Bispecific antibodies: a mechanistic review of the pipeline

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Abstract | The term bispecific antibody (bsAb) is used to describe a large family of molecules designed to recognize two different epitopes or antigens. BsAbs come in many formats, ranging from relatively small proteins, merely consisting of two linked antigen-binding fragments, to large immunoglobulin G (IgG)-like molecules with additional domains attached. An attractive bsAb feature is their potential for novel functionalities — that is, activities that do not exist in mixtures of the parental or reference antibodies. In these so-called obligate bsAbs, the physical linkage of the two binding specificities creates a dependency that can be temporal, with binding events occurring sequentially, or spatial, with binding events occurring simultaneously, such as in linking an effector to a target cell. To date, more than 20 different commercialized technology platforms are available for bsAb creation and development, 2 bsAbs are marketed and over 85 are in clinical development. Here, we review the current bsAb landscape from a mechanistic perspective, including a comprehensive overview of the pipeline.

The original concept of a man-made antibody-based molecule with two different antigen-binding sites — a bispecific antibody (bsAb) — was first described by Nisonoff and co-workers more than 50 years ago[1] and paralleled the first insights into antibody architecture[2]. Using mild re-oxidation to couple rabbit antigen-binding fragments (Fabs) of different specificities, they demonstrated agglutination of two different cell types mediated by bispecific fragments[3]. The successive conceptual and technical innovations in generating bsAbs subsequently evolved alongside the landmark advances in the fields of antibody engineering and antibody biology (FIG. 1), leading to the extensive collection of over 100 bsAb formats known today[4–6]. About one-quarter of these have been developed into technology platforms and are being commercialized by biotech and pharma companies for the generation of novel and differentiated therapeutics[7].

Dual-targeting concepts enabled by bsAbs hold great therapeutic promise, but translation of these concepts into treatments has proved challenging. For instance, the archetypical bsAb application — T cell redirection and engagement — was first described in the mid-1980s[8,9] but did not reach patients until 2009 with the European Union approval of catumaxomab for the intraperitoneal treatment of malignant ascites[10]. Intravenous administration with catumaxomab (a T lymphocyte antigen CD3 × epithelial cell adhesion molecule (EpCAM) bsAb; × denotes the combination of the two antigen specificities) was not feasible and induced fatal toxicity at low doses, which was linked to Fc-mediated off-target T cell activation in the liver[11]. Despite the recent market withdrawal of catumaxomab in 2017 for commercial reasons, the impressive clinical results of another approved T cell-engaging bsAb (bsTCE), blinatumomab (CD3 × B lymphocyte antigen CD19)[12,13], sparked renewed interest and investment in this concept. This is reflected in the 43 T cell-redirecting bsAbs currently in clinical development for haematological and solid tumour indications. The success has also stimulated further evolution of the concept and exploration of alternative trigger molecules for T cell engagement (for example, the Vγ9Vδ2 T cell receptor (TCR)[14]). In addition, combination therapies are being investigated and compared with other successful T cell-activating strategies (for example, checkpoint inhibitors and chimeric antigen receptor (CAR) T cell approaches).

In addition to cancer, inflammatory disorders have generally been the focus of the clinical development of bsAbs. The first market entry of a bsAb in a non-cancer indication, however, occurred in November 2017 with the US Food and Drug Administration (FDA) approval of emicizumab (coagulation factor IXa (FIXa) × FX) for the treatment of haemophilia A[15]. In addition, other disease areas outside cancer and inflammatory disorders are being explored, with clinical candidates in diabetes and HIV infection in phase I trials and an increasing number of preclinical proof-of-concept studies in various other diseases, including several viral and bacterial infections, Alzheimer disease, osteoporosis and regenerative medicine. The mechanisms by which bsAb concepts

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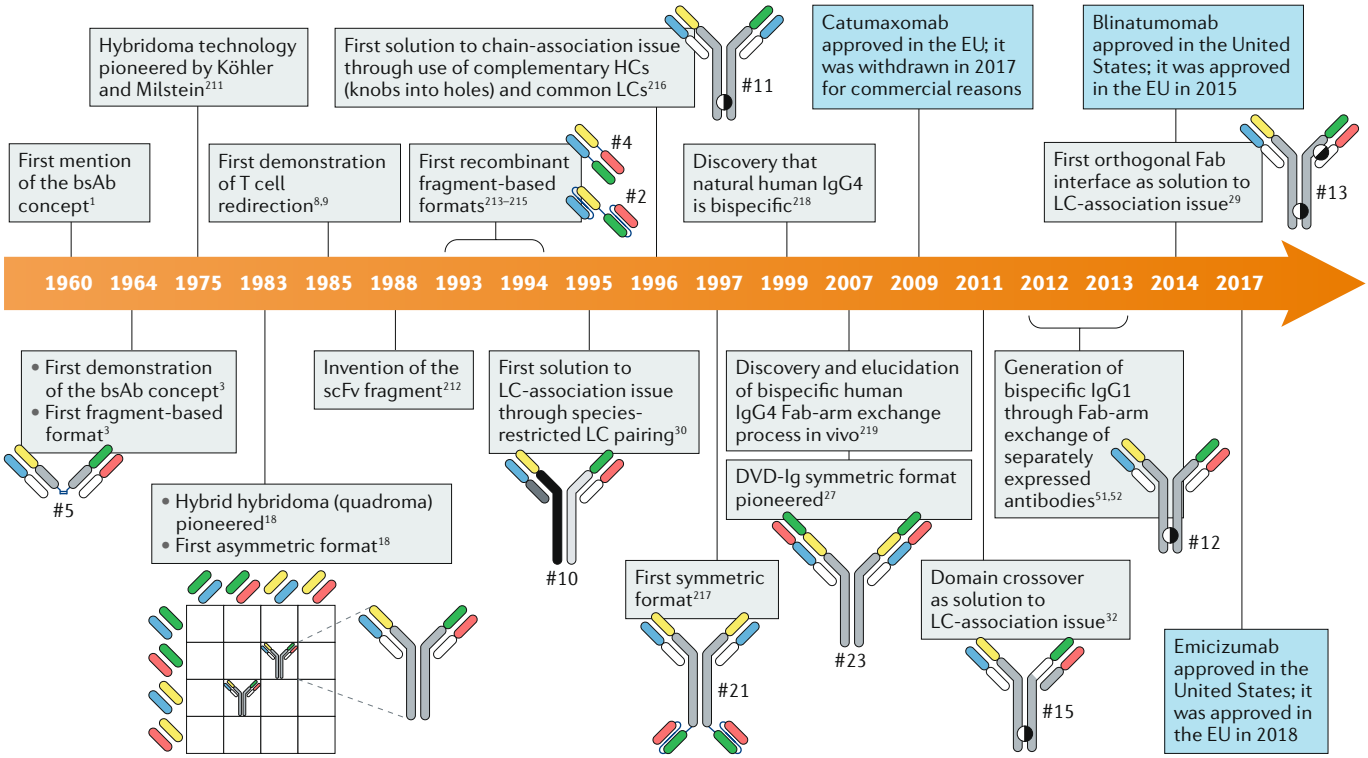

Fig. 1 | **Timeline of conceptual and technical innovations contributing to the development of the therapeutic bsAb landscape.** Formats are illustrated where relevant with hashtags referring to formats exemplified in FIG. 2. The quadroma is accompanied by a matrix illustrating the chain-association issue. Random chain association yields 16 possible combinations of which only 2 represent the functional bispecific antibody (bsAb). The year of first approval for all marketed bsAbs is indicated. DVD, dual variable domain; EU, European Union; Fab, antigen-binding fragment; HC, heavy chain; Ig, immunoglobulin; LC, light chain; scFv, single-chain variable fragment.

differentiate from the mere mixing of antibodies in these dual-targeting approaches are surprisingly diverse, as highlighted in a number of key examples below.

BsAbs addressing the multifactorial nature of complex diseases are being developed, for instance, through combinatorial bsAbs targeting redundancy in disease-mediating ligands and receptors or bsAbs targeting crosstalking signalling cascades. Conceptually, however, one could argue that combinatorial strategies do not utilize the full spectrum of differentiation that bsAbs may provide. One of the attractive features of bsAbs is their potential to display activity that is not present in any combination of parent antibodies (that is, generate a new functionality dependent on the physical linkage of the two specificities). Because of this acquired novel activity, such antibodies have been dubbed obligatory bsAbs[6,16], although we prefer the term obligate bsAbs[17]. In this Review, we describe recent advances and emerging applications of bsAbs using a mechanistic framework, with a focus on obligate concepts. We also discuss advances in the translation of therapeutic bsAb concepts into the clinic and provide future perspectives for this field.

Combinatorial bsAbs
Bispecific antibodies (bsAbs) that display an activity or functionality that can also be obtained by combining separate antibodies with the same specificities (for example, a parental or reference antibody mixture).

Obligate bsAbs
Bispecific antibodies (bsAbs) that display an activity or functionality that is dependent on the physical linkage of the two specificities (and cannot be obtained by combining separate antibodies with the same specificities). The dual-targeting concepts mediated by these bsAbs are considered obligate concepts.

Formats

In natural bivalent antibodies, the two antigen-binding sites are identical and composed of determinants from both heavy (H) and light (L) chain variable domains. Therefore, one of the initial challenges in bsAb development (through co-expression of two different H and two different L chains) was obtaining the functional bsAb from the mixture of ten possible H_2L_2 recombinations[18], commonly referred to as the chain-association issue.

Over the past decades, numerous strategies have been developed to either circumvent or address the issue, with the particular objective of increasing the homogeneity and yield of the desired end-product. The different design features or functional properties introduced by these strategies can all be used to classify the extensive collection of resulting bsAb formats. For the purpose of this Review, having a mechanistic focus, we will adhere to an architectural classification[4] and briefly discuss opportunities and limitations. Additional differentiation based on the number of binding sites (valency) will further subcategorize formats, as this affects dual-targeting and potential multi-targeting applications (such as avidity or crosslinking-induced agonism) (FIG. 2).

Fragment-based formats

The first class, which represents the minimalistic approach to designing bispecific molecules, simply combines multiple antigen-binding moieties (that is, antibody fragments) in one molecule without an Fc region, thereby circumventing the chain-association issue. This lack of complexity and the absence of a (glycosylated) Fc region allow relatively simple production of these formats through (co-)expression of 1–2 polypeptide chains in lower eukaryotic and prokaryotic expression systems[19], offering the advantage of high yields and reduced costs.

However, Fc-deficient formats have a relatively short plasma half-life, as they lack protection from catabolism by the neonatal Fc receptor, FcRn. Reformatting to Fc-fusion proteins or the introduction of a human serum albumin (HSA)-targeting moiety may be necessary for clinical development depending on the intended therapeutic application (see below). Their lack of Fc-mediated effector functions or the ability to tailor such functions for specific needs might represent another drawback. In addition, the fragment-based products can suffer from stability and aggregation issues, requiring re-engineering of the end-product[20,21].

Owing to the modular nature of this class, the valency of the two specificities can be customized to fit the application, and thus formats with 1 + 1, 1 + 2 and 2 + 2 designs (that is, designating the number of binding sites for each specificity) are being evaluated in the clinic (FIG. 2; see below). Other permutations — for example, 1 + 3 (REF.[22]) or 3 + 3 (REFS[23,24]) — incorporating additional binding sites may follow in the future.

Symmetric formats

An alternative design strategy to circumvent the chain-association issue, while retaining the Fc region, is to incorporate both specificities in a single polypeptide chain or single HL pair. The resulting format includes the above-mentioned Fc-fusion proteins of fragment-based formats (to improve pharmacokinetic properties or effector functions) and formats in which antibody fragments are fused to regular antibody molecules. The symmetric design allows for the production of these formats through (co-)expression of 1–2 polypeptide chains. Symmetric formats more closely resemble native antibodies but differ in size and architecture. These differences can negatively affect favourable properties associated with native antibodies (such as stability and solubility) and may thus impair the physicochemical and/or pharmacokinetic properties of these agents[25,26].

Because of the symmetric nature of this class, most formats in clinical development have tetravalent 2 + 2 designs. The close proximity of antigen-binding sites, however, may impair optimal engagement of both targets simultaneously, potentially reducing functional valency, and may require optimization (for example, linker length and domain position) in individual lead candidates[27].

Asymmetric formats

Most approaches used to generate the formats in this third class seek to retain the native architecture of natural antibodies as closely as possible in order to preserve the associated functional characteristics and favourable quality attributes. This infers that the chain-association issue needs to be addressed and the symmetry of the H_2L_2 assembly broken. As a consequence, most asymmetric formats result from strategies forcing correct HL chain pairing[28–34] and/or promoting H chain heterodimerization[35–44] during co-expression of the four polypeptide chains (or three if common L chains[40] or H chains[45] are employed). In addition, the asymmetry can be leveraged to isolate the desired end-product by designing purification strategies based on differential protein A binding[30,46], sequential affinity chromatography[45] or size differences[42]. Alternative strategies to circumvent HL chain mispairing make use of separate expression of both specificities as half-molecules[47–50] or parental antibodies[51,52], followed by post-production assembly or recombination of antibody half-molecules, respectively, driven by mutations promoting H chain heterodimerization (for example, controlled Fab-arm exchange (cFAE))[50].

As most asymmetric formats closely resemble natural antibodies and lack additional non-native antibody domains or linker sequences, they are thought to have the lowest potential for immunogenicity. However, the elaborate engineering that may be involved in solving the chain-association issue may counteract this advantage in some of these formats. The asymmetric nature of this class also infers that bsAbs with regular immunoglobulin G (IgG) architecture (usually) become functionally monovalent for each target (1 + 1). It is noted that reduced avidity of asymmetric formats compared with formats allowing multivalent target binding may affect potency for certain applications[53].

Mechanistic review of the bsAb pipeline

As of March 2019, the commercial clinical pipeline included over 85 bsAbs (FIG. 3). Reflecting the substantial recent interest in the development of bsAbs for cancer therapy (FIG. 4), ~86% were being evaluated in patients with cancer. BsAbs that bridge cells as their obligate mechanism of action represent the largest group, with T cell redirection as the most common denominator (TABLES 1,2).

The strength of obligate bsAbs is their ability to unlock novel functionalities that require two binding specificities to be connected in the same molecule. This can be exploited for innovative therapeutic concepts, for instance, to bridge two cell types (in-trans binding) or to engage two molecules on the membrane of one cell (in-cis binding). These concepts require simultaneous binding of the two specificities, while other obligate concepts are based on sequential binding of the two binding domains[53]. Examples of obligate bsAb concepts and their design and mechanism of action are summarized in FIG. 5 and are discussed in this section.

Bridging cells (in-trans binding)

T cell redirection: a historical perspective. The archetypical obligate bsAb concept is the redirection of the cytotoxic activity of effector T cells to specifically eliminate tumour cells. By this approach, T cells are physically linked with tumour cells via bsAbs that are composed of a T cell-binding domain and a tumour-binding domain (FIG. 5a). These bsTCEs mostly activate T cells through binding of CD3ε in the TCR complex, thereby bypassing major histocompatibility complex (MHC) restriction and causing activation independent of the epitope specificity of the TCR. Although proof-of-concept bsTCEs showed high potency in co-culture assays[8,9], in the clinic, these molecules induced rapid and uncontrolled T cell-mediated cytokine release at very low doses, while no lasting clinical activity was noted[54,55]. Together with complications that were encountered with the production

Chain-association issue
The co-expression of two different heavy (H) and two different light (L) chains results in a complex mixture of sixteen possible H_2L_2 recombinations, representing ten different antibodies. Only one of these antibodies (represented by two possible H_2L_2 recombinations) corresponds with the desired bispecific antibody (maximal yield 12.5% in the mixture). This issue is addressed by strategies forcing cognate HL-pairing and/or promoting heterodimerization of the two different H chains.

Valency
The number of antigen-binding sites in an antibody molecule. The design of a bispecific antibody (bsAb) format influences the number of binding sites per target. A bivalent bsAb with one binding site for each target is denoted as 1 + 1. Incorporating additional binding sites can lead to trivalent (2 + 1) and tetravalent (2 + 2 or 1 + 3) designs.

Antibody fragments
The antibody molecule consists of different domains that can be expressed separately and used as modular building blocks. The domains involved in antigen recombination are often used as binding moieties in the design of antibody-based therapeutics. Examples include domain antibodies (heavy chain-only variable domain (V_{HH})) and single-chain Fv fragments (scFvs), antigen-binding fragments (Fabs), single-chain Fab fragments (scFabs) and, more recently, single-chain Fc fragments (scFcs).

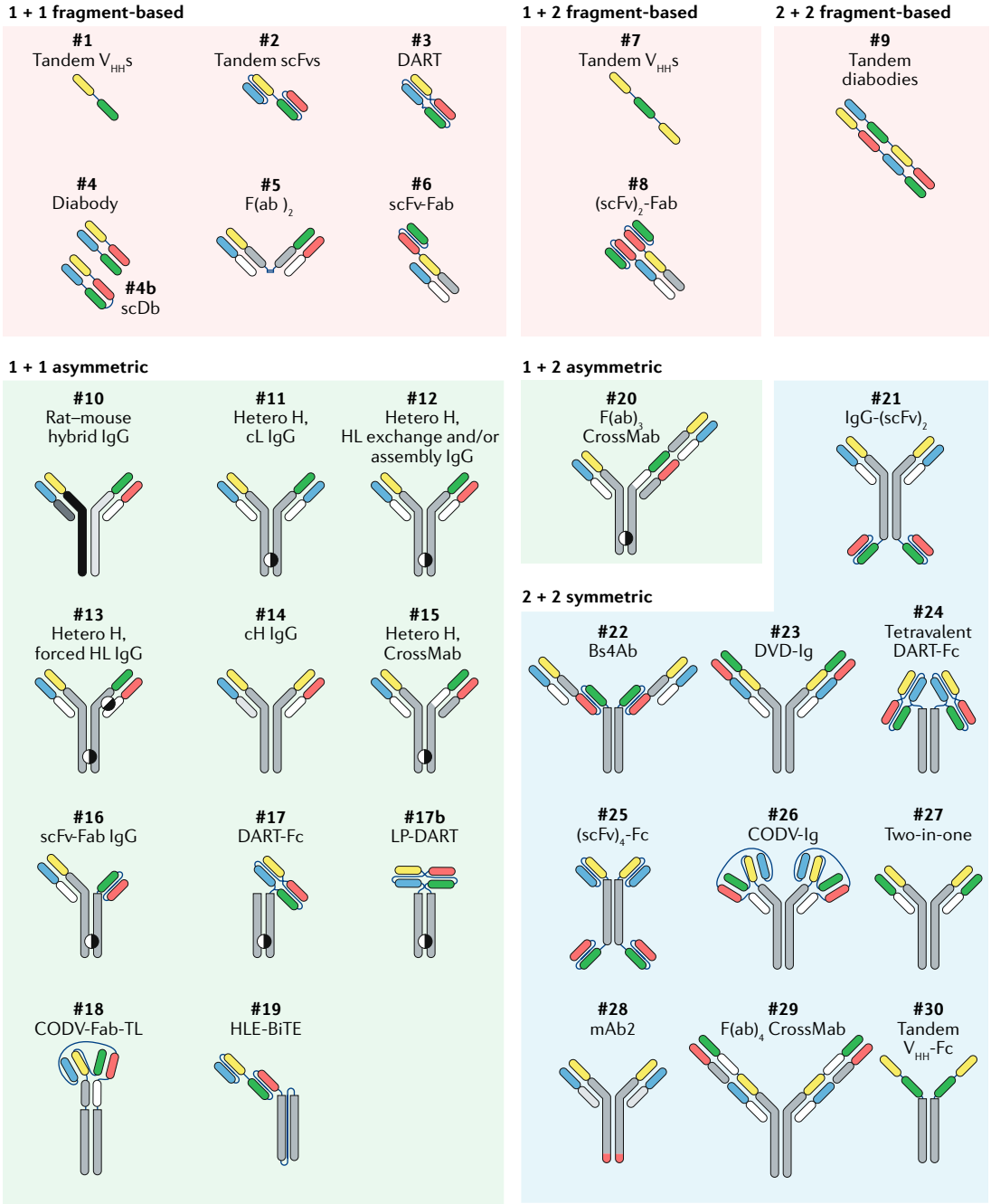

Fig. 2 | **A selection of bsAb formats.** Bispecific antibodies (bsAbs) are categorized by increasing valency for each of the two target specificities from left to right and their format class. Formats are classified as fragment-based (without an Fc domain) and asymmetric or symmetric Fc-bearing molecules. The molecules may contain mutations that affect chain pairing and other manufacturability parameters, Fc-mediated effector functions and half-life. The hashtags correspond to the detailed descriptions of architecture and mutations for the molecules in clinical development listed in TABLES 1–4. Exemplary technology platforms include #1 and #7, nanobody; #2, bispecific T cell engager (BiTE); #3, #17(b) and #24, dual-affinity re-targeting (DART); #6 and #8, immunotherapy antibody (ITab); #9, tandem diabody (TandAb); #10, Triomab; #11, asymmetric reengineering technology immunoglobulin (ART-Ig) and biclonics; #12, DuoBody and knobs into holes (KiH); #13, DuetMab; #14, κλ body; #16, Xmab, YBODY and bispecific engagement by antibodies based on the T cell receptor (BEAT); #15, #20 and #29, CrossMab; #18 and #26, crossover dual variable (CODV); #23, dual variable domain (DVD); #25, Adaptir; and #27, two-in-one and dual-action antigen-binding fragment (Fab) (DAF). Antibody domains are coloured according to their architecture: orange, variable heavy (H) chain specificity 1; green, variable H chain specificity 2; blue, variable light (L) chain specificity 1; red, variable L chain specificity 2; grey, H chain constant region; white, L chain constant region; light grey, alternative L chain constant region; format #10: dark grey and black, rat L chain and immunoglobulin G2b (IgG2b) H chain; and white and light grey, mouse L chain and IgG2a H chain. cH common heavy; cL, common light; HLE, half-life extended; scDb, single-chain diabody; scFv, single-chain variable fragment; V_{HH}, heavy chain-only variable domain.

and stability of these bsAbs, this considerably reduced the appetite to further develop such agents at that time.

The interest in bsTCEs, however, was revived when the first clinical data with blinatumomab were presented[56]. Blinatumomab is a small antibody-fragment-based bsAb with a molecular mass of ~55 kDa that lacks an Fc domain and has a short plasma half-life (1.25 ± 0.63 hours) in vivo[57,58]. When blinatumomab was given by continuous intravenous infusion to achieve desired trough levels, impressive responses were observed at very low doses in patients with non-Hodgkin lymphoma (NHL)[56]. Later, when the focus was shifted to relapsed and/or refractory (r/r) acute lymphoblastic leukaemia (ALL), an outstanding complete response rate of 43% was reported[59]. The results from this study formed the basis for approval of blinatumomab for the treatment of r/r ALL by the FDA in 2014.

These exciting clinical data prompted companies to find solutions to efficiently generate and produce stable bsAbs (see Formats). As a result, plenteous developable bsAb formats became available, and many entered clinical trials. At present, approximately half of the bsAbs undergoing evaluation in clinical trials are bsTCEs (51%; $n = 44/86$ (FIG. 3; TABLE 1)).

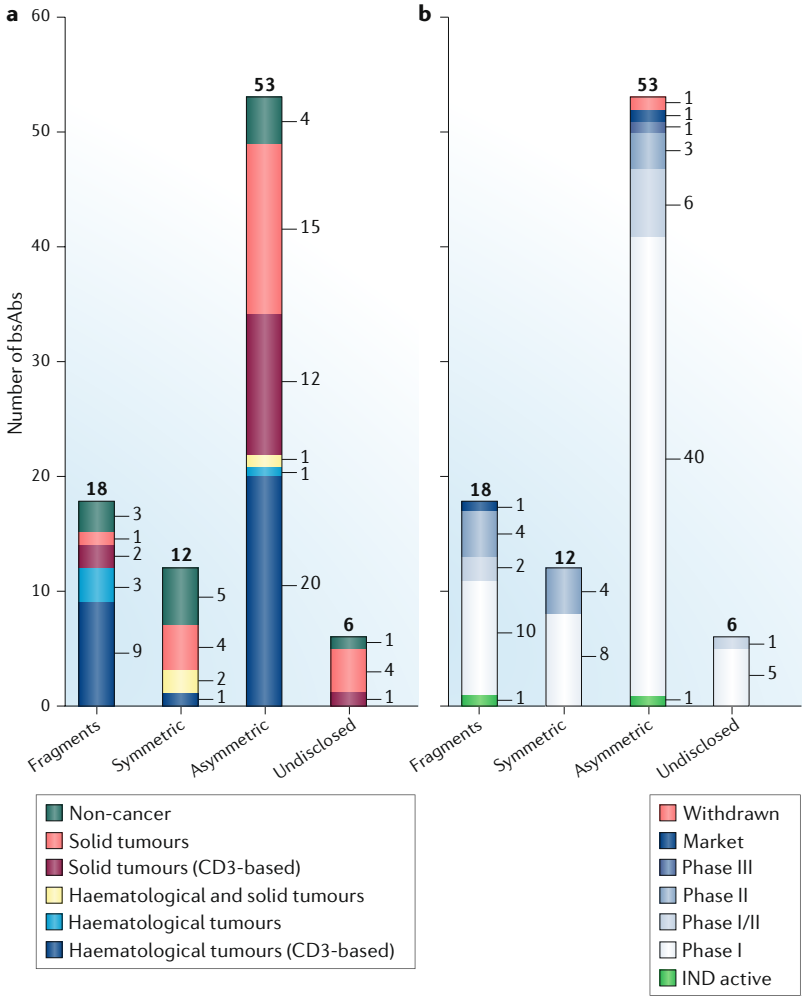

Fig. 3 | **Overview of the clinical development pipeline for bsAbs.** The agents are classified by format class and divided by indication and CD3-mediated concepts (T cell engagement) in panel **a** and development stage in panel **b**. bsAb, bispecific antibody; IND, investigational new drug.

The Fc region in bsTCEs: active, suppressed or absent. The clinical experience with the monospecific, murine anti-human CD3 antibody OKT3 (also known as muromonab-CD3) and the pioneering bsTCE catumaxomab provided important lessons for the safe use of CD3-targeting antibodies.

OKT3 has been used as an immunosuppressant in transplantation medicine but showed unleashed T cell activation and severe cytokine release in patients, particularly at the first dosing[60]. A major mechanism of the observed cytokine release involves CD3 clustering on T cells via the binding of Fc regions of CD3 antibodies to Fc receptors on other immune cells. To prevent this unrestricted clustering of CD3, a second-generation, humanized CD3 antibody with reduced FcγR binding (and complement factor C1q binding) was developed (huOKT3γ1 (L234A-L235A))[61] and showed significantly reduced cytokine release[62]. Similarly, the severe adverse events that were induced upon intravenous administration of the CD3 × EpCAM bsTCE catumaxomab were attributed to off-target binding of its active Fc region to FcγR-expressing Kupffer cells in the liver. As a result, robust, local cytokine release and T cell-mediated hepatotoxicity were induced, which were fatal for one patient[11].

In addition to these safety issues, we recently showed that CD3-targeting bsAbs containing an effector function-competent backbone also had diminished therapeutic activity in a syngeneic model in vivo[63]. This suggests that Fc-mediated effector mechanisms may hamper tumour-specific T cell redirection and tumour cell killing.

Together, this indicates that CD3-targeting bsTCEs require preferably complete suppression of Fc-mediated effector functions in order to minimize off-target toxicity and to maximize therapeutic efficacy. Indeed, all CD3-targeting bsAbs that are currently in the clinic have engineered Fc domains to reduce FcγR binding or are bispecific antibody fragments that lack the Fc region by design. It should be noted, however, that the mutations used to suppress FcγR binding vary between the different formats, and some Fc-containing bsTCEs are more inert than others. Indeed, a highly inert Fc region without residual FcγR and C1q interactions but in which FcRn binding is retained is preferred[64,65].

T cell targeting. Although alternative T cell targets, such as the αβTCR[66] and T cell surface glycoprotein CD5 (REF.[67]), have been explored to redirect or engage T cells, bsTCEs targeting CD3ε are the most advanced. Most of the CD3ε-binding units disclosed so far are derived from a limited number of murine antibody clones — often the non-human primate cross-reactive monoclonal antibody (mAb) SP34 (REF.[68]) — that have been humanized, deimmunized and/or affinity matured.

Several studies have shown that the affinity for CD3 dramatically affects the biodistribution of bsTCEs. Although bsTCEs with high CD3 affinity (dissociation constant (K_D) <1 nmol per litre) showed superior effectivity in co-culture assays in vitro[69], lower affinity

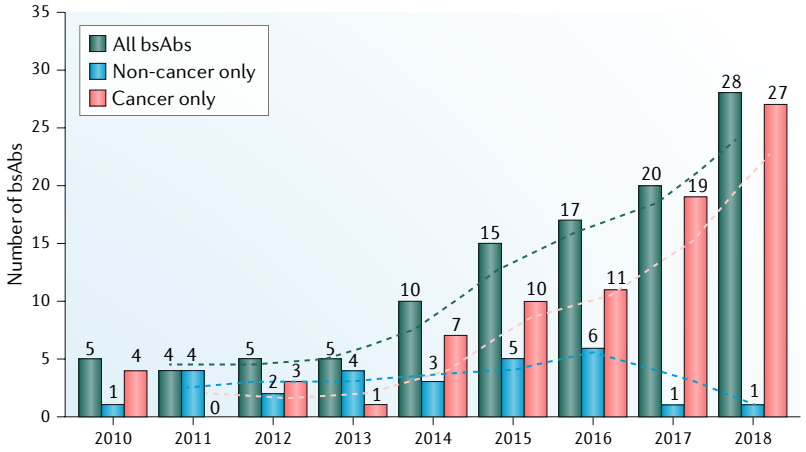

Fig. 4 | **Year of clinical study initiation for bsAbs.** Data include only bispecific antibodies (bsAbs) sponsored in clinical studies by commercial firms. The dotted lines are 2-year moving averages. Cellular therapies incorporating bsAbs and bispecific molecules that include non-antibody-derived binding sites were excluded.

(K_D = ~50–200 nmol per litre) of the CD3-binding arm is preferable to allow efficient tumour distribution in vivo without rapid CD3-mediated plasma clearance or trapping of the antibody in T cell-containing tissues, such as spleen and lymph nodes[69–72]. In these studies, the presence of a tumour-specific arm was required to drive tumour distribution, whereas in the absence of a tumour-associated antigen (TAA)-binding arm, no tumour-specific distribution was observed[72].

In addition to affinity, the valency for CD3 binding may affect the activity of the molecule. While bivalent CD3 binding is key for antigenic modulation and tolerance induced by monospecific CD3 antibodies[73], in the context of bsTCEs, the use of a monovalent CD3 arm may be desired. While monovalent binding is sufficient to induce tumour-specific T cell activation, it may prevent antigenic modulation or cytokine release as a result of crosslinking of CD3 molecules on the surface of T cells. Although the majority of bsTCEs in development indeed contain a single CD3-binding arm, a few clinical-stage bispecific molecules have two CD3-binding domains, yet it is unclear whether these formats also functionally bind CD3 bivalently. Examples include the CD3 × CD33 (also known as SIGLEC-3)-directed tandem diabody (TandAb) AMV-564 (Amphivena Therapeutics), the CD3 × CD19 TandAb AFM11 (Affimed) and the CD3 × CD123 Adaptir molecule APVO436 (Aptevo Therapeutics). These molecules showed efficient T cell activation that was strictly dependent on the presence of antigen-expressing cells in co-culture assays[74,75]. The safety and efficacy of these bsTCEs are currently being evaluated in phase I clinical trials (TABLE 1).

Tumour cell targeting. BsTCEs directly couple T cells and tumour cells to form an immune synapse, resulting in TCR activation, release of granzymes and perforin and eventually target cell lysis[76]. This essentially resembles the mechanism of TCR–peptide-loaded MHC (pMHC) interaction-mediated lytic synapse formation, including sequence of events, molecular composition and signalling[76]. A possible difference may be the larger size and higher number of synapses that could be induced by bsTCEs than by natural TCR–pMHC complexes. This may be the result of a greater number of contact points that could be established by bsTCEs[77]. In comparison, T cell activation can be induced by less than ten TCR–pMHC complexes[78]. Similarly, for high-affinity (~30–300 pM) soluble TCR × CD3 bispecific molecules, killing was observed at low antigen density levels of 10–150 molecules per cell[79]. For bsTCEs, in theory, between 50 and 100,000 contact points may be established, depending on expression levels of the TAA.

Some in vitro studies indicate that the activity of bsTCEs correlates with target expression levels, as shown for bsTCEs directed against carcinoembryonic antigen (CEA)[80], CD33 (REF.[81]) and human epidermal growth factor receptor 2 (HER2; also known as ERBB2)[82], while others have shown no such correlation, for instance, with bsTCEs targeting erythropoietin-producing hepatoma receptor tyrosine kinase A2 (EPHA2)[83] or prostate-specific membrane antigen (PSMA)[84]. Despite these contrasting results, it is likely that a certain threshold of target expression is required for the cytotoxic activity of bsTCEs. Such a threshold presumably differs for each antigen. For instance, it has been suggested that binding of as few as 50 bsTCE molecules targeting the myeloma antigen Fc receptor-like protein 5 (FcRL5) was sufficient to induce effective T cell activation and target cell apoptosis by the FcRL5-directed bsTCE RG6160 (REF.[85]), while at least 10,000 CEA-binding sites were required for the cytotoxic activity of the CEA bsTCE cibisatamab (Roche)[84].

The activity of bsTCEs also depends on properties of the antigen other than its expression level on the cell surface, such as its mobility in the membrane[86]. In addition, the dimensions of the TAA molecule and the epitope distance to the target cell membrane are known to critically affect the cytotoxic potential of bsTCEs[85,87]. In fact, a small target size and binding to a membrane-proximal epitope allow close intercellular membrane–membrane vicinity to be established by the bsTCE, resulting in optimal synapse formation and effective T cell-mediated cytotoxicity.

It has been suggested that the use of a bivalent tumour-targeting arm may induce enhanced potency and tumour selectivity of bsTCEs as a result of increased binding avidity[82,88,89]. Several tetravalent bsTCEs are in development, including the aforementioned Adaptir and TandAb compounds, which have two CD3 and two TAA-binding units. Alternatively, trivalent 1 + 2 bsTCEs are in development that are monovalent for CD3 and bivalent for TAA binding. For instance, RG6026 (also known as RO7082859; Roche) has such a 1 + 2 design, which may afford increased tumour antigen avidity, T cell activation and tumour cell killing compared with other bsTCE formats. It is composed of two anti-B lymphocyte antigen CD20 Fabs derived from obinutuzumab, one anti-CD3 Fab fused to one of the anti-CD20 Fabs via a short flexible linker and a heterodimeric Fc domain that was engineered to prevent binding to Fc receptors and C1q[90]. In addition to RG6026, four other bsTCEs targeting CD20 and CD3 are now in phase I clinical trials (GEN3013, mosunetuzumab, REGN1979

Table 1 | **Clinical-stage bsAbs for cancer indications: obligate concepts**

BsAb names (sponsors)	Targets	Format[a] and engineering	Disease area (selected indications)	Status (selected trials)
Bridging cells (in-trans): T cell redirection and/or activation				
Orlotamab, MGD009 (Macrogenics)	CD3 x B7-H3	• Tandem domain-exchanged Fv-Fc(G1) (#17, DART-Fc, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), L234A-L235A (Fc-silencing), H435R (purification)	Solid malignancies (NSCLC and melanoma)	Phase I (NCT02628535 and NCT03406949)
AMG420, BI 836909 (Boehringer Ingelheim)	CD3 x BCMA	• Tandem scFv (#2, BiTE, FRAG, 1 + 1)	Haematological malignancies (MM)	Phase I (NCT02514239 and NCT03836053)
AMG701 (Amgen)	CD3 x BCMA	• Tandem scFv-scFc(G1) (#19, HLE-BiTE, ASYM, 1 + 1) • Possibly N297G (Fc-silencing); G446del-K447del (reduction charge-heterogeneity); R292C-V302C (CH2 domain stabilization)	Haematological malignancies (MM)	Phase I (NCT03287908)
CC-93269, EM801 (Celgene)	CD3 x BCMA	• Fab-Fc(G1) x Fab-Fab-Fc(G1) (#20, CrossMab, ASYM, 1 + 2) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), HL-pairing: CrossMab, L234A-L235A-P329G (Fc-silencing)	Haematological malignancies (MM)	Phase I (NCT03486067)
JNJ-64007957 (Janssen)	CD3 x BCMA	• Hetero H, HL exchanged IgG4 (#12, DuoBody, ASYM, 1 + 1) • Hetero HH: F405L-R409K x WT (R409), S228P (hinge-stabilization), F234A-L235A (Fc-silencing)	Haematological malignancies (MM)	Phase I (NCT03145181)
PF-06863135 (Pfizer)	CD3 x BCMA	• Hetero H, HL assembly IgG (#12, ASYM, 1 + 1) • Hetero HH: C223R-E225R-P228R-K409R x C223E-(E225)-P228E-L368E; IgG2Δa (A330S-P331S; Fc-silencing)	Haematological malignancies (MM)	Phase I (NCT03269136)
REGN5458 (Regeneron)	CD3 x BCMA	• Hetero H, cL IgG4 (#11, ASYM, 1 + 1)[b] • Hetero HH: WT x H435R-Y436F (purification), HL-pairing: cL, E233P-F234V-L235A-G236del (Fc-silencing)	Haematological malignancies (MM)	Phase I/II (NCT03761108)
APVO436 (Aptevo Therapeutics)	CD3 x CD123	• scFv-Fc(G1)-scFv (#25, Adaptir, SYM, 2 + 2) • L234A-L235A-G237A-K322A (Fc-silencing)	Haematological malignancies (AML and MDS)	Phase I (NCT03647800)
Flotetuzumab, MGD006 (Macrogenics, City of Hope Medical Center, National Cancer Institute)	CD3 x CD123	• Tandem domain-exchanged Fv (#3, DART, FRAG, 1 + 1)	Haematological malignancies (AML, MDS and CML)	Phase II pending (NCT02152956 and NCT03739606)
JNJ-63709178 (Janssen)	CD3 x CD123	• Hetero H, HL exchanged IgG4 (#12, DuoBody, ASYM, 1 + 1) • Hetero HH: F405L-R409K x WT (R409), S228P (hinge-stabilization), F234A-L235A (Fc-silencing)	Haematological malignancies (AML)	Phase I (NCT02715011)
SAR440234 (Sanofi)	CD3 x CD123	• VH1-VH2-CH1-Fc1(G1) x VL2-VL1-CL-Fc2(G1) (#18, CODV-Fab-TL1, ASYM, 1 + 1) • L234A-L235A (Fc-silencing), H435R-Y436F (purification)	Haematological malignancies (AML, B-ALL and MDS)	Phase I/II (NCT03594955)
Vibecotamab, Xmab14045 (Xencor)	CD3 x CD123	• Fab-Fc(G1) x scFv-Fc(G1) (#16, Xmab, ASYM, 1 + 1) • Hetero HH: L368D-K370S x E357Q-S364K, E233P-L234V-L235A-G236del-S267K (Fc-silencing)	Haematological malignancies (AML, B-ALL and CML)	Phase I (NCT02730312)
A-319 (Generon)	CD3 x CD19	• scFv-Fab (#6, ITab, FRAG, 1 + 1)	Haematological malignancies (ALL and B-ALL)	IND active
AFM11 (Affimed)	CD3 x CD19	• Tandem diabodies (#9, TandAb, FRAG, 2 + 2)	Haematological malignancies (NHL and ALL)	Phase I suspended (NCT02106091 and NCT02848911)
AMG562 (Amgen)	CD3 x CD19	• Tandem scFv-scFc(G1) (#19, HLE-BiTE, ASYM, 1 + 1) • Possibly N297G (Fc-silencing); G446del-K447del (reduction charge-heterogeneity); R292C-V302C (CH2 domain stabilization)	Haematological malignancies (lymphoma)	Phase I (NCT03571828)
Blinatumomab, Blincyto, MT103, MEDI-538, AMG103 (Amgen)	CD3 x CD19	• Tandem scFv (#2, BiTE, FRAG, 1 + 1)	Haematological malignancies (ALL and B-ALL)	Marketed

Table 1 (cont.) | **Clinical-stage bsAbs for cancer indications: obligate concepts**

BsAb names (sponsors)	Targets	Format[a] and engineering	Disease area (selected indications)	Status (selected trials)
Bridging cells (in-trans): T cell redirection and/or activation (cont.)				
GEN3013, DuoBody-CD3xCD20 (Genmab)	CD3 x CD20	• Hetero H, HL exchanged IgG1 (#12, DuoBody, ASYM, 1 + 1) • Hetero HH: F405L x K409R, L234F-L235E-D265A (Fc-silencing)	Haematological malignancies (DLBCL, FL and MCL)	Phase I/II (NCT03625037)
Mosunetuzumab, RG7828, RO7030816, BTCT4465A (Roche)	CD3 x CD20	• Hetero H, HL assembly IgG1 (#12, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), N297G (Fc-silencing)	Haematological malignancies (CLL, NHL and DLBCL)	Phase I/II (NCT03677141 and NCT03677154)
Plamotamab, XmAb13676 (Xencor)	CD3 x CD20	• Fab-Fc(G1) x scFv-Fc(G1) (#16, Xmab, ASYM, 1 + 1) • Hetero HH: L368D-K370S x E357Q-S364K, E233P-L234V-L235A-G236del-S267K (Fc-silencing)	Haematological malignancies (NHL and CLL)	Phase I (NCT02924402)
REGN1979 (Regeneron)	CD3 x CD20	• Hetero H, cL IgG4 (#11, ASYM, 1 + 1) • Hetero HH: WT x H435R-Y436F (purification), HL-pairing: cL, E233P-F234V-L235A-G236del (Fc-silencing)	Haematological malignancies (FL, CLL and NHL)	Phase II pending (NCT03888105, NCT02651662 and NCT02290951)
RO7082859, RG6026, CD20-TCB (Roche)	CD3 x CD20	• Fab-Fc(G1) x Fab-Fab-Fc(G1) (#20, CrossMab, ASYM, 1 + 2) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), HL-pairing: CrossMab, L234A-L235A-P329G (Fc-silencing)	Haematological malignancies (NHL)	Phase I (NCT03075696 and NCT03533283)
AMG330 (Amgen)	CD3 x CD33	• Tandem scFv (#2, BiTE, FRAG, 1 + 1)	Haematological malignancies (AML)	Phase I (NCT02520427)
AMG673 (Amgen)	CD3 x CD33	• Tandem scFv-scFc(G1) (#19, HLE-BiTE, ASYM, 1 + 1) • Possibly: N297G (Fc-silencing), G446del-K447del (reduction charge-heterogeneity), R292C-V302C (CH2 domain stabilization)	Haematological malignancies (AML)	Phase I (NCT03224819)
AMV-564 (Amphivena Therapeutics)	CD3 x CD33	• Tandem diabodies (#9, TandAb, FRAG, 2 + 2)	Haematological malignancies (AML and MDS)	Phase I (NCT03144245 and NCT03516591)
GEM333 (GEMoaB Monoclonals)	CD3 x CD33	• scDb (#4b, FRAG, 1 + 1)	Haematological malignancies (AML)	Phase I (NCT03516760)
AMG424, Xmab13551 (Amgen)	CD3 x CD38	• Fab-Fc(G1) x scFv-Fc(G1) (#16, Xmab, ASYM, 1 + 1) • Hetero HH: L368D-K370S x E357Q-S364K, E233P-L234V-L235A-G236del-S267K (Fc-silencing)	Haematological malignancies (MM)	Phase I (NCT03445663)
GBR1342 (Glenmark Pharmaceuticals)	CD3 x CD38	• Fab-Fc(G1) x scFv-Fc(G1) (#16, BEAT, ASYM, 1 + 1) • Hetero HH: BEAT (A) x BEAT (B), L234A-L235A (Fc-silencing)	Haematological malignancies (MM)	Phase I (NCT03309111)
Cibisatamab, RG7802, RO6958688, CEA-TCB (Roche)	CD3 x CEA	• Fab-Fc(G1) x Fab-Fab-Fc(G1) (#20, CrossMab, ASYM, 1 + 2) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), HL-pairing: CrossMab, L234A-L235A-P329G (Fc-silencing)	Solid malignancies (NSCLC)	Phase I/II (NCT03337698)
Tepoditamab, MCLA-117 (Merus)	CD3 x CLEC12A	• Hetero H, cL IgG1 (#11, Biclonics, ASYM, 1 + 1) • Hetero HH: L351D-L368E x L351K-T366K, HL-pairing: cL; L235G-G236 R (Fc-silencing)	Haematological malignancies (AML)	Phase I (NCT03038230)
AMG757 (Amgen)	CD3 x DLL3	• Tandem scFv-scFc(G1) (#19, HLE-BiTE, ASYM, 1 + 1) • Possibly N297G (Fc-silencing); G446del-K447del (reduction charge-heterogeneity); R292C-V302C (CH2 domain stabilization)	Solid malignancies (small-cell lung cancer)	Phase I (NCT03319940)
AMG596 (Amgen)	CD3 x EGFRvIII	• Tandem scFv (#2, BiTE, FRAG, 1 + 1)	Solid malignancies ($EGFRvIII^+$ glioblastoma)	Phase I (NCT03296696)
A-337 (Generon)	CD3 x EpCAM	• scFv-LC x scFv-Fd (#8, ITab, FRAG, 1 + 2)	Solid malignancies (NSCLC)	Phase I
Catumaxomab, removab	CD3 x EpCAM	• Rat 2b–mouse 2a hybrid IgG (#10, Triomab, ASYM, 1 + 1)	Solid malignancies (malignant ascites owing to epithelial carcinomas)	Withdrawn from the market

Table 1 (cont.) | **Clinical-stage bsAbs for cancer indications: obligate concepts**

BsAb names (sponsors)	Targets	Format[a] and engineering	Disease area (selected indications)	Status (selected trials)
Bridging cells (in-trans): T cell redirection and/or activation (cont.)				
RG6160, RO7187797, BFCR4350A (Genentech)	CD3 x FcRH5 (CD307)	• Tandem scFv (#2, BiTE, FRAG, 1 + 1)	Haematological malignancies (MM)	Phase I (NCT03275103)
AMG427 (Amgen)	CD3 x FLT3	• Tandem scFv-scFc(G1) (#19, HLE-BiTE, ASYM, 1 + 1) • Possibly N297G (Fc-silencing); G446del-K447del (reduction charge-heterogeneity); R292C-V302C (CH2 domain stabilization)	Haematological malignancies (AML)	Phase I (NCT03541369)
ERY974 (Chugai)	CD3 x GPC3	• Hetero H, cL IgG4 (#11, ART-Ig, ASYM, 1 + 1) • Hetero HH: E356K x K439E, HL-pairing: cL, L235R-S239K-N297A (Fc-silencing); K196Q (pI-engineering); G446del-K447del (reduction charge-heterogeneity); S228P (hinge-stabilization)	Solid malignancies (gastric cancer and squamous cell oesophageal carcinoma)	Phase I (NCT02748837)
MGD007 (Macrogenics)	CD3 x gpA33	• Tandem domain-exchanged Fv-Fc(G1) (#17, DART-Fc, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), L234A-L235A (Fc-silencing), H435R (purification)	Solid malignancies (CRC)	Phase I/II (NCT03531632 and NCT02248805)
JNJ-64407564 (Janssen)	CD3 x GPRC5D	• Hetero H, HL exchange IgG4 (#12, DuoBody, ASYM, 1 + 1) • Hetero HH: F405L-R409K x WT (R409), S228P (hinge-stabilization), F234A-L235A (Fc-silencing)	Haematological malignancies (MM)	Phase I (NCT03399799)
GBR1302 (Glenmark Pharmaceuticals)	CD3 x HER2	• Fab-Fc(G1) x scFv-Fc(G1) (#16, BEAT, ASYM, 1 + 1) • Hetero HH: BEAT (A) x BEAT (B), L234A-L235A (Fc-silencing)	Solid malignancies (HER2$^+$ cancers)	Phase I (NCT02829372)
M802 (YZYBio)	CD3 x HER2	• Fab-Fc(G1) x scFv-Fc(G1) (#16, YBODY, ASYM, 1 + 1) • Hetero HH: D356K-D399K-Y407A x K392D-K409D-T366W	Solid malignancies (breast cancer and gastric cancer)	Phase I
RG6194, BTRC4017A (Genentech)	CD3 x HER2	• Undisclosed	Solid malignancies (locally advanced or metastatic HER2-expressing cancers)	Phase I (NCT03448042)
REGN4018 (Regeneron)	CD3 x MUC16	• Hetero H, cL IgG4 (#11, ASYM, 1 + 1) • Hetero HH: WT x H435R-Y436F (purification), HL-pairing: cL, E233P-F234V-L235A-G236del (Fc-silencing)	Solid malignancies (ovarian, fallopian tube or peritoneal cancers)	Phase I (NCT03564340)
PF-06671008 (Pfizer)	CD3 x P-cadherin	• Tandem domain-exchanged Fv-Fc (#17b, LP-DART, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), possibly Fc-silenced	Solid malignancies (TNBC, NSCLC and CRC)	Phase I (NCT02659631)
AMG160 (Amgen)	CD3 X PSMA	• Tandem scFv-scFc(G1) (#19, HLE-BiTE, ASYM, 1 + 1) • Possibly: N297G (Fc-silencing), G446del-K447del (reduction charge-heterogeneity), R292C-V302C (CH2 domain stabilization)	Solid malignancies (prostate cancer)	Phase I (NCT03792841)
MOR209, APVO414, ES414	CD3 x PSMA	• scFv-Fc(G1)-scFv (#25, Adaptir, SYM, 2 + 2) • L234A-L235A-G237A-K322A (Fc-silencing)	Solid malignancies (prostate cancer)	Discontinued after phase I[c]
Pasotuxizumab A212, BAY2010112	CD3 x PSMA	• Tandem scFv (#2, BiTE, FRAG, 1 + 1)	Solid malignancies (prostate cancer)	Discontinued after phase I[c]
Tidutamab, XmAb18087 (Xencor)	CD3 x SSTR2	• Fab-Fc(G1) x scFv-Fc(G1) (#16, Xmab, ASYM, 1 + 1) • Hetero HH: L368D-K370S x E357Q-S364K, E233P-L234V-L235A-G236del-S267K (Fc-silencing)	Solid malignancies (neuroendocrine and GIST)	Phase I (NCT03411915)
Bridging cells (in-trans): NK cell redirection and/or activation				
AFM13 (Affimed)	CD16A x CD30	• Tandem diabodies (#9, TandAb, FRAG, 2 + 2)	Haematological malignancies (Hodgkin lymphoma and cutaneous lymphoma)	Phase II (NCT02321592 and NCT03192202)
GTB-3550, OXS-3550 (GT Biopharma)	CD16 x CD33	• Tandem scFv fusion protein (#2 fused to IL-15, FRAG, 1 + 1)	Haematological malignancies (MDS, AML and systemic mastocytosis)	Phase I/II pending (NCT03214666)

Table 1 (cont.) | **Clinical-stage bsAbs for cancer indications: obligate concepts**

BsAb names (sponsors)	Targets	Format[a] and engineering	Disease area (selected indications)	Status (selected trials)
Bridging cells (in-trans): immune cell redirection and/or activation				
ABBV-428 (AbbVie)	CD40 x MSLN	• scFv-hinge-Fc(G1)-linker-scFv (#25, SYM, 2 + 2) • Fc-silenced; possibly V263L or V273E	Solid malignancies	Phase I (NCT02955251)
INBRX-105 (Inhibrx)	PD-L1 × 4-1BB	• Tandem V_{HH}-Fc(G1) (#30, SYM, 2 + 2) • E233del-L234del-L235del (Fc-silencing)	Haematological and solid malignancies (lymphoma and solid tumours)	Phase I (NCT03809624)
MCLA-145 (Merus, Incyte)	PD-L1 × 4-1BB	• Hetero H, cL IgG (#11, Biclonics, ASYM, 1 + 1) • Hetero HC: L351D-L368E x L351K-T366K, HL-pairing: cL; possibly Fc-silenced	Solid malignancies	IND active
Bridging receptors (in-cis)				
Zenocutuzumab, MCLA-128, PB4188 (Merus)	HER2 x HER3	• Hetero H, cL IgG1 (#11, Biclonics, ASYM, 1 + 1) • Hetero HH: L351D-L368E x L351K-T366K, HL-pairing: cL, glycoengineered: low-fucose (ADCC-enhanced)	Solid malignancies (breast cancer)	Phase II (NCT03321981)
JNJ-61186372 (Janssen R&D)	EGFR x MET	• Hetero H, HL exchange IgG1 (#12, DuoBody, ASYM, 1 + 1) • Hetero HH: F405L x K409R, glycoengineered: low-fucose (ADCC-enhanced)	Solid malignancies (NSCLC)	Phase I (NCT02609776)
XmAb23104 (Xencor)	PD-1 x ICOS	• Fab-Fc(G1) x scFv-Fc(G1) (#16, Xmab, ASYM, 1 + 1) • Hetero HH: L368D-K370S x E357Q-S364K, E233P-L234V-L235A-G236del-S267K (Fc-silencing), M428L-N434S (Xtend in vivo half-life extension)	Solid malignancies	Phase I pending (NCT03752398)

Data available as of 1 March 2019. Molecules are ordered on the basis of the antigens in the second column. ADCC, antibody-dependent cellular cytotoxicity; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; ART-Ig, asymmetric reengineering technology immunoglobulin; ASYM, asymmetric; B7-H3, B7 homologue 3 (CD276); B-ALL, B cell acute lymphoblastic leukaemia; BCMA, B cell maturation antigen; BEAT, bispecific engagement by antibodies based on the T cell receptor; BiTE, bispecific T cell engager; bsAb, bispecific antibody; CEA, carcinoembryonic antigen; cL, common light; CLEC12A, C-type lectin domain family 12 member A; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; CODV-Ig, crossover dual variable Ig-like; CRC, colorectal cancer; DART, dual-affinity re-targeting; DLBCL, diffuse large B cell lymphoma; DLL3, delta-like ligand 3; EGFR, epidermal growth factor receptor; EGFRvIII, EGFR variant III; EpCAM, epithelial cell adhesion molecule; Fab, antigen-binding fragment; FcRH5, Fc receptor homologue 5 (CD307); FL, follicular lymphoma; FLT3, FMS-like tyrosine kinase 3; FRAG, fragment-based; GIST, gastrointestinal stromal tumour; GPC3, glypican 3; GPRC5D, G protein-coupled receptor family C group 5 member D; H, heavy; HER2, human epidermal growth factor receptor 2; HLE, half-life extended; ICOS, inducible T cell co-stimulator; Ig, immunoglobulin; IND, investigational new drug; ITab, immunotherapy antibody; KiH, knobs into holes; L, light; MCL, mantle cell lymphoma; MDS, myelodysplastic syndrome; MM, multiple myeloma; MSLN, mesothelin; MUC16, mucin 16; NHL, non-Hodgkin lymphoma; NK, natural killer; NSCLC, non-small-cell lung cancer; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand 1; pI, isoelectric point; PSMA, prostate-specific membrane antigen; scDb, single-chain diabody; scFc, single-chain Fc fragment; scFv, single-chain variable fragment; SSTR2, somatostatin receptor 2; SYM, symmetric; TandAb, tandem diabody; TNBC, triple-negative breast cancer; V_{HH}, heavy chain-only variable domain; WT, wild-type. [a]Format data provided in the first bullet point in cells in the third column include the bsAb format number (#) in FIG. 2, technology trade name, class and valency; see FIG. 2 for additional information on format class (FRAG, SYM or ASYM) and valency. Engineering data provided in the second bullet point in cells in the third column include additional constant region mutations, which were obtained from public documents (scientific literature, abstracts, posters and patent publications). [b]Based on format disclosure for REGN1979. [c]Molecules active in 2018 that were discontinued by March 2019.

and plamotamab; TABLE 1), which are all asymmetric, full-length IgG bispecifics with a 1 + 1 design, though they are developed and produced using different technologies. Of note, the subcutaneous formulation of GEN3013 is designed to reduce peak cytokine levels while retaining efficacy in B cell depletion[91], and in this respect it differs from the other CD20 bsTCEs in development. It will be exciting to see how these differences in antibody design and formulation compare in the clinic.

Targeting haematological versus solid malignancies with bsTCEs. The majority of clinical-stage bsTCEs are being developed for the treatment of haematological malignancies (67%; $n = 29/43$) (FIG. 3; TABLE 1). While some address novel targets such as C-type lectin domain family 12 member A (CLEC12A) (tepoditamab, MCLA-117; Merus), FcRL5 (RG6160; Genentech/Roche) or G protein-coupled receptor family C group 5 member D (GPRC5D) (JNJ-64407564; Janssen), many of these products target well-known, validated B cell or myeloid antigens, including CD19, CD20, CD33, CD38 (also known as ADPRC1), CD123 or B cell maturation antigen (BCMA; also known as TNFRSF17).

There is a striking overlap between the compounds directed at the same target for the treatment of haematological tumours. In addition to the five bsTCEs targeting CD20 that were already discussed above, six are directed against BCMA, five against CD123, four against CD33 and two against CD38 (TABLE 1). Although the targets are the same, the formats generally differ between these bsTCE products. For instance, various different formats were used to design the clinical-stage CD123 bsTCEs, representing a fragment-based format (flotetuzumab, Macrogenics/Servier), a tetravalent, symmetric format (APVO436, Aptevo Therapeutics) and three asymmetric formats (vibecotamab, Xencor/Novartis; JNJ-63709178,

Janssen Pharmaceuticals; and SAR440234, Sanofi). JNJ-63709178 and vibecotamab have encountered FDA clinical holds owing to potentially related grade 3 adverse events and deaths in trials. Closer analysis of the clinical data will reveal how these different formats directed at the same antigen perform in terms of safety and efficacy.

Targets of bsTCEs developed for the treatment of haematological malignancies are commonly also expressed on normal B and plasma cells, but depletion of these cells can be tolerated without inducing severe adverse events. By contrast, many solid tumour antigens are expressed at low levels in critical tissues and may therefore induce adverse events by on-target T cell reactivity towards tissues expressing the antigen, complicating the development of bsTCEs for solid tumours. Furthermore, the development of antibody therapeutics for solid tumours may be more challenging than for haematological malignancies owing to factors unique to these cancers, which inter alia include the immunosuppressive tumour microenvironment, disordered tumour vasculature and limitations in tumour penetration of the antibody (and the effector cells). Nevertheless, ~14 bsTCEs are being evaluated in the clinic that target solid tumour antigens, such as HER2, epidermal growth factor receptor (EGFR) variant (v)III, PSMA and EpCAM, among others (TABLE 1).

Preclinical studies in animal models addressing both efficacy and safety are complex because of a general lack of cross reactivity with (rodent) antigens and effector T cells, but promising antitumour activity in solid tumour models using a variety of bsTCEs has been observed in humanized mouse models. For instance, a CEA-targeting bsTCE induced a highly inflamed tumour microenvironment, even in poorly infiltrated tumours, and regression of CEA-expressing tumours in humanized mice (that is, NOG mice xenografted with a human colon carcinoma cell line mixed with human peripheral blood mononuclear cells (PBMCs))[92]. Tumour-specific inflammation was also induced by bsTCEs against P-cadherin in established subcutaneous solid tumour models supplemented with human PBMCs injected intraperitoneally and glypican 3 (GPC3) with human GPC3-transfected murine tumour cells in human CD3 transgenic mice[93,94]. Interestingly, in a syngeneic mouse melanoma model, treatment with a bsTCE targeting mouse tyrosinase-related protein 1 (TRP1) and mouse CD3 did not induce substantial cytokine release and toxicity provided that the bsTCE contained an inert Fc fragment fully devoid of Fc-mediated effector functions[63]. The bsTCE furthermore turned established melanomas into inflammatory sites, with an influx not only of T cells but also of natural killer (NK) cells and inflammatory macrophages. However, the bsTCE failed to induce long-term immunity in this model, as shown by similar tumour growth rates in untreated and long-term surviving mice that initially cleared the tumour upon bsTCE treatment[95].

Resistance to bsTCE therapy. The antitumour activity of bsTCEs is often constrained by the development of resistance. Studies into potential resistance mechanisms indicated that downregulation of bsTCE-specific TAAs on tumour cells is one of the mechanisms of tumour escape. For example, $CD19^-$ relapses have been noted in blinatumomab-treated patients with ALL and prevented further activity of the bsTCE[96].

Other mechanisms of resistance may involve immune suppression by regulatory T cells or immune checkpoints. Enhanced programmed cell death 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1) expression, for instance, which may be induced upon bsTCE treatment, limits their activity, while combined inhibition of the PD-1–PD-L1 axis was shown to enhance the preclinical antitumour activity of bsTCEs[97–100]. Preliminary clinical results showed early signs of enhanced clinical activity and a manageable safety profile for the CEA bsTCE cibisatamab in combination with the PD-L1 antibody atezolizumab in metastatic colorectal cancer[101], as well as for blinatumomab in combination with the PD-1 antibody nivolumab in r/r ALL[102]. Early data also suggested acceptable safety for the combination of the PD-1 antibody cemiplimab and the CD20 bsTCE REGN1979 (REF.[103]). However, these data are still premature, and longer follow-up will reveal whether such combination strategies translate into improved patient outcomes.

Alternative bispecific approaches for activating T cells. Prompted by the clinical success of antibodies against the immune checkpoints cytotoxic T lymphocyte antigen 4 (CTLA4), PD-1 and PD-L1, bsAbs targeting these and other immune checkpoints are emerging. Reflecting the substantial interest in these immune checkpoint inhibitors, the PD-1–PD-L1 axis is frequently targeted, with at least nine bsAbs targeting one of these antigens along with either CTLA4, lymphocyte-activation gene 3 (LAG3) or T cell immunoglobulin mucin 3 (TIM3; also known as HAVCR2) now in early-stage clinical studies (TABLE 2). A rationale for simultaneously targeting two immune checkpoints is provided by the improved clinical benefit observed in combination studies with mAbs targeting these checkpoints. For example, the treatment of patients with melanoma with ipilimumab (anti-CTLA4) plus nivolumab (anti-PD-1) induced improved survival outcomes compared with treatment with ipilimumab alone[104]. However, the increased antitumour activity was associated with significantly increased immune-related adverse events[104].

To improve the safety profile of combined targeting of PD-1 and CTLA4, Fc-silenced bsAbs have been designed to suppress the PD-1 pathway through high-affinity PD-1 binding while inhibiting CTLA4 with a low-affinity binding arm. This design favours CTLA4 inhibition in PD-1–CTLA4 double-positive tumour-infiltrating lymphocytes while reducing binding to CTLA4-expressing peripheral T cells, which may translate into more favourable safety and tolerability[105,106]. Currently, the safety and early efficacy of four PD-1 × CTLA4 bsAbs are being evaluated in early clinical trials (TABLE 2). The concept of blocking two immune checkpoint inhibitors is also being clinically evaluated for other target combinations, such as PD-1 × LAG3, PD-1 × TIM3 and PD-L1 × CTLA4 (TABLE 2), while many others are in preclinical development.

Table 2 | **Clinical-stage bsAbs for cancer: combinatorial concepts**

BsAb names (sponsors)	Targets	Format[a] and engineering	Disease area (selected indications)	Status (selected trials)
Targeting tumour heterogeneity				
OXS-1550, DT2219ARL (GT Biopharma)	CD19 x CD22	• Tandem scFv fusion protein (#2 fused to modified diphtheria toxin, BLT, FRAG, 1 + 1)	Haematological malignancies (B cell lymphoma and leukaemia)	Phase I/II (NCT02370160)
EMB01 (Epimab Biotherapeutics)	EGFR x MET	• Tandem Fab-Fc(G1) (#29, Fit-Ig, SYM, 2 + 2)	Solid malignancies	Phase I (NCT03797391)
MCLA-158 (Merus)	EGFR x LGR5	• Hetero H, cL IgG1 (#11, Biclonics, ASYM, 1 + 1) • Hetero HH: L351D-L368E x L351K-T366K, HL-pairing: cL, glycoengineered: low-fucose (ADCC-enhanced)	Solid malignancies (CRC)	Phase I (NCT03526835)
Targeting ligand redundancy				
BI 836880 (Ablynx/ Boehringer Ingelheim)	ANG2 x VEGF	• Tandem V_{HH} (#1, nanobody, FRAG,1+1, anti-HSA for half-life extension)	Solid malignancies (NSCLC)	Phase I (NCT02689505)
Vanucizumab, RO5520985 (Roche)	ANG2 x VEGF	• Hetero H IgG1 (#15, CrossMab, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), HL-pairing: CrossMab	Solid malignancies	Phase I (NCT02715531)
Dilpacimab, ABT-165 (AbbVie)	DLL4 x VEGF	• Tandem Fv-IgG1 (#23, DVD-Ig, SYM, 2 + 2) • L234A-L235A (Fc-silencing)	Solid malignancies (CRC)	Phase I (NCT03368859 and NCT01946074)
Navicixizumab, OMP-305B83 (Celgene/Oncomed)	DLL4 x VEGF	• Hetero H, cL IgG2 (#11, ASYM, 1 + 1) • Hetero HH: L368E-Y407E x T357K-D399K, HL-pairing: cL	Solid malignancies (ovarian, peritoneal or fallopian tube cancers)	Phase I (NCT03030287)
NOV1501, ABL001, TR009 (ABL Bio)	DLL4 x VEGF	• IgG-scFv (#21, SYM, 2 + 2) • Isotype and engineering undisclosed	Solid malignancies	Phase I (NCT03292783)
Targeting multiple checkpoints				
AK104 (Akesobio AU)	PD-1 x CTLA4	• Undisclosed	Solid malignancies (gastric or gastroesophageal junction adenocarcinoma)	Phase I/II (NCT03852251 and NCT03261011)
MGD019 (Macrogenics)	PD-1 x CTLA4	• Tandem domain-exchanged Fv-Fc(G1) (#17, DART-Fc, ASYM, 1 + 1)	Solid malignancies	Phase I (NCT03761017)
XmAb20717 (Xencor)	PD-1 x CTLA4	• Fab-Fc(G1) x scFv-Fc(G1) (#16, Xmab, ASYM, 1 + 1) • Hetero HH: L368D-K370S x E357Q-S364K, E233P-L234V-L235A-G236del-S267K (Fc-silencing), M428L-N434S (Xtend in vivo half-life extension)	Solid malignancies	Phase I (NCT03517488)
MEDI5752 (AstraZeneca)	PD-1 x CTLA4	• Hetero H, forced HL IgG1 (#13, DuetMab, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), HL-pairing: orthogonal Fab (HC: F126C, LC: S121C), H435R-Y436F (purification)	Solid malignancies	Phase I (NCT03530397)
MGD013 (Macrogenics)	PD-1 x LAG3	Tandem domain-exchanged Fv-Fc(G4) (#24, DART-Fc, SYM, 2 + 2)	Solid and haematological malignancies	Phase I (NCT03219268)
RO7121661, RG7769 (Roche)	PD-1 x TIM3	• Hetero H IgG1 (#15, CrossMab, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), HL-pairing: CrossMab	Solid malignancies (NSCLC and melanoma)	Phase I (NCT03708328)
KN046 (Alphamab)	PD-L1 x CTLA4	• Hetero H, cL IgG1 (#11, CRIB, ASYM, 1 + 1)[b] • Hetero HH: S354C-T366W-K409A x Y349C-T366S-L368A-Y407-F405K, HL-pairing: cL	Solid and haematological malignancies (TNBC, NSCLC and lymphoma)	Phase II pending (NCT03838848, NCT03872791, NCT03529526 and NCT03733951)
FS118 (F-Star)	PD-L1 x LAG3	• IgG1 (#28, mAb2, SYM, 2 + 2)	Solid malignancies	Phase I (NCT03440437)
LY3415244 (Eli Lilly)	PD-L1 x TIM3	• Undisclosed	Solid malignancies	Phase I (NCT03752177)
Undisclosed				
IBI318 (Innovent Biologics, Eli Lilly)	PD-1 x undisclosed TAA	• IgG1 (format undisclosed)	Solid malignancies	Phase I pending (NCT03875157)

Table 2 (cont.) | **Clinical-stage bsAbs for cancer: combinatorial concepts**

BsAb names (sponsors)	Targets	Format[a] and engineering	Disease area (selected indications)	Status (selected trials)
Targeting checkpoint and tumour antigens				
TG-1801, NI-1701 (NovImmune, TG Therapeutics)	CD47 x CD19	• cH IgG1 (#14, κλ body, ASYM, 1 + 1) • Hetero HH: cH, HL-pairing: κ and λ light chain (purification)	Haematological malignancies (B cell lymphoma)	Phase I (NCT03804996)
Increasing avidity: biparatopic bispecific antibodies				
KN026 (Alphamab)	HER2 x HER2	• Hetero H, cL IgG1 (#11, CRIB, ASYM, 1 + 1) • Hetero HH: S354C-T366W-K409A x Y349C-T366S-L368A-Y407-F405K, HL-pairing: cL	Solid malignancies (breast and gastric cancer)	Phase I (NCT03619681 and NCT03847168)
MBS301 (Beijing Mabworks Biotech)	HER2 x HER2	• Hetero H, HL assembly IgG1 (#12, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), glycoengineering: afucosylated (ADCC-enhanced)	Solid malignancies ($HER2^+$ solid tumours)	Phase I pending (NCT03842085)
ZW25 (Zymeworks)	HER2 x HER2	• scFv-Fc(G1) x Fab-Fc(G1) (#16, Azymetric, ASYM, 1 + 1) • Hetero HH: T350V-L351Y-F405A-Y407V x T350V-T366L-K392L-T394W	Solid malignancies ($HER2^+$ solid tumours)	Phase I (NCT02892123)
ZW49 (Zymeworks)	HER2 x HER2 ADC	• scFv-Fc(G1) x Fab-Fc(G1) (#16, Azymetric, ASYM, 1 + 1, conjugated to auristatin) • Hetero HH: T350V-L351Y-F405A-Y407V x T350V-T366L-K392L-T394W	Solid malignancies ($HER2^+$ solid tumours)	Phase I (NCT03821233)

Data available as of 1 March 2019. ADC, antibody–drug conjugate; ADCC, antibody-dependent cellular cytotoxicity; ANG2, angiopoietin 2; ASYM, asymmetric; BLT, bispecific ligand-directed toxin; bsAb, bispecific antibody; cH, common heavy; cL, common light; CRC, colorectal cancer; CRIB, charge repulsion-induced bispecific; CTLA4, cytotoxic T lymphocyte antigen 4; DART, dual-affinity re-targeting; DLL4, delta-like ligand 4; DVD, dual variable domain; EGFR, epidermal growth factor receptor; Fab, antigen-binding fragment; Fit-Ig, Fabs-in-tandem; FRAG, fragment-based; H, heavy; HER2, human epidermal growth factor receptor 2; Ig, immunoglobulin; KiH, knobs into holes; L, light; LAG3, lymphocyte-activation gene 3; LGR5, leucine-rich repeat-containing G protein-coupled receptor 5; NSCLC, non-small-cell lung cancer; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand 1; scFv, single-chain variable fragment; SYM, symmetric; TAA, tumour-associated antigen; TIM3, T cell immunoglobulin mucin 3; TNBC, triple-negative breast cancer; VEGF, vascular endothelial growth factor; V_{HH}, heavy chain-only variable domain. [a]Format data provided in the first bullet point in cells in the third column include the bsAb format number (#) in FIG. 2, technology trade name, class and valency; see FIG. 2 for additional information on format class (FRAG, SYM or ASYM) and valency. Engineering data provided in the second bullet point in cells in the third column include additional constant region mutations, which were obtained from public documents (scientific literature, abstracts, posters and patent publications). [b]Based on format disclosure for KN026.

Data that will become available from clinical studies will guide further optimization of bsAb concepts targeting immune checkpoints while ensuring an acceptable safety profile. The mechanisms of action of bsAbs targeting two checkpoint blocking molecules are expected to be mostly combinatorial, as the blocking activity may also be readily achieved by combining two mAbs. These bsAbs nevertheless may be designed with obligate features by combining low-affinity and high-affinity binding arms in a single molecule, for example, with an aim to increase safety or improve pharmacokinetics.

Alternatively, pathways that positively regulate T cell activation, such as the inducible T cell co-stimulator (ICOS; also known as CD278) and tumour necrosis factor receptor superfamily member 4 (TNFRSF4; also known as OX40 or CD134) pathways, are being targeted by bsAbs, including some that are in clinical development (TABLE 1). The required conditional activation of immune cells via checkpoint agonists can be achieved by the use of obligate bsAb concepts. INBRX-105 (PD-L1 × 4-1BB; also known as TNFRSF9 or CD137) is an example that is designed to only (re)activate T cells via the checkpoint agonist molecule 4-1BB in the tumour environment where it encounters PD-L1 while simultaneously abrogating inhibition via the PD-1–PD-L1 axis. Other concepts combine the immune regulatory binding arm with a tumour-targeting arm such as mesothelin (MSLN).

Beyond classical T cells: bridging alternative effector cell types. Despite their promise, CD3-based bsTCEs have demonstrated a number of disadvantages, including a potential high toxicity, particularly for targets with broad tissue expression, such as EGFR and EpCAM. Lutterbuese and colleagues[107], for example, reported a very steep dose–response with extreme toxicities at higher doses for an EGFR bsTCE in cynomolgus monkeys. EGFR bsTCEs were furthermore shown to be able to induce killing of $EGFR^-$ bystander cells[108]. Kebenko and colleagues[109] assessed safety and antitumour activity for solitomab (also known as AMG110), an EpCAM bsTCE in a phase I study in patients with solid tumours, and observed serious adverse events with dose-limiting toxicities across all dose levels that prevented escalation to therapeutic levels. A feature of CD3-based bsAbs is that they activate all T cells irrespective of lineage, which, next to provoking toxicity, may also limit efficacy. For example, Duell and colleagues[110] demonstrated that blinatumomab also activates regulatory T cells, thereby inhibiting cytotoxic T cell proliferation and tumour cell killing. High regulatory T cell numbers indeed were shown to predict non-responsiveness to blinatumomab in patients with r/r B cell ALL[110].

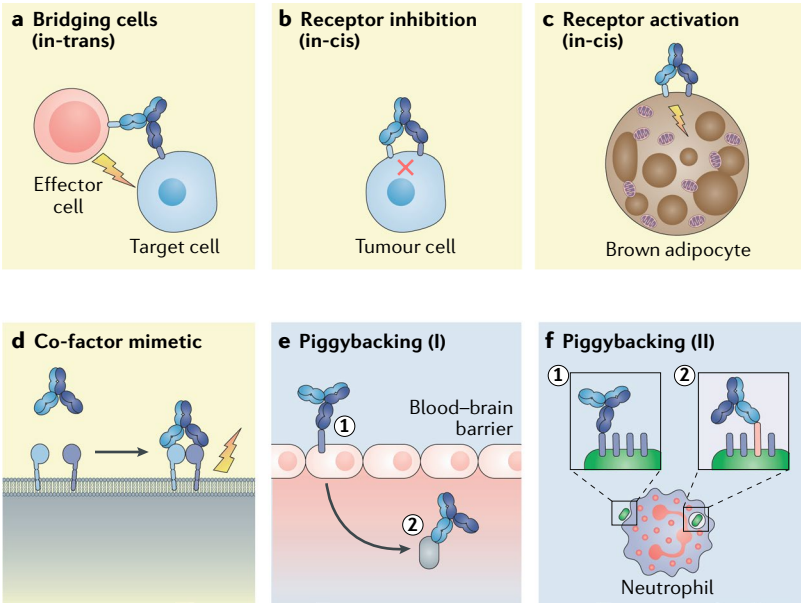

Fig. 5 | **Examples of obligate mechanisms of action of bsAbs. a** | Bridging cells is an important obligate mechanism of action in which the bispecific antibody (bsAb) brings cells in close proximity, leading to exclusive activation of the effector cell in the presence of the target cell. Examples include bispecific effector cell engagers that retarget cytotoxic T cells or natural killer (NK) cells for tumour cell killing. The concept is also being used to (re)activate T cell immunity in the tumour microenvironment by bridging tumour antigens with agonist checkpoint molecules. In infectious disease, T cell redirection with bsAbs is being employed to eliminate virus-infected cells. **b**,**c** | The bridging of receptors on cells (in-cis) is an obligate mechanism in which the crosslinking of specific cell surface receptors leads to their inactivation (for example, to reduce tumour growth in cancer applications) or activation (for example, to conditionally activate a growth factor receptor for the treatment of diabetes). **d** | BsAbs may be designed for the precise positioning of an enzyme and a substrate as a cofactor mimetic, such as a bsAb to replace a critical clotting factor in the treatment of haemophilia. **e**,**f** | Piggybacking allows the translocation of an active binding arm to an otherwise inaccessible compartment, such as central nervous disease targets protected by the blood–brain barrier (example I) or bacterial or viral antigens with a role in endosomal escape (example II). Yellow boxes show spatial obligate mechanisms, in which binding of the bispecific moieties occurs simultaneously. Blue boxes show temporal obligate mechanisms of action, in which binding of the bispecific moieties occurs sequentially.

These observations support the notion that recruiting specific T cell subsets for tumour cell killing could provide advantages over existing approaches. In this respect, Vγ9Vδ2 T cells are of particular interest, as they represent a potent class of pro-inflammatory cells involved in natural immune surveillance. The monomorphic Vγ9Vδ2 TCR senses the presence of phosphoantigens derived from infectious agents or from metabolic dysregulation that often occurs in tumour cells[111]. Vγ9Vδ2 T cells are prevalent in a large number of haematological and solid tumours, and their presence in tumours was shown to correlate with a favourable outcome[112,113]. The first bispecific γδTCEs targeting HER2 and EGFR have been reported[14,220]. The latter molecule induced lysis of patient-derived colorectal carcinoma cells and was shown to have minimal activity against primary EGFR$^+$ keratinocytes, thus holding the promise for an increased therapeutic window[14].

Other approaches are focusing on retargeting and activating NK cells, such as through a trispecific molecule consisting of a single-chain variable fragment (scFv) against CD16A (also known as FcγRIIIa) on NK cells connected to an anti-TAA scFv via an IL-15 linker[114,115]. Furthermore, a CD16 × HER2 bispecific molecule was shown to induce killing through both NK and γδT cells expressing the CD16 receptor[116]. AFM13, a TandAb against CD16A and CD30 that was able to trigger NK cell-mediated killing of CD30$^+$ NHL cells, represents another example[117,118]. This bsAb is composed of a linear array of four antibody variable fragments coupled via linkers, with two binding sites for CD30 situated between two binding sites for CD16A. The tumour cell CD30 antigen thus interacts with the middle of the molecule, while both ends of the molecule remain available for effector cell binding. It should be noted that CD16A also shows expression on circulating and tissue-resident macrophages and that the mechanism of action is therefore unlikely to be NK cell-specific.

Finally, CD47 (also known as IAP) has been investigated for its role in modulating effector cell-mediated killing. CD47 serves as a negative regulator of phagocytosis by inhibiting effector cells expressing signal regulatory protein-α (SIRPα; also known as SHPS1). However, the ubiquitous expression of CD47 makes it a difficult target. Dheilly and colleagues developed a solution using their κλ bsAb platform in which a low-affinity CD47 antibody is combined with a high-affinity antibody against a tumour antigen, which ensures that CD47 is engaged by the bsAb only on tumour cells co-expressing both antigens. Proof of concept was provided by a CD47 × CD19 bsAb that induced increased Fc-mediated phagocytosis and retained its activity in the presence of high amounts of non-tumour-associated CD47 (REF.[119]). Interestingly, the affinity-engineering approach allows the generation of an obligate bsAb targeting two molecules on the same cell, as a similar tumour specificity could not be achieved by simply combining the parental antibodies.

Beyond oncology: T cell redirection in infectious diseases. Virus-specific bsTCEs are designed to redirect CD8$^+$ T cells to infected cells expressing viral surface antigens and have been described in hepatitis B virus[120], cytomegalovirus[121] and HIV-1 infection[122]. In the case of HIV, gp120 envelope glycoprotein (Env)-specific bsTCEs (primarily assessed in fragment-based 1 + 1 formats) were able to induce killing in vitro of cells (CD4$^+$ T cells and macrophages) infected with diverse HIV isolates and were shown to inhibit HIV replication ex vivo in HIV-infected cells obtained from subjects on continuous anti-retroviral therapy[123–126]. In addition, engagement of the CD3 receptor present on latently infected cells could reactivate viral replication, thereby inducing the expression of viral antigens and enabling the CD8$^+$ T cells to also kill these cells[123]. Together, these studies show the therapeutic potential of HIV-specific bsTCEs for the in vivo elimination of HIV, especially in combination with complementary interventions such as latency-reversing agents to address virus reservoirs[125–127]. One such bsTCE, MGD014 (an asymmetric 1 + 1 format), is being evaluated in a phase I study in HIV-infected individuals on anti-retroviral therapy (TABLE 3).

Beyond oncology: T cell redirection in regenerative medicine. Intravenous administration of stem and progenitor cells after ischaemia–reperfusion (IR) injury in order to restore tissue damage has shown therapeutic potential in acute myocardial infarction models[128,129]. The low homing efficiency of these cells to the site of injury, however, is considered one of the major limitations for successful clinical translation[130]. Enhancing the targeted delivery of stem and progenitor cells is thought to improve the efficacy of tissue regeneration, and bsAb-based redirection approaches are thus currently under investigation[131,132].

As activated platelets accumulate in the heart after IR injury, these are targeted for delivery of the cells with regenerative potential. In this context, the active conformations of the major platelet integrin glycoprotein (GP) GPIIb/IIIa (also known as αIIb/βIIIa or CD41/CD61) or CD41 alone have been investigated as targeting antigens[131,132]. The bsAb-mediated delivery of endothelial progenitor cells, through binding to stem cell marker CD34, to $CD41^+$ platelets resulted in effective heart repair in a mouse model of acute myocardial infarction, as confirmed by cardiac function, heart morphometry and immunohistochemistry[131]. The delivery of a subset of PBMCs to the site of IR injury, through a bsAb targeting stem cell antigen 1 (SCA1, also known as LY6A.2/LY6E.1; a murine haematopoietic stem cell marker) and the activated conformation of GPIIb/IIIa on platelets, resulted in a significant decrease in infiltrating inflammatory cells[132]. These $SCA1^+$ PBMCs decreased fibrosis, increased capillary density and restored cardiac function, supposedly through the modulation of cardiac repair mechanisms.

Bridging receptors (in-cis binding)

Targeted inhibition of oncogenic receptor tyrosine kinases (RTKs), such as EGFR and HER2, is a successful anticancer approach, but the development of resistance is a major limitation of such therapies. Resistance often involves upregulation of other RTKs that bypass specific receptor inhibition to activate parallel signalling pathways. For instance, upregulation or activation

Table 3 | **Clinical-stage bsAbs for non-cancer indications: obligate concepts**

BsAb names (sponsors)	Targets	Format[a] and engineering	Disease area (selected indications)	Status (selected trials)
Bridging cells (in-trans): T cell redirection and/or activation				
MGD014 (Macrogenics)	CD3 x HIV-1 Env	• Tandem domain-exchanged Fv-Fc(G1) (#17, DART-Fc, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), L234A-L235A (Fc-silencing), H435R (purification)	HIV-1 infection	Phase I (NCT03570918)
Bridging receptors (in-cis)				
MGD010, PRV-3279 (Macrogenics)	CD32b x CD79b	• Tandem domain-exchanged Fv (#3, DART, FRAG, 1 + 1)	Immune-mediated disorders (phase I in healthy volunteers)	Phase I (NCT02376036)
BFKB8488A, RG7992 (Genentech)	FGFR1 x KLB	• Hetero H, HL assembly IgG1 (#12, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), N297G (Fc-silencing)	Diabetes (phase I in overweight volunteers with likely insulin resistance, patients with type 2 diabetes mellitus and patients with NAFLD)	Phase I (NCT02593331 and NCT03060538)
Cofactor mimetic				
Emicizumab, Hemlibra, ACE910, RO5534262 (Chugai, Roche)	FIXa x FX and/or FXa	• Hetero H, cL IgG4 (#11, ART-Ig, ASYM, 1 + 1) • Hetero HH: E356K x K439E, HL-pairing: cL, S228P (hinge-stabilization), G446del-K447del (reduction charge-heterogeneity), K196Q-F296Y (pI-engineering), H435R (purification)	Routine prophylaxis of patients with haemophilia A with and without FVIII inhibitors	Marketed
Piggyback				
MEDI3902 (AstraZeneca)	Psl x PcrV	• Fab-scFv-Fc(G1) (#22, SYM, 2 + 2)	Prevention of *Pseudomonas aeruginosa* pneumonia in mechanically ventilated subjects	Phase II (NCT02255760 and NCT02696902)
Piggyback (bispecific molecules for half-life extension)				
Vobarilizumab, ALX-0061 (Ablynx)	IL-6 R x HSA	• Tandem V_{HH} (#1, nanobody, FRAG, 1 + 1, anti-HSA for half-life extension)	SLE and rheumatoid arthritis	Phase II (NCT02518620 and NCT02437890)
Ozoralizumab, ATN103 (Ablynx)	TNF x HSA	• Tandem V_{HH} (#7, nanobody, FRAG, 1 + 2, anti-HSA for half-life extension)	Rheumatoid arthritis	Phase II (NCT01063803)

Data available as of 1 March 2019. ART-Ig, asymmetric reengineering technology-immunoglobulin; ASYM, asymmetric; bsAb, bispecific antibody; cL, common light; DART, dual-affinity re-targeting; Env, gp120 envelope glycoprotein; Fab, antigen-binding fragment; FGFR1, fibroblast growth factor receptor 1; FIXa, activated coagulation factor IX; FRAG, fragment-based; HSA, human serum albumin; Ig, immunoglobulin; IL-6R, IL-6 receptor; KiH, knobs into holes; KLB, β-klotho; NAFLD, non-alcoholic fatty liver disease; PcrV, *Pseudomonas aeruginosa* needle tip protein of the serotype-independent type III secretion system; pI, isoelectric point; Psl, *P. aeruginosa* persistence factor; scFv, single-chain variable fragment; SLE, systemic lupus erythematosus; SYM, symmetric; TNF, tumour necrosis factor; V_{HH}, heavy chain variable domain. [a]Format data provided in the first bullet point in cells in the third column include the bsAb format number (#) in FIG. 2, technology trade name, class and valency; see FIG. 2 for additional information on format class (FRAG, SYM or ASYM) and valency. Engineering data provided in the second bullet point in cells in the third column include additional constant region mutations, which were obtained from public documents (scientific literature, abstracts, posters and patent publications).

of the tyrosine-protein kinase MET pathway confers resistance of non-small-cell lung cancer (NSCLC) tumours to treatment with EGFR tyrosine kinase inhibitors[133]. This provided the rationale for the development of bsAbs that co-target multiple RTKs (FIG. 5b), a number of which are in clinical studies (TABLES 1,2). Although targeting multiple RTKs is a combinatorial concept, some molecules display obligate features. For instance, JNJ-61186372 (Janssen Pharmaceuticals) is an EGFR × MET bsAb generated through cFAE that blocks EGFR and MET signalling through inhibition of ligand-induced activation and receptor degradation[134]. JNJ-61186372 has antibody-dependent cellular cytotoxicity (ADCC) activity, which has been increased by producing the antibody with a low-fucose-containing Fc carbohydrate[134]. In addition to ADCC, Fc interactions appeared to be required for downmodulation of EGFR and MET[135]. Recently, preliminary clinical activity against EGFR-driven NSCLC was reported, while the safety profile was manageable[136].

The most effective and potent bispecific antibodies cannot be predicted on the basis of the characteristics of the parental antibodies. Therefore, the selection of the best bsAb is often performed empirically by functional screening of panels of bsAbs generated from a matrix of antibody pairs. JNJ-61186372 was selected from a panel of EGFR × MET bsAbs on the basis of superior functional activity. The importance of selecting the optimal Fab arm combination is demonstrated by the fact that JNJ-61186372 was the only bsAb in the screen with all desired activities, including the absence of undesired MET and/or EGFR receptor activation[135]. JNJ-61186372 indeed represents an interesting example of how the activity of a bsAb may differ from a mixture of the parental antibodies. Bivalent MET antibodies induce MET crosslinking and tumour cell activation, thwarting their use as cancer therapeutics. Combination of a single (non-activating) MET-binding arm with an appropriate EGFR-binding arm in an asymmetric 1 + 1 design ensured the generation of a molecule with an ability to block both EGFR and MET signalling. In another example, unbiased phenotypic screening of a panel of 545 bsAbs against HER2 and HER3 identified a HER2 × HER3 bsAb (PB4188) that potently inhibited tumour cell growth in vitro and in vivo[137]. This antibody binds with high affinity to HER2, thereby increasing the local concentration of HER3 Fabs, which results in binding to HER3 and inhibition of heregulin (HRG)-mediated activation of HER2 and/or HER3 signalling, even at high HRG concentrations[137]. By contrast, combinations of HER2 and HER3 mAbs were unable to inhibit HER3 signalling at high HRG concentrations. Zenocutuzumab (MCLA-128), which is the ADCC-enhanced clinical candidate derived from PB4188, is currently undergoing clinical evaluation in various indications for which HRG–HER3 pathway activation has been reported (TABLE 1).

Targeting ligand redundancy

Targeting redundancy for multiple growth or angiogenesis factors represents an area of interest for bsAbs. The bsAbs in this group are mostly combinatorial in nature. Dilpacimab (also known as ABT-165), a bsAb with dual variable domains (DVD-Ig) that bind vascular endothelial growth factor (VEGF) and delta-like ligand 4 (DLL4)[138], has progressed furthest. It is in a phase II clinical trial, in which it is being compared with the anti-VEGF mAb bevacizumab in patients with previously treated metastatic colorectal cancer who are also receiving chemotherapy (TABLE 2).

In non-cancer indications, romilkimab, composed of an anti-IL-4 antibody fused with a variable domain targeting IL-13, is being evaluated in a phase II study of patients with diffuse cutaneous systemic sclerosis (TABLE 4). The only bsAb in phase III studies, faricimab (also known as RO6867461), is a human IgG1-based CrossMab that binds VEGF with one arm and angiopoietin 2 with the other arm. All four of the protein chains comprising this molecule are different. In addition, to optimize for use in ophthalmic indications, the Fc region was modified to abolish binding to IgG Fc receptors including FcRn, which eliminated effector functions and increased systemic clearance[139]. Faricimab is currently in two phase III studies of patients with diabetic macular oedema and was previously evaluated in three phase II studies in neovascular age-related macular degeneration (TABLE 4). Patients with vision loss from diabetic macular oedema treated with faricimab demonstrated clinically meaningful visual acuity gains compared with baseline and statistically significant improvements in visual acuity compared with patients treated with the approved anti-VEGF mAb fragment ranibizumab.

Biparatopic bsAbs

Instead of targeting two different proteins, bsAbs may be designed to simultaneously bind to two non-overlapping epitopes on the same target. Biparatopic targeting builds on increasing binding strength through antigen crosslinking and aggregation, thereby mimicking effects observed for antibody mixtures and polyclonal antibodies. Biparatopic bsAbs are therefore essentially a combinatorial concept.

ZW25 is a bsAb designed to biparatopically bind to HER2 to increase avidity, resulting in more potent silencing of HER2 signalling (TABLE 2). In addition, it causes the removal of HER2 protein from the cell surface, and it has enhanced effector function. These combined mechanisms of action translated into antitumour activity in preclinical models that was more potent than that induced by trastuzumab[140]. Preliminary clinical data showed manageable safety and promising early efficacy in patients with advanced HER2$^+$ cancers, including gastroesophageal and breast cancers[141]. A biparatopic antibody–drug conjugate based on the ZW25 backbone, which may benefit from more effective toxin release through improved internalization and degradation in lysosomes, as described by Li and colleagues[142], is also being developed.

Agonistic bispecific antibodies

As opposed to blocking pathogenic signalling by inhibitory antibodies, some therapeutic concepts require the activation of receptor signalling by agonistic antibodies, such as that described for checkpoint agonists above. Obligate bsAbs are also particularly suited to activate

Table 4 | **Clinical-stage bsAbs for non-cancer indications: combinatorial concepts**

BsAb names (sponsors)	Targets	Format[a] and engineering	Disease area (selected indications)	Status (selected trials)
Targeting ligand redundancy				
AMG570, MEDI0700 (Amgen, AstraZeneca)	BAFF x B7RP1	• IgG–peptide fusion; IgG with carboxy-terminal BAFF-binding peptide (SYM, 2 + 2)	SLE and rheumatoid arthritis	Phase I (NCT02618967 and NCT03156023)
Tibulizumab, LY3090106 (Eli Lilly)	BAFF x IL-17A	• IgG4-$(scFv)_2$ (#21, SYM, 2 + 2) • S228P (hinge-stabilization)	Sjögren syndrome	Phase I (NCT03736772 and NCT02614716)
RO7040547, BITS7201A, RG7990 (Genentech)	IL-17 x IL-13	• Undisclosed	Asthma	Discontinued after phase I[b]
IL-23 × CGRP bsAb (Eli Lilly)	IL-23 x CGRP	• Undisclosed	Autoimmune diseases	Phase I
Romilkimab, SAR156597 (Sanofi)	IL-4 x IL-13	• Tandem Fv-IgG4 (#23,DVD-Ig, SYM, 2 + 2) • S228P (hinge-stabilization), L235E (Fc-silencing)	Diffuse cutaneous systemic sclerosis	Phase II (NCT02921971)
MEDI7352 (AstraZeneca)	NGF x TNF	• TNFR2-Fc fusion with carboxy-terminal anti-NGF scFv (SYM, 2 + 2)	Painful osteoarthritis of the knee and painful diabetic neuropathy	Phase II (NCT02508155 and NCT03755934)
Faricimab, RO6867461, RG7716 (Roche)	VEGFA x ANG2	• IgG1 (#15, CrossMab, ASYM, 1 + 1) • Hetero HH: T366W x T366S-L368A-Y407V (KiH), HL-pairing: CrossMab, L234A-L235A-P329G (Fc-silencing), I253A-H310A-H435A (FcRn knockout to increase plasma clearance)	Neovascular wet age-related macular degeneration and diabetic macular oedema	Phase III (NCT03622593, NCT03622580, NCT03823300 and NCT03823287)

Data available as of 1 March 2019. ANG2, angiopoietin 2; ASYM, asymmetric; B7RP1, B7-related protein 1; BAFF, B cell-activating factor; bsAb, bispecific antibody; CGRP, calcitonin gene-related peptide; DVD, dual variable domain; FcRn, neonatal Fc receptor; Ig, immunoglobulin; KiH, knobs into holes; NGF, nerve growth factor; scFv, single-chain variable fragment; SLE, systemic lupus erythematosus; SYM, symmetric; TNF, tumour necrosis, factor; VEGFA, vascular endothelial growth factor A. [a]Format data provided in the first bullet point in cells in the third column include the bsAb format number (#) in FIG. 2, technology trade name, class and valency; see FIG. 2 for additional information on format class (SYM or ASYM) and valency. Engineering data provided in the second bullet point in cells in the third column include additional constant region mutations, which were obtained from public documents (scientific literature, abstracts, posters and patent publications). [b]Molecules active in 2018 that were discontinued by March 2019.

multicomponent receptor complexes in which concurrent binding of a receptor and co-receptor is required for activation (FIG. 5c).

Activation of the fibroblast growth factor 21 (FGF21) pathway has been reported to ameliorate obesity and diabetes[143,144]. However, recombinant FGF21 has poor pharmacokinetic properties, and chronic treatment carries risks of adverse effects[145]. The agonistic bsAb BFKB8488A (Roche) has therefore been designed to activate this metabolic pathway by selectively targeting the fibroblast growth factor receptor 1C (FGFR1C)–β-klotho (KLB) receptor complex[146]. While KLB is selectively expressed in liver, adipose and pancreas tissues, FGFR1C has broad tissue distribution[147]. Hence, co-targeting of these receptors may restrict signalling activation only to tissues that co-express KLB and FGFR1C and limit undesirable consequences of broad FGFR activation, such as induction of cell proliferation. Preliminary results from an ongoing first-in-human trial showed an improvement of the cardio-metabolic profile in obese subjects with insulin resistance[148] (TABLE 3).

Cofactor mimetics

BsAbs against enzyme–substrate complexes that act as enzymes or cofactors provide a strong yet challenging obligate opportunity in which spatial aspects — that is, optimal position of the enzyme and substrate — are critical (FIG. 5d). Sampei and colleagues set out to generate a bsAb to replace FVIII as a potential treatment for haemophilia A in order to prevent episodes of bleeding resulting from FVIII dysfunction. The bsAb was envisaged to mimic FVIIIa, the activated form of FVIII, in its ability to bring FIXa and FX together and thereafter enhance the catalytic activity of FIXa[149]. In an extensive screening effort, 40,000 asymmetric bsAbs were generated from 200 mAbs against FIXa or FX, resulting in a hit rate of bsAbs with FVIII mimetic activity of ~0.3%. To resolve the chain-association issue, each of the hits was expressed with only one of the cognate L chains, after which the most potent bsAb was selected and a common L chain further optimized by framework region and complementarity-determining region (CDR) shuffling. Additional rounds of optimization included humanization to reduce immunogenicity risk, CDR mutagenesis to further increase enzyme activity, charge engineering of the variable region to improve solubility and pharmacokinetics, and isoelectric point engineering to facilitate separation of the bsAb from homodimeric contaminants in ion-exchange purification[149]. The final candidate, termed ACE910 or emicizumab, exhibited a micromolar binding affinity for both FIXa and FX and/or FXa such that in plasma only a small fraction forms the active trimolecular complex at active doses of the drug (50–90 μg per millilitre)[150–152]. Prophylaxis of patients with haemophilia A with emicizumab was shown to effectively reduce bleeding in patients with and without inhibitors (that is, anti-FVIII antibodies)[151,152]. Emicizumab was approved by the FDA for routine prophylaxis to reduce bleeding in patients with haemophilia A with FVIII inhibitors in 2017 (TABLE 3). A further approval in October 2018 also included

prophylaxis with emicizumab in patients without FVIII inhibitors. Emicizumab was first approved in Europe in March 2018.

Piggyback approaches

Approaches that exploit the first specificity of a bsAb purely as a transport modality for the second specificity are obligate in nature and require sequential binding. These approaches, termed 'piggyback' or 'hijacking' approaches, have been described for gaining access to (or escaping) restricted (cellular) compartments. One of the first examples of the specific delivery of proteins using the hijacking concept was described by Raso and colleagues[153], who demonstrated that chemically linked bispecific Fabs against the B cell receptor and ricin A induced toxicity by mediating internalization of the toxin. Later studies demonstrated that the delivery of (mutant) diphtheria toxins with bsAbs displaying pH-dependent binding could be used to increase toxin release in endosomes (at low pH), thereby improving cell killing[154].

A more recent example is the hijacking of the transferrin transcytosis pathway to cross the blood–brain barrier and gain access to the immune-privileged brain compartment[155] (FIG. 5e). Through targeting of the transferrin receptor (TfR) with one binding arm of the bsAb, investigators have demonstrated enhanced brain delivery of a second binding arm specific for β-secretase 1 (BACE1) in preclinical mouse and monkey models for Alzheimer disease[156]. Using a TfR affinity-optimized asymmetric 1 + 1 format, the TfR × BACE1 bsAb was shown to reduce amyloid-β (Aβ) peptide levels, the enzymatic product of BACE1, in both brain tissue and cerebral spinal fluid[156]. Similarly, an asymmetric TfR × Aβ bsAb (with 1 + 2 design), was shown to significantly reduce plaque numbers in both the brain cortex and hippocampus in a chronic mouse model of Alzheimer disease pathology[157].

A third piggyback approach has been described in Ebola virus (EBOV) infection, where a broadly conserved and extracellularly exposed epitope, present on the uncleaved EBOV GP, was targeted to gain access to the endosomal compartment during viral uptake[53]. Sequestered in the late endosome, proteolytic cleavage of GP then reveals the cryptic receptor binding site (RBS) for the Niemann–Pick C1 (NPC1) intracellular receptor, which is required to enter the cytoplasm. Thus, through endosomal delivery of broadly neutralizing second binding domains, against either the cryptic RBS or NPC1, by the GP-specific first binding domain, Wec and colleagues demonstrated that bsAbs could neutralize all known EBOV strains in vitro and could confer post-exposure protection against lethal challenge with multiple viruses in mice[53]. Both symmetric 2 + 2 and asymmetric 1 + 1 formats were shown to be effective in this approach, although the 1 + 1 design displayed reduced potency, possibly owing to loss of avidity.

The Psl × PcrV bsAb MEDI3902, a full-length IgG1 antibody with scFvs inserted between the Fabs and the Fc in a symmetric 2 + 2 format[158] (TABLE 3), has been shown to employ a similar piggyback mechanism to enhance the killing of *Pseudomonas aeruginosa* by neutrophils (FIG. 5f). This bsAb targets the persistence factor Psl and the needle tip protein PcrV of the serotype-independent type III secretion system virulence factor of *P. aeruginosa*. Psl is a major extracellular polysaccharide and *P. aeruginosa* biofilm component that has been implicated in inhibiting complement deposition and reducing recognition and phagocytosis of bacteria by neutrophils. Inhibition of Psl by MEDI3902 indeed led to increased internalization by neutrophils. PcrV plays a role in reducing phagosome acidification and bacterial killing. PcrV is expressed by internalized bacteria as a component of the type III secretion injectisome in which it has a role in *P. aeruginosa* survival by preventing relocation to acidic vacuoles. The anti-PcrV arm of MEDI3902 inhibits PcrV activity and increases localization of the ingested bacteria to such low pH vesicles. The physical linkage between anti-Psl and anti-PcrV in the bsAb therefore facilitates increased access of anti-PcrV by piggybacking on bacteria via the anti-Psl binding arm[159]. In a mouse model of bacteraemic infection, the bsAb provided enhanced protection compared with the combination of an anti-PcrV and an anti-Psl antibody, suggesting that bispecificity does indeed confer a mechanistic advantage[160]. The observation that MEDI3902 also provided protection against strains lacking the PcrV virulence factor[161], however, demonstrates that this obligate feature is not absolute.

By dual-targeting CD63 (also known as LAMP3), involved in lysosomal trafficking, and HER2, a model antigen for tumour specificity, the intracellular delivery of an antibody–drug conjugate to the lysosome could be improved[162]. In this case, the tumour-specific arm (HER2) together with a low-affinity CD63-binding arm was used to ensure tumour specificity (also see cross-arm binding efficiency) and reduce toxicity. Indeed, by hijacking lysosomal trafficking in this way, the investigators demonstrated efficacy, both in vitro and in vivo in a xenograft mouse model, only if both targets were expressed and targeted[162].

Finally, escaping the endosomal compartment through dual-targeting or multi-targeting represents a piggyback approach that is pursued for fragment-based formats to overcome the lack of an Fc region. For example, vobarilizumab and ozoralizumab are H chain-only variable domain (V_{HH})-based bsAbs currently in phase II studies, targeting the IL-6 receptor and tumour necrosis factor, respectively (TABLE 3). To extend half-life, these molecules additionally target HSA in order to hijack the FcRn salvage pathway and escape lysosomal degradation, a property normally provided by the antibody Fc region[163,164]. The designation as obligate arguably is somewhat arbitrary, as sustained functionality of these bsAbs might also be obtained by continuous dosing (as for blinatumomab) or by expressing the tandem V_{HH} with an Fc fragment.

Advancing dual-targeting concepts

Compared with therapeutic mAbs, the increased complexity associated with dual-targeting concepts can provide additional challenges during the different stages of discovery and development. Recent advances

Cross-arm binding efficiency
An increase in apparent affinity when a bispecific antibody binds to the second target or receptor following its binding to the first target or receptor on the same cell.

in addressing selected challenges, benefiting the overall advancement of bsAbs, are discussed in this section.

How format and design affect development strategy

The bsAb format class can influence the repertoire of antigen-binding domains to be explored in dual-targeting concepts and vice versa. For instance, formats requiring a common L (cL) chain or cH chain may limit the use of (pre-existing) antibody panels to those combinations that tolerate these restrictions. As a consequence, complementary technologies, such as cL chain transgenic animals[165–167] and cL chain[168] or cH chain[45] phage-display libraries, have been developed to compensate for these limitations and increase antibody repertoire during the discovery phase. Similarly, antibody fragments may not consistently yield full-length IgGs with similar (functional) properties upon reformatting, thus limiting their value as antibody sources in most asymmetric formats. However, the compatibility of antibody fragments with library selection approaches to tailor defined antibody properties (such as affinity maturation, modulating species cross reactivity and removal of potential manufacturing liabilities) represents a very attractive feature during development[169]. To utilize the advantages of library selection in full-length antibody-based formats, strategies that integrate the reformatting of entire antibody fragment repertoires early in the discovery pipeline are being explored[170].

The development of dual-targeting concepts that require simultaneous binding to both targets is not straightforward, as design parameters (such as affinity, valency, epitope specificity and format architecture) may display interdependency. For instance, when targeting two membrane antigens on the same cell, the valency of target engagement can affect not only monospecific interaction through avidity but also the interaction with the second specificity through cross-arm binding efficiency[171,172]. Furthermore, format architecture and relative paratope orientation can additionally influence optimal target engagement (dependent on the relative epitope topology or target distribution) and could affect therapeutic activity[27,173]. Thus, for these applications, the selection (and optimization) of the right combination of binding arms could require screening of up to thousands of binding pairs for the right biological activity[137,149,174]. This requires an integrated discovery process that interrogates the full array of design parameters and target binding repertoires through empirical selection strategies to facilitate the successful identification of the most effective bsAbs. Screening in the final product format or in formats that can easily be adapted to the final product format is thought to further expedite successful development[51,137].

By contrast, in applications where the therapeutic activity of both specificities occurs sequentially and does not require simultaneous binding, the relative paratope orientation is likely to play a subordinate role and can thus be addressed by bsAb formats of different design[53]. Moreover, the independent activity of both specificities allows for selection and optimization of individual binding arms during the discovery phase, followed by designed recombination in different bsAb format classes and final product selection based on the desired end-product requirements.

Additional considerations affecting the selection of bsAb format may include the flexibility of the format to be used with alternative IgG isotype backbones (for example, IgG2 (REFS[175,176]) or IgG4 (REFS[46,94,177])) to reduce Fc-mediated toxicity. Moreover, compatibility of the bsAb format with additional optimization strategies, established for regular antibody-based therapeutics, could further contribute to their successful development. In this context, Fc-engineering strategies that alter the pharmacokinetic properties[139], tailor the functional profile (for example, glycoengineering to enhance ADCC[134,178] or introducing mutations to silence effector functions[139,179]) or facilitate the manufacturing process (for example, isoelectric point engineering[94,149,180] or removing charge heterogeneity[179]) have all been described.

Translational tools to increase success

Establishing proof of concept in vivo is an important step in the translational stage of clinical development of therapeutic (bs)Abs. With increasingly complex (dual-targeting) concepts, however, the presence of an intact immune system and disease tissue microenvironment, including disease-associated immune cells, is essential to accurately capture the relevant biology being targeted. As a consequence, the efficacy and safety of these complex concepts are often studied in immuno-competent mice using syngeneic models in combination with (surrogate) mouse antibodies to maximize the (potential) use of effector functions and avoid anti-drug antibody responses[181–183]. In addition, genetically engineered mouse models, in which the relevant human targets are introduced or the equivalent mouse targets replaced by their human counterparts, are being explored to complement in vivo validation efforts[94,97,184].

In the case of surrogate bsAbs, fragment-based formats can easily be used in translational studies[185–188]; however, their poor pharmacokinetic properties and lack of Fc-mediated effector functions restrict their translational value to therapeutic concepts that tolerate these limitations. Surrogate symmetric[189] and asymmetric[63,97,190] bsAb formats, on the basis of a single murine subclass, are expected to have regular pharmacokinetic properties and potentially the most native functional characteristics. Using such surrogate bsAbs, investigators have modelled different translational aspects of T cell redirection, such as the requirement for an inert backbone[63], the effect of CD3 affinity on biodistribution[72], the mechanisms of T cell recruitment[191] or a rationale for combination therapy[97].

Beyond dual-targeting

Adding extra specificities to bispecific molecules can introduce additional functions, potentially increasing their therapeutic activity. Accordingly, multi-specific antibody-based formats, containing three or more different antigen-binding sites, are being explored in different therapeutic areas.

From a design point of view, the most straightforward approach to creating multi-specificity is to engineer additional antibody fragments into the existing fragment-based or symmetric bsAb formats[192–198]. Alternatively, however, symmetric designs can be combined with asymmetric H chain heterodimerization or forced HL chain pairing strategies to introduce the extra specificities[199–203]. More elaborate engineering efforts, such as combining multiple forced HL chain pairing strategies in one molecule, have also been described[204].

In oncology, simultaneous targeting of more disease-mediating receptors or crosstalking signalling cascades is thought to increase the chance of effectively addressing receptor redundancy or heterogeneity and lower the risk of escape. As a consequence, many multi-specific formats have adopted this concept and target multiple members of the same receptor families, such as EPHA2, EPHA4 and EPHB4 (REF.[192]) or EGFR and HER3 in combination with insulin-like growth factor 1 receptor (IGF1R) and MET[199] or HER2 and VEGF[203]. As with bsAbs, however, one could argue that these concepts are not strictly obligate and could be achieved by combination therapies. From a development point of view, however, the argument that regulatory approval for one molecule is faster (and more cost-effective) than seeking approval for each individual antibody becomes more relevant for multi-specific formats but should still be offset against the lack of freedom in dosing of the individual components for optimal efficacy. The significantly increased toxicity with limited efficacy benefit observed with combination therapies of cetuximab and bevacizumab in the clinic, for example, highlights dosing challenges that may be difficult to address in multispecific formats.

Multi-specificity is also being explored as a treatment option for viral diseases to cover genetic diversity and prevent acquired resistance. In HIV-1 infection, trispecific (1 + 1 + 1) formats exhibited higher neutralizing potency and exceptional breadth (> 98% coverage) compared with (combinations of) single broadly neutralizing antibodies or bsAbs in vitro[196,200,201]. Similar results were obtained in EBOV infection[195]. One HIV-1 study further demonstrated in vivo protection by a trispecific anti-HIV-1 antibody against a mixture of two differentially sensitive chimeric simian–human (SH)IV isolates in non-human primates[201].

What is on the horizon?

Looking ahead, promising conceptual innovations that are currently in the early stages of development represent exciting possibilities for the near future. One example of such a novel concept, being pioneered by Keyt and colleagues[205], exploits the natural architecture of antibody classes carrying a J chain, such as IgM and IgA, to which an effector cell-targeting arm is attached. This would allow for bispecific formats with 1 + 10 design in the case of pentameric IgM (or formats with 1 + 4 design in the case of dimeric IgA), which could mediate high-avidity targeting of pathogenic drivers expressed at very low levels. Industrial-scale production of IgM, however, still remains technically challenging[206].

Another promising concept is the non-protein delivery of therapeutic bsAbs using either mRNA-encoded or DNA-encoded formats[120,207,208]. For instance, Stadler and colleagues[208] achieved sustained in vivo production of an optimized, nucleoside-modified mRNA encoding a fragment-based bsTCE (a 1 + 1 design targeting CD3 and tight-junction protein claudin 6 (CLDN6)). Through polymer-based and/or lipid-based formulation, the investigators ensured targeting and translation in the liver after intravenous administration and demonstrated elimination of advanced tumours as effectively as the corresponding purified protein-delivered bsAb. Likewise, Petal et al.[207] showed sustained in vivo expression of a DNA-encoded symmetric bsAb format upon intramuscular administration and electroporation. In this study, the DNA-delivered version of MEDI3902 (a symmetric 2 + 2 design targeting *P. aeruginosa* proteins PcrV and Psl[159]; discussed above) exhibited indistinguishable potency compared with its protein-delivered version and protected against lethal challenge in a pneumonia mouse model. Because manufacturing of pharmaceutical-grade mRNA and DNA is fast, the investigators argue that this approach could accelerate clinical development of novel bsAbs. Furthermore, in the case of DNA, the temperature stability would allow for long-term storage, easier transport and thus administration to broader populations. The concept of DNA-mediated antibody transfer has particular promise for infectious disease applications with respect to ease of the supply chain, the potential for long-term in vivo antibody production and cost-effectiveness[209]. Genetically transferred obligate bsAbs introduce new opportunities for targeting viral or bacterial vulnerabilities and thereby may provide useful alternatives for vaccines against challenging microorganisms.

In another interesting combination approach, the combination of an oncolytic adenovirus armed with a bsTCE transgene (targeting CD3 and EGFR) and a folate receptor-α (FRα)-specific CAR T cell was shown to display synergistic antitumour effects in vivo[210]. In this study, Wing and colleagues[210] demonstrated enhanced T cell activation and prolongation of survival in mouse models of cancer, providing a rationale for additional clinical evaluation of this concept.

Conclusions

Therapeutic bsAbs are a rapidly expanding group of diverse molecules. Two bsAbs have obtained regulatory approval and are currently marketed: blinatumomab, a fragment-based bsTCE in cancer; and emicizumab, a full-size bispecific IgG with natural architecture used as an enzyme-cofactor replacement in a bleeding disorder. Over 85 bsAbs are progressing through clinical development in a wide variety of indications. Currently, there is a strong focus on cancer, which can in part be explained by the high potential of bsAbs in immuno-oncology approaches, with 43 bsTCEs and 15 bsAbs targeting immune checkpoint molecules in development.

BsAbs come in many formats (FIG. 2), which affect manufacturing, valency, Fc-mediated effector functions and in vivo half-life. Choosing the right bsAb format is

therefore strongly guided by the desired target product profile and clinical indication. Despite the wide variety, it appears quite useful to categorize bsAbs from a mechanistic perspective. BsAb design can be combinatorial, in which the bsAb is intended as an alternative for an antibody mixture (for example, to reduce cost), but a drawback of this approach is that the ratio of antibody binding domains is fixed early in development, and so toxicity, pharmacokinetics and pharmacodynamics cannot be optimized by discordant dosing. Obligate bsAbs are those in which the physical linkage of binding domains creates a novel functionality; that is, a function which cannot be accomplished by an antibody mixture. In temporal obligates, the mechanism of action is mediated by sequential binding events in which binding of the first domain facilitates activity of the second domain, for example, by providing access to a distal site. In spatial obligates, the mechanism of action depends on simultaneous binding in which the bsAb mediates its positioning, for example, in targeting an effector cell to a tumour or an enzyme to its substrate. The design of spatial obligates is particularly challenging, as it requires the precise 3D positioning of the two binding domains with their target epitopes. Unbiased phenotypic screening of large bsAb libraries may be required to obtain optimal candidates. It should be noted, however, that the mechanistic distinction between bsAbs is not absolute, and bsAbs in development therefore represent a spectrum from combinatorial to temporal and spatial obligate mechanisms.

In conclusion, bsAbs provide exciting opportunities for novel drug design and development, and we anticipate that the continuing developments of the platforms and concepts described above will provide a lasting therapeutic impact.

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Author contributions
All authors contributed substantially to all aspects of the article.

Competing interests
A.F.L. and M.L.J. are employees of Genmab, a biotechnology company that develops therapeutic antibodies including bispecific antibodies and bispecific antibody technology. They own warrants and/or stock. P.W.H.I.P. is an employee of Lava Therapeutics, a start-up biotechnology company that develops therapeutic antibodies including bispecific antibodies and bispecific antibody technology. He obtains stock options as part of his employment.