OptimAbs)[75] and ADCs (OptimADCs)[76].

Chimeric, humanized and human antibodies. During the development of new drugs, the World Health Organization (WHO) assigns a unique International Nonproprietary Name (INN) to each pharmaceutical substance. mAb INNs comprise a '-mab' suffix that is preceded by a substem that broadly indicates the antibody origin and 'human-ness' — for example, 'xi-' (for chimeric mAbs), 'zu-' (for humanized mAbs) or 'u-' (for fully human mAbs) — with the implication that this is related to immunogenicity. However, the WHO recently revised INN definitions for antibodies to be based on amino acid sequence identity. These new definitions lead to inconsistent classification of somatically mutated human antibodies, humanized antibodies and antibodies derived from semi-synthetic or synthetic libraries, and transgenic animals[77,78]. For example, brentuximab and indatuximab are chimeric mAbs as originally defined. However, coltuximab, mirvetuximab and vadastuximab are mAbs that are humanized as originally defined but would be classified as chimeric ('-xi-' substem) under the new WHO definitions. Dialogue between the WHO, the INN Expert Group and key stakeholders such as the Antibody Society (a non-profit organization) is ongoing, with the aim of developing a new INN system for antibodies to avoid confusion and miscommunication between researchers and clinicians who are prescribing antibody-based drugs.

Isotype selection. Most currently approved mAbs are selected from three human IgG isotypes, which are defined by different heavy-chain amino acid sequences (IgG1, IgG2 or IgG4). IgG3 isotypes are not used as therapeutics owing to a significantly faster clearance rate (up to three times faster). Disulfide bonds (16 for IgG1 and IgG4 and 18 for IgG2) and non-covalent interactions maintain the three-dimensional antibody structure. The heavy and light chains are linked by one disulfide bond, and the heavy chains are linked by two (for IgG1 and IgG4) or four (for IgG2) disulfide bonds that are located in a short, highly flexible hinge region. The other 12 disulfide bonds are intramolecular and delimit 6 different globular domains: 1 variable (V_L) and 1 constant domain for the light chains (C_L), as well as 1 variable (V_H) and 3 constant domains for the heavy chains (C_H1, C_H2 and C_H3).

Like unconjugated therapeutic mAbs, the large majority of ADCs (including brentuximab vedotin[79] and trastuzumab emtansine[80]) are based on the IgG1 isotype (chimeric, humanized or human). IgG1 is considered to be easier to develop than IgG2, which has distinct disulfide isomeric structures that are linked to a more-complex hinge region[81], and IgG4, which may form half antibodies (one heavy and one light chain; 75 kDa) and bispecific antibodies *in vivo* when not stabilized by a serine-to-proline mutation in the hinge region[82].

Human IgGs of different isotypes differ in their ability to support secondary immune functions, ADCC and complement-dependent cytotoxicity (CDC). IgG1 can

Table 4 | **Selected site-specific conjugation technologies and associated stabilization chemistries**

Engineering technology (mAb anchor position)	Conjugation chemistry	Technology name	Owner (collaborators)	Refs
Engineered cysteine and selenocysteine*				
Engineered cysteine (heavy chain-A114C and light chain-V2015C (first generation), light chain-V2015C (second generation), and adjacent basic amino acid)	Thiol plus maleimide	THIOMABs	Genentech	13,14
Engineered cysteine (heavy chain-S239C)	Thiol plus self-hydrolyzing maleimide‡	Engineered cysteine mAbs	Seattle Genetics	43,44
Engineered cysteine	Thiol plus maleimide	NA	Pfizer	169
Engineered cysteine (heavy chain-S239C and S442C)	Thiol plus maleimide	NA	MedImmune/AstraZeneca	36
Engineered cysteine (hinge cysteine to serine mutation)	Thiol plus maleimide	ThiofleximAbs	MedImmune/AstraZeneca	170
Engineered cysteine (light chain-Q124C)	Thiol plus maleimide	Actibody	Kyowa Kirin	120
Engineered cysteine	Thiol plus maleimide	SAP	Novartis	91
Engineered cysteine and selenocysteine	Thiol plus phenyloxadiazole sulfone	Selenomabs and Thioselenomabs	Scripps Research Institute	93,171
Unnatural amino acid engineering*				
Engineered pAcPhe; UAG amber stop codon (heavy chain-A114)	Oxime ligation, alkoxyamine-to-keto-group reaction	EuCODE	Ambrx (Agensys, Merck and Co., Pfizer)	95,172
Engineered pAMF	SPAAC, copper-free click chemistry	Xpress CF+	Sutro Biopharma (Celgene, Merck KGaA)	96,173
Engineered N_6-((2-azidoethoxy)carbonyl)-L-lysine	SPAAC, copper-free click chemistry	AzAbs	Allozyne (MedImmune/AstraZeneca, Spirogen)	97
Enzyme-assisted ligation (formylglycine-generating enzyme, transglutaminase and sortase)				
Engineered Lys-Cys-X-Pro-X-Arg tag (various positions) plus FGE treatment	Cysteine oxidized to formylglycine, HIPS ligation	SMARTag	Catalent/Redwood (Eli Lilly, Roche, Sanofi, Takeda)	99
Engineered glutamine [Lys-Lys-Gln-Gly] tag (various positions) plus BTG treatment	Ligation of γ-carboxyamide group from glutamine residues plus primary amines	BTG	Pfizer	100, 174
Glutamine [Lys-Lys-Gln-Gly] tag (heavy chain-Q295) and engineered glutamine tag (heavy chain-N297 plus PGNase F treatment or heavy chain-N297Q) plus BTG treatment	Ligation of γ-carboxyamide group from glutamine residues plus primary amines	TG-ADC	ETH Zurich and Innate Pharma (Sanofi)	102
Lys-Pro-Glu-Thr-Gly tag (C termini of heavy and light chains) plus SrtA treatment	Ligation LPETG plus primary amine of polyglycine motif	SMAC	NBE Therapeutics	103
Glycan remodelling: metabolic engineering, chemical oxidation and glycoengineering				
Glycan metabolic engineering (fucose in glycans linked to N297) and incorporation of 6-thiofucose peracetate plus fucosyltransferase VIII treatment	Maleimide plus 6-thiofucose	NA	Seattle Genetics	107
Fucose periodate oxidation (fucose in glycans linked to N297)	Fucose-specific conjugation of hydrazide	NA	Philogen	108
Glycan engineering (sialic acids in glycans linked to N297), and galactosyl- and sialyltransferase treatments	Periodate oxidation (aldehyde) plus amino-oxy-payload, oxime ligation	NA	Genzyme/Sanofi	109
Glycan engineering (azido-modified sialic acids in glycans linked to N297), and galactosyl- and sialyltransferase treatments	Strain-promoted alkyne–azide cycloaddition, copper-free click chemistry	NA	University of Georgia	110
Galactosyltransferase treatment plus UDP-keto-galactose incorporation	C2-keto-gal oximation	NA	US National Cancer Institute	111
Glycan engineering (GlcNAc in glycans linked to N297), and endoglycosidase and glycosyltransferase treatment and azide tagging	Strain-promoted alkyne–azide cycloaddition, copper-free click chemistry	GlycoConnect, HydraSpace	Synaffix	112
Amino-terminal engineered serine				
Engineered serine (N-terminal light chain)	Site selective aldehyde oxidation plus oxime ligation	NA	MedImmune/AstraZeneca	113
Engineered serine (N-terminal light or heavy chain)	Site selective aldehyde oxidation plus oxime ligation	SeriMabs	ImmunoGen	175

Table 4 (cont.) | **Selected site-specific conjugation technologies and associated stabilization chemistries**

Engineering technology (mAb anchor position)	Conjugation chemistry	Technology name	Owner (collaborators)	Refs
Ligation at the Fab nucleotide-binding sites				
Nucleotide-binding pocket in Fab arms of IgGs	Oxime ligation NH2 plus indole-based 5-difluoro-2,4-dinitrobenzene derivatives	NA	University of California, Davis	114
Cysteine rebridging[§]				
Cysteine chemical rebridging (native hinge interchain cysteine crosslinking)	Thiol plus bis-sulfone	ThioBridge	Abzena/PolyTherics	115
Cysteine chemical rebridging (native hinge interchain cysteine crosslinking)	Thiol plus dibromomaleimide	NGM	University College London/ThioLogics	116
Cysteine chemical rebridging (native hinge interchain cysteine crosslinking)	Thiol plus dibromomaleimide	SNAP	Igenica Biotherapeutics	117
Avoiding or limiting retro-Michael drug deconjugation				
Basic pH-driven succinimide ring-opening (native or engineered cysteine)	Thiol plus maleimide followed by pH 9.2 treatment (45 °C, 48 hours)	Succinimide ring hydrolysis	Pfizer	119
Basic amino group adjacent to the maleimide (native or engineered cysteine)	Thiol plus maleimide	Self-hydrolysing maleimides	Seattle Genetics	89
Ring-opened linker and *N*-substituted succinimide thioethers (native or engineered cysteine)	Thiol plus maleimide	NA	ProLynx	121
Ring-opened linker (native or engineered cysteine)	Thiol plus maleimide	NA	MedImmune/AstraZeneca	122
Ring opening by anion exchange chromatography (engineered cysteine, light chain-Q124C)	Thiol plus maleimide	Actibody, AEX	Kyowa Kirin	120
Maleimide replacement (native or engineered cysteine)	Thiol plus arylpropionitrile	CBTF	Syndivia	123
Self-hydrolysable hydrophilic maleimidomethyl dioxane-based linker (native or engineered cysteine)	Thiol plus maleimide	MTDF	Syndivia	124

AzAbs, azide antibodies; BTG, bacterial transglutaminase; CBTF, sodium 4-((4-(cyanoethynyl)benzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate; EWM, electron-withdrawing maleimides; FGE, formylglycine-generating enzyme; GlcNAc, *N*-acetylglucosamine; HIPS, hydrazino-iso-Pictet–Spengler; MDTF, sodium 4-(maleimidomethyl)-1,3-dioxane-5-carbonyloxy-2,3,5,6-tetrafluorobenzenesulfonate; NA; not available; NAM, *N*-aryl maleimides; NGM, next-generation maleimide; pAcPhe, p-acetylphenylalanine; pAMF, para-azidomethyl-L-phenylalanine; PGNase F, peptide:*N*-glycosidase F; SAP, spatial aggregation propensity; SMAC, sortase-enzyme mediated antibody conjugation; SPAAC, strain-promoted azide–alkyne cycloaddition; SrtA, sortase A; THIOMABs, thio-engineered monoclonal antibodies; UDP, uridine diphosphate. *Vector engineering. [‡]Promotes slower deconjugation. [§]No need for antibody or cell engineering; drug–antibody ratio of 1, 2, 3 or 4.

usually support ADCC and CDC, whereas IgG2 and IgG4 are typically inefficient or limited in their effector functions[83]. Human IgG4 may sometimes support ADCP[84].

An example of an antibody that supports ADCC is trastuzumab. Interestingly, trastuzumab emtansine was reported to retain the mechanisms of action of unconjugated trastuzumab — that is, binding to HER2 and prevention of HER2 shedding, inhibition of the phosphoinositide 3-kinase (PI3K)–AKT signalling pathway and immunoglobulin Fcγ receptor (FcγR)-mediated engagement of immune cells, which results in ADCC linked to the IgG1 isotype[85]. Trastuzumab emtansine also has activity against cell lines and tumours that are resistant to lapatinib[85], which is a small-molecule inhibitor of HER2 and epidermal growth factor receptor (EGFR) pathways. As a result, this ADC is indicated for the treatment of patients with HER2$^+$ metastatic breast cancer who previously received trastuzumab and a taxane (either separately or in combination).

By contrast, some companies consider that ADCC in addition to the cytotoxic warhead may be too toxic. For example, Agensys selected IgG2 isotypes (such as in glembatumumab vedotin and ASG-5ME), and Wyeth and Biotest selected IgG4 isotypes (for example, for gemtuzumab ozogamicin, inotuzumab ozogamicin and indatuximab ravtansine). IgG2 offers the theoretical possibility to conjugate more payloads, because it contains four interchain disulfide bridges compared with two in IgG1 and IgG4 (REF. 86). Nevertheless, IgG2 and IgG4 hinges are more difficult to reduce than IgG1 and, as a result, cysteine-based ADCs are more difficult to produce. So, to attenuate the effector function of IgG1, Fc-mutated variants of IgG1 isotypes (E233P, L234V and/or L235A) have been designed (so-called Fc-silent antibodies)[83]. The ADC MEDI4276, which is in phase I trials, has three mutations in its Fc domain (E234F, S239C and S442C) (FIG. 1d; TABLE 1) to reduce FcγR binding and to minimize thrombocytopenia, as seen with trastuzumab emtansine. Conversely, the first glycoengineered afucosylated antagonistic ADC to enter clinical trials, J6M0-mc–MMAF — which targets B cell maturation antigen (BCMA; also known as TNFRSF17)[87] — was designed to have enhanced ADCC activity by increasing the binding affinity of its Fc domain for FcγRIIIa, which is expressed on effector cells[88].

Novel conjugation strategies

Second-generation ADCs are all controlled mixtures of different drug-loaded species (from 0 to 8 drug molecules per antibody) and have a typical average DAR of 3.5 (for example, trastuzumab emtansine; FIG. 1b) or 4 (for

example, brentuximab vedotin; FIG. 1c). Unconjugated species are generally not active and are in competition with the drug-loaded species for binding to the antigen. This does not seem to be problematic for brentuximab vedotin and trastuzumab emtansine, in which around 5% of the antibody is unconjugated, but is more problematic for gemtuzumab ozogamicin, in which 50% of the antibody is unconjugated. In addition, species that have a DAR of more than 4 have been shown to lead to lower tolerability, higher plasma clearance rates and decreased efficacy *in vivo*[72].

Most of the ADCs that are currently on the market and in clinical trials share common structural features, such as a thiosuccinimide linkage, which is formed through the reaction of thiols and alkyl maleimides. This type of chemistry is widely used because the reaction of maleimides and thiols is very rapid under physiological conditions and is quantitative (without a large excess of both original species). However, thiosuccinimide formation is slowly reversible under physiological conditions. Nearly two-thirds of the ADCs in clinical development, including the two approved ADCs, contain alkyl maleimides that can result in measurable drug loss during prolonged circulation. The pharmacological consequences of this maleimide elimination from ADCs (via a retro-Michael reaction; discussed further below) include diminished antitumour activity due to reduced exposure to the antibody-conjugated form of the drug and greater toxicity, which arises from the non-targeted release of the drug and the linker. This has been described both for cysteine-linked ADCs[89] and lysine-linked ADCs via the thioether linker SMCC[11]. These issues can be solved by site-specific conjugation and alternative conjugation chemistries[15,90], as described below.

Engineered cysteines. ADCs with additional cysteines engineered into different sites of the IgG that have different solvent accessibility and local charge, as developed by companies such as Genentech[13], Seattle Genetics[44], Novartis[91], MedImmune[92], Kirin and Pfizer, have been successfully investigated in preclinical studies of ADCs (TABLE 4). All of these ADCs show a uniform stoichiometry with DARs of near 2 or 4, depending on how many cysteines were engineered per antibody molecule (FIG. 1d,e); in experiments with rats and cynomolgus monkeys, these ADCs were better tolerated than conventional ADCs[14]. However, the highly solvent-accessible site rapidly lost conjugated thiol-reactive linkers in the plasma owing to maleimide exchange with reactive thiols that are present in albumin, free cysteine or glutathione[14]. This was shown for the first generation of TDCs and improved in the second-generation TDCs (through a V205C mutation in the light chain), based on the observation that a partially accessible site within a positively charged environment promoted hydrolysis of the succinimide ring in the linker, thereby preventing the maleimide exchange reaction[14].

Vadastuximab talirine comprises a humanized anti-CD33 mAb with engineered cysteines in the heavy chain (S239C), which is conjugated to a PBD dimer via a protease-cleavable linker (valine-alanine), with a near homogeneous average DAR of 2 (REF. 43) (FIG. 1e). Vadastuximab talirine was the first publicly disclosed ADC with site-specific conjugation that reached clinical trials. Seattle Genetics has also produced SGN-CD70A, and more recently SGN-C19B, based on the same technology, both of which have entered clinical trials (see above).

The incorporation of C-terminal selenocysteines provides an alternative means to introduce site-specific conjugation sites into mAbs. Selenocysteine can be co-translationally inserted into proteins by recoding the stop codon UGA from termination to selenocysteine insertion. The nucleophilic selenol group of selenocysteine displays a chemical reactivity that allows regiospecific conjugation in the presence of the other natural amino acids[93].

Unnatural amino acid engineering. Genetically encoded unnatural amino acids (UAAs) with bioorthogonal chemical reactivity (that is, allowing a chemical reaction that can occur inside living systems without interfering with native biochemical processes) can be used to create site-specific ADCs. This is achieved by engineering tRNA synthetases to recognize UAAs that are supplemented to the culture media and charge-engineered tRNAs, thereby allowing for the genetic coding of the UAA[94].

For example, auristatins have been conjugated to 2 UAA sites engineered into IgG molecules without conjugation to any of the 20 canonical amino acid side chains[95]. Antibodies that contain para-acetylphenylalanine (pAcPhe) were expressed in mammalian cells, with yields comparable to the corresponding wild-type proteins. An oxime ligation reaction that was optimized to afford high coupling efficiencies resulted in conjugation of auristatin molecules to pAcPhe. The reaction is chemically defined, efficient and scalable, and the oxime linkage is highly stable, which should reduce toxicity related to the release of the free toxin *in vivo*.

A cell-free protein expression system for the production of ADCs through site-specific incorporation of the optimized UAA para-azidomethyl-L-phenylalanine (pAMF) has also been developed[96], which facilitates near-complete conjugation of dibenzocyclooctyne – PEG–MMAF using copper-free click chemistry (a strain-promoted azide–alkyne cycloaddition (SPAAC) reaction).

Azide antibodies (AzAbs; developed by Allozyme) contain site-specific engineered azide handles that are designed for bioconjugation[97]. Each azide handle is incorporated into an UAA that is engineered into the target mAb by placing a specific stop codon at the desired sites. A key component of the technique is the difference in reactivity, which occurs only at the site of the azide, and there is no detectable reactivity at any other positions on the antibody. Stability of the linker is one of the most important considerations for ADCs given that poor linker stability can lead to toxicity issues in the clinic, as was the case for first-generation ADCs such as gemtuzumab ozogamicin. Upon completion of click bioconjugation to the AzAb, the aromatically stabilized linker offers approximately tenfold higher stability than ADC linkers formed through maleimide or thioester chemistries.

Strain-promoted azide–alkyne cycloaddition (SPAAC). A bioorthogonal non-toxic ligation reaction that allows site-specific conjugation.

Enzyme-assisted ligation. Site-specific chemical protein conjugation can also be achieved by using genetically encoded amino acid tags that are inserted in the mAb sequence, which are specifically recognized by enzymes such as formylglycine-generating enzyme (FGE; also known as SUMF1), transglutaminases or sortases.

SMARTag (developed by Redwood, now part of Catalent) is a technology that uses FGE, which — upon recognition of a specific amino acid sequence tag (Cys-X-Pro-X-Arg) that can be inserted into an antibody in a site-specific manner — changes cysteine to formylglycine[98]. The modified antibody can then react selectively with aldehyde-specific warheads through a reaction that is based on the hydrazino-Pictet–Spengler ligation[99].

Bacterial transglutaminases (BTGs) catalyse the formation of a covalent bond between a glutamine side chain and a primary amine. The conjugation site has a significant impact on ADC stability and pharmacokinetics in a species-dependent manner (for example, it is different in mice, rats or primates). These differences are attributed to the position of the linkage rather than the chemical instability that was observed with a maleimide linkage[100]. Pfizer uses this technology for PF-06664178, which is a TROP2-specific ADC that entered phase I trials in patients with advanced or metastatic solid tumours[101].

Innate Pharma has also developed ADCs using BTG-mediated conjugation of MMAE to an IgG1, which resulted in stable-bond formation between the heavy-chain residue glutamine 295 and the linker–drug moiety. This procedure requires the enzymatic removal of *N*-linked glycans from the antibody and yields a defined DAR of 2. Alternatively, a mutant aglycosylated IgG1 variant may be generated by site-directed mutagenesis. The mutation introduces an additional glutamine and yields a DAR of 4 after coupling[102].

The bacterial enzyme sortase A (SrtA) may be used to catalyse a transpeptidation reaction[103], which modifies heavy and light chains at their C termini by addition of the SrtA recognition motif Lys-Pro-Glu-Thr-Gly. In a second step, tubulin polymerization inhibitors, such as MMAE or maytansine, that are coupled to a pentaglycine peptide may be linked to the mAb by a similar transpeptidation reaction, which is a technology that has been developed by NBE Therapeutics.

Glycan remodelling and glycoconjugation. The native *N*-glycosylated 'Asn297' (based on EU numbering)[104] of IgGs represents an interesting, specific modification site that is distant from the antigen-binding sites of the variable domain[105]. Making modifications at this site could allow the risks of impairing the binding affinity of the antibody to be minimized, which can be a potential pitfall when constructing ADCs using chemical conjugation via lysines. Numerous strategies to target the *N*-glycan for bioconjugation have been developed, including metabolic engineering, chemical oxidation, and enzymatic and chemo-enzymatic modification, as discussed below[106].

Metabolic glycoengineering of mAbs has been suggested to improve conjugate-coupling homogeneity. Instead of fucose, unnatural fucose derivatives (such as 6-thiofucose) can be added to the culture media and incorporated in the *N*-glycan moiety of the mAbs[107]. This unnatural thio-glycan is then used for conjugation using maleimide chemistry to produce ADCs with improved homogeneity compared with ADCs in which the drug is attached via hinge cysteines.

Chemical approaches to glycan modification use sodium periodate ($NaIO_4$) to oxidize *cis*-glycol groups of carbohydrates such as galactose or sialic acid, which results in the formation of an aldehyde functionality, followed by hydrazone condensation with a linker–drug moiety[108].

In addition, numerous glycoengineering techniques have been described that use enzymes for the preparation of site-specific ADCs[109]. These include the preparation of homogeneous ADCs through glycan remodelling and SPAAC[110], as well as chemoenzymatic approaches[111,112].

Amino-terminal engineered serine. MedImmune and ImmunoGen have recently described homogeneous and hydrolytically stable ADCs that have serine residues engineered into the amino terminus of their light and heavy chains. The hydroxyl groups of these serine residues are used for mild and selective oxidation. The resulting aldehydes can then be used for oxime ligation[113].

Ligation to Fab nucleotide-binding sites. Researchers at the University of California, Davis, in the United States have recently shown that the nucleotide-binding pocket (NBP) in the Fab arms of IgG can be specifically targeted by conjugated indole-based 5-difluoro-2,4-dinitrobenzene linker–drug moieties. Ligation can occur at any one of the few lysine residues that are located at the NBP sites[114].

Native cysteine rebridging. Companies such as PolyTherics (now Abzena), ThioLogics, Igenica Biotherapeutics, Sorrento Therapeutics and the University of Tours, France, have described several strategies that use bis-alkylation conjugation at reduced interchain disulfides. One of the main advantages of these strategies is the ability to produce stable and homogeneous ADCs without the need to specifically engineer the antibody for conjugation[115–117]. The resulting conjugates retain antigen-binding capabilities, are stable in serum, and have demonstrated potent and antigen-selective cell killing in *in vitro* and *in vivo* cancer models.

Avoiding retro-Michael deconjugation. As discussed above, Michael addition of a thiol to a maleimide is commonly used for bioconjugation of drugs to antibodies. Indeed, both currently approved ADCs (brentuximab vedotin and trastuzumab emtansine) contain maleimide–thiol adducts. *In vivo*, such adducts undergo cleavage by thiol exchange[118], which can compromise the efficacy of a conjugate as well as leading to toxicity due to the released drug. However, if the succinimide moiety of a maleimide–thiol conjugate is hydrolysed, the ring-opened product is stabilized against deconjugation, and a number of new technologies have recently been proposed to achieve this. As a direct result of these observations,

Glycan remodelling
Enzymatic tailoring of the oligosaccharides of an antibody to enable the introduction of reactive groups that are exploited for the site-specific attachment of cytotoxic drugs.

methods for succinimide hydrolysis on ADCs have been reported, such as ADC incubation at pH 9.2, which has a positive impact on ADC potency, stability, exposure and efficacy[119], as well as purification by anionic exchange chromatography[120]. As an alternative, self-hydrolysing maleimides have been designed to improve the stability and pharmacological properties of ADCs[71] and achieve long-term stabilization of maleimide–thiol conjugates[121]. To avoid the relative instability of maleimide conjugates in blood, the use of aryl maleimide-coupling agents has been reported[122], and new coupling reagents have been developed, such as sodium 4-((4-(cyanoethynyl) benzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate (CBTF)[123] and 2-(maleimidomethyl)-1,3-dioxanes, which are a serum-stable self-hydrolysable hydrophilic alternative to SMCC linkers[124].

Highly loaded ADCs. Highly loaded ADCs such as the conjugates with a DAR of 8, which are obtained by conventional conjugation, can display a shorter pharmacokinetic half-life, higher toxicity and a lower therapeutic index than the ADCs with a DAR of 4 (REF. 72). However, site-specific, highly loaded ADCs demonstrated efficacy in a mouse xenograft model in which expression levels of the target antigen were low, whereas no or low efficacy could be achieved with similarly loaded conventional molecules or site-specific conjugates with lower loading[100]. These findings might have a major clinical impact, as they suggest a way to target tumours for which low target expression, slow internalization or inefficient intracellular processing of the ADC would otherwise not allow the delivery of sufficient drug levels to achieve a therapeutic response[125]. Interestingly, Mersana Therapeutics has developed a polymer-based ADC platform (Fleximers), which allows the conjugation of up to 15 payloads on IgG molecules[126] and has recently entered phase I trials.

Enhancing the potency of ADCs

In addition to careful target, linker and warhead selection, several additional strategies are used to enhance the efficacy of ADCs. Such strategies may be designed to avoid potential resistance against the warhead, to enhance tumour penetration through the use of smaller protein scaffolds or to enhance efficacy by combining ADCs with the recently approved mAb-based immune checkpoint inhibitors.

Overcoming resistance to ADCs. As ADCs are increasingly used in the clinic, it is expected that resistance development will be a key issue. 'Classic' mechanisms of resistance to certain warheads, such as tubulin-binding agents, include the increased expression of ATP-binding cassette (ABC) efflux pumps (such as MDR1, as discussed above), altered microtubule composition and alterations in the levels of pro-apoptotic proteins. Besides these well-described mechanisms, resistance to ADCs can also result from a downregulation of antigen expression, downregulation of antigen–ADC internalization and enhanced complex recycling on the cell surface, as well as through reduced intracellular trafficking or drug release[127].

Although there are only a few publications of preclinical models of resistance to ADCs, the reports available shed light on the heterogeneity of resistance mechanisms. For example, it was shown that resistance to trastuzumab emtansine is associated with the expression of multidrug-resistance genes (including *MDR1*) and a decrease in HER2 expression[128]. ABC protein expression has also been suggested to be important for calicheamicin-based ADCs[129]. Moreover, *in vitro* experiments showed decreased CD30 expression in two out of three lymphoid lines that were exposed to brentuximab vedotin[130]. Importantly, resistance to a given ADC was not associated with cross-resistance to an ADC that contains a conjugate with a different mechanism of action[131], which opens up new research avenues. Although documentation of resistance mechanisms in clinical samples is expected with much interest, there is clearly a need to develop additional preclinical models of resistance to different ADCs that contain different types of conjugates. It is likely that, besides tumour cell-related mechanisms of resistance, the tumour microenvironment also contributes to resistance to these agents[132].

Barriers to effective tumour penetration. Tumour and antigen accessibility is a critical factor and often a major hurdle for effective ADC delivery. Because of limited tumour penetration by the antibody and hence reduced drug delivery, highly potent payloads are of paramount importance. It has been reported that only 0.001–0.01% of an injected unmodified tumour-specific antibody — and, by analogy, a tumour-specific ADC — actually binds to tumour cells in humans[133].

An increasing number of formats for next-generation ADCs have also recently been reported[134], including non-IgG scaffolds such as designed ankyrin repeat proteins (DARPins)[135] and non-internalizing mAb scaffolds that are coupled to cytotoxic drugs by means of disulfide linkers, which are then selectively cleaved in the tumour microenvironment[136].

Combining ADCs with immuno-oncology antibodies. Antibodies directed against molecules that downregulate immune responses (such as cytotoxic T lymphocyte antigen 4 (CTLA4), programmed cell death protein 1 (PD1) or programmed cell death 1 ligand 1 (PDL1)), which are also known as immune checkpoint inhibitors, have shown spectacular effects in several tumour types[137]. Therefore, it will be important to determine how ADCs might be of use in combination with this family of agents. Hodgkin lymphoma is an interesting example, as both an ADC (brentuximab vedotin)[138] and anti-PD1 antibodies such as nivolumab (developed by BMS)[139] have shown activity in patients with heavily pretreated disease. As treatment with ADCs that have dolastatin-based warheads induces maturational changes in dendritic cells, which affect their homing properties and activate cellular antitumour immune responses in patients[140], the combination of brentuximab vedotin with checkpoint inhibitors may prove to be highly active. A clinical trial to evaluate the combination of brentuximab vedotin, nivolumab and the anti-CTLA4 mAb ipilimumab (developed by BMS)

as a potential treatment option for patients with relapsed or refractory Hodgkin lymphoma is ongoing (ClinincalTrials.gov identifier: NCT01896999). A second trial will be carried out in patients with T cell NHL, and relapsed or refractory B-NHL, including DLBCL.

Checkpoint inhibitors, which activate immune responses in a non-antigen-specific manner, seem to be more likely to be active in patients with tumours that contain a large number of mutations, as recently demonstrated in colorectal cancer with mismatch-repair deficiency[141]. Some cytotoxic drugs that are used in ADCs have been shown to induce immunogenic cell death and can induce dendritic cell activation and maturation[142]. Therefore, it is possible that the targeting of tumour cells with ADCs could contribute to enhancing responses to immunomodulating antibodies[142].

In orthotopic mouse models of HER2$^+$ breast cancer, despite primary resistance to immunotherapy, combined treatment with trastuzumab emtansine and anti-CTLA4 and anti-PD1 mAbs was curative, because it enhanced innate and adaptive antitumour immune responses[143]. Tumour rejection was accompanied by massive T cell infiltration, T helper 1 (T_H1) cell polarization and, notably, a substantial increase in regulatory T cells. Depletion of regulatory T cells resulted in inflammation and tissue damage, which implies that these cells have an essential role in protecting the host during therapy. This study provides insights into the mechanisms underlying the therapeutic activity of trastuzumab emtansine and a rationale for potential combination strategies with immunotherapeutic agents[143].

ADC for non-oncology indications

Few applications for ADCs have been explored outside the field of oncology using non-cytotoxic drugs[144]. Among these is an ADC that is directed against C-X-C chemokine receptor type 4 (CXCR4), which is an antigen that is selectively expressed on haematopoietic cells. This ADC has immunosuppressive activity, as it selectively delivers dasatinib, which is an inhibitor of the tyrosine kinase LCK, to human T lymphocytes and thereby suppresses T cell receptor-mediated T cell activation[145].

Besides targets on human cells, an antibody–antibiotic conjugate (AAC) that targets intracellular *Staphylococcus aureus* has recently been reported[146]. This AAC (DSTA4637S; developed by Roche) consists of an anti-*S. aureus* THIOMAB antibody that is site-specifically conjugated to a highly efficacious antibiotic (dmDNA31) via an intracellular protease-sensitive peptide linker, which is cleaved only in the proteolytic environment of the lysosome. DSTA4637S has reached phase I trials (NCT02596399)[147].

Conclusion and future directions

The development of ADCs has benefited from general improvements in the design of therapeutic mAbs and from specific improvements in methods for conjugate synthesis through which enhanced homogeneity can be achieved. Diversification of linking strategies and warheads has provided new opportunities to improve drug delivery to tumours while reducing drug exposure to normal tissues. Indeed, it is essential to gain a better understanding of the determinants of toxicity of ADCs, either as single agents or in combination with other therapies. To increase the therapeutic index of ADCs, improvements have to be made either in the potency of the cytotoxic agent to lower the minimum effective dose or in tumour selectivity to increase the maximum tolerated dose. As discussed, the synthesis and characterization of more-homogeneous and stable ADCs with medicinal chemistry-like control over their macromolecular structure is of paramount importance for the clinical success of next-generation ADCs.

Protein structural characterization tools such as mass spectrometry are allowing better understanding of ADC biotransformations *in vivo*. This knowledge and the development of quantitative bioanalytical assays will contribute to the identification of early-developability criteria for all of the ADC components (antibody, drug and linker). For example, off-target hepatic toxicities have been reported for several ADCs and were found to be due to the expression of a mannose receptor on the cell surface of hepatocytes[100]. Therefore, antibodies with low mannose content should be selected for the ADC. Another example is the emergence of alternatives to maleimide conjugation chemistries, which limit the drug deconjugation in the serum that has been observed with most of the second-generation ADCs that are currently on the market or in clinical trials.

Because of the hybrid nature of ADCs, product quality attributes for both the biological component (the mAb) and the small-molecule components (the drug and drug–linker combination) must be considered[148]. Therefore, early-developability assessment requires state-of-the-art analytical[149] and structural methods, such as native and ion mobility mass spectrometry[79,80,150], two-dimensional liquid chromatography[151–154] and capillary electrophoresis[155,156] coupled to mass spectrometry. These emerging methods allow a deep insight into important structural features that are related to ADC functions.

In addition, recent ADC development has created a renewed interest in cytotoxic natural products, which are typically highly potent cytotoxic agents but often have unacceptable toxicities. In the future, breakthroughs in the efficacy of ADCs are likely to involve warheads with novel mechanisms of action[157].

Moreover, alternative formats to mAbs, such as protein scaffolds (DARPins, nanobodies, single-chain variable fragments (scFvs), peptide–drug conjugates), antibody–dual-drug conjugates (ADDCs), Fabs[61] and Probodies (developed by CytomX)[158], are being investigated at the discovery and preclinical stages. Interestingly, the first biparatopic ADC, which targets two non-overlapping epitopes on the same antigen (HER2; see above) has entered phase I trials in patients who are refractory to or ineligible for HER2-targeted therapies[36]. These new formats will have to be compared with full-format mAbs in terms of toxicity, efficacy and pharmacokinetics to determine the therapeutic indications in which they may possess added therapeutic value in comparison with conventional mAbs.

There are several possible indications for ADCs: as single agents in patients with refractory or relapsing disease; in palliative settings, for consolidation or maintenance; and in combination with other agents as first line-therapy or in relapsed patients. As the results of randomized trials that compare ADC-based regimens with other regimens are becoming available, the main criteria that will determine regulatory and commercial success are the relative efficacy in comparison with other available combinations, the toxicity profile and the cost. Although there is no general rule as to what level of efficacy is required or what level of toxicity is acceptable — these levels being dependent on the context — ADC-based regimens will be compared with the best available alternatives and will have to present a significant advantage in at least one clinically meaningful parameter, which can be associated with tumour response, survival or quality of life.

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Competing interests statement
The authors declare competing interests: see Web version for details.

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