Bispecific antibodies (bsAbs) combine the functionality of two antibodies in one molecule. Two bsAb-drugs are currently on the market (one recently approved) and more are in clinical development. Driven by large pharma, bsAbs are emerging as next-generation biologics.

Bispecific antibodies

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Bispecific antibodies (bsAbs) combine specificities of two antibodies and simultaneously address different antigens or epitopes. BsAbs with 'two-target' functionality can interfere with multiple surface receptors or ligands associated, for example with cancer, proliferation or inflammatory processes. BsAbs can also place targets into close proximity, either to support protein complex formation on one cell, or to trigger contacts between cells. Examples of 'forced-connection' functionalities are bsAbs that support protein complexation in the clotting cascade, or tumor-targeted immune cell recruiters and/or activators. Following years of research and development (R&D), the first bsAb was approved in 2009. Another bsAb entered the market in December 2014 and several more are in clinical trials. Here, we describe the potentials of bsAbs to become the next wave of antibody-based therapies, focusing on molecules in clinical development.

Recombinant bispecific antibodies

The concept of recombinant bispecific prototype immunoglobulin (Ig)-G-like antibodies was devised more than two decades ago. Morrison and colleagues fused flexible linker peptides to the C termini of the heavy chains of IgG followed by single-chain variable domains with different binding specificities [1]. The molecules could be differentiated from 'normal' antibodies because they had dual functionalities. Technical hurdles initially hampered further development, causing bsAbs to remain a topic of R&D primarily in the academic and biotech environment. However, rapidly evolving technologies that enabled the engineering, production, and development of recombinant protein derivatives, combined with renewed interest from the pharmaceutical industry, then jump-started the bsAb research field. Today, many different bsAb formats suitable for the development of therapeutic proteins are available [2–17]. Originating frequently in academic environments, several protein formats have proven to be robust enough to support their clinical application. This has subsequently triggered significant interest and 'buy-in' from pharmaceutical companies (including Roche, Pfizer, Genentech, Sanofi, Abbvie, Chugai, Amgen, and others), which are now developing bsAb therapeutics.

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Initially, bsAb were generated by chemical conjugation of two different, purified monoclonal antibodies (mAbs) or by fusing two hybridomas resulting in a quadroma cell line producing, among others, bispecific IgG molecules [18]. Over the past two decades, genetic engineering has resulted in a range of recombinant bispecific antibody formats, with over 50 different formats now available [2]. This has revolutionized the development of bsAb for therapeutic and diagnostic applications, enabling researchers to adjust the size, valency, flexibility, half-life, and biodistribution of bsAb to fit the desired target–product profile.

Generally, bsAb can be divided into two major classes, those bearing an Fc region and those lacking an Fc region, the latter normally being smaller than the IgG and IgG-like bispecific molecules comprising an Fc (Fig. 1). The Fc region facilitates purification of the bsAb, using protocols established for IgG molecules, and can contribute to improved solubility and stability. Furthermore, the presence of an Fc region also has consequences for Fc-mediated effector functions, which might be desirable add-ons for therapeutic applications, such as antibody-dependent cellular cytotoxicity (ADCC), complement fixation (CDC), and the long half-life resulting from their larger size and FcRn-mediated recycling processes [19]. These functions can further be adopted by genetic engineering, for example, by eliminating ADCC and/or CDC while maintaining the long half-life. By contrast, bsAb lacking an Fc region rely entirely on their antigen-binding capacity to exert their therapeutic activities.

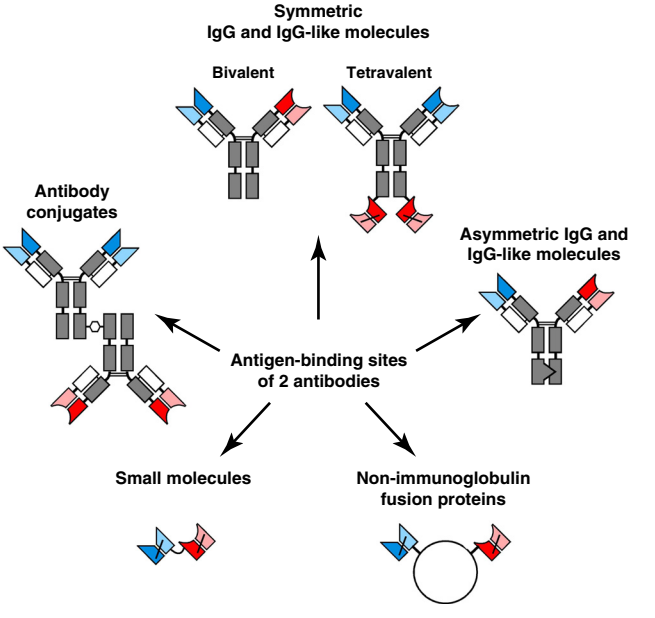

FIG. 1

Schematic overview of the different strategies used to generate bispecific antibodies (bsAbs) derived from the antigen-binding sites of two different antibodies. Symmetric bsAb are generated by the assembly of antibodies with unmodified heavy chain constant regions, such as by the heterodimerization of heavy chains from two different antibodies or homodimerization of heavy chains extended by an additional binding site resulting in bivalent or tetravalent molecules. Using heavy chains modified to force heterodimerization (e.g., using a knobs-into-holes strategy) results in asymmetric bsAb. Alternatively, two different antibody fragments, such as scFv, can be fused to a non-immunoglobulin protein, such as albumin. Furthermore, two antigen-binding fragments can be directly fused, resulting in small bsAb molecules. Finally, bsAb can also be generated by chemical conjugation of two different antibodies.

Bispecific IgG molecules can be assembled from two different heavy and light chains expressed in the same producer cell. However, because of random assembly of the different chains, this results in a substantial number of nonfunctional molecules in respect to bispecificity. A simple solution to this problem is the fusion of a second binding moiety, for example a single-chain Fv fragment or a domain antibody to the N or C terminus of the heavy or light chain, respectively, of an antibody, resulting in tetravalent molecules with two binding sites for each antigen (Fig. 1). Double-variable domain (DVD)-Igs also belong to this class of symmetric bispecific IgG and IgG-like molecules. Here, a second variable heavy chain domain (VH) is fused to the VH of a heavy chain and a second variable light chain domain (VL) is fused to the light chain (Fig. 2) [20]. Alternatively, dual recognition can be achieved by selecting VH and VL domains capable of binding to two different antigens (Fig. 2). These two-in-one antibodies are bivalent and indistinguishable from normal IgG molecules.

A breakthrough in the generation of bivalent, bispecific IgG molecules was the development of the knobs-into-holes technology [12]. Here, heavy-chain heterodimerization was forced by introducing different mutations into the two CH3 domains, resulting in asymmetric antibodies. Variations of this general approach have been established over recent years, for example using alternative mutations [21], electrostatic steering effects [22], or hybrid CH3 domains derived from IgG and IgA [13]. However, all these approaches suffer from the so-called 'light-chain problem', that is, random pairing of the two different light chains with the two chains of the heavy chain heterodimer. This can be circumvented by the use of a common light chain, which enables binding to both antigens. However, this might not be possible for all antibodies. In such cases, bacterial expression and assembly of knob- or hole-containing half-antibodies, each containing the matching light-heavy chain combination, can be applied [15]. An elegant solution was provided by CrossMab technology [16]. Here, correct pairing of the light chains is achieved by exchanging the CH1 domain of one heavy chain with the constant (CL) domain of the corresponding light chain (Fig. 2). More recently, mutations were also introduced into the CH1–CL as well as the VH–VL interface of the Fab fragments, thus enforcing correct pairing of the light chains with the corresponding heavy chains [23]. Of note, asymmetric heavy chains can also serve as building blocks to generate trivalent, bispecific, or even trispecific antibodies by fusing a further antigen-binding moiety to one of the heavy chains [6].

Small bsAb lacking an Fc region either comprise the variable VH and VL domains of two antibodies, or are based on Fab fragments. A simple format, which is utilized for example in the Bispecific T cell Engager (BiTE®) technology, is the genetic fusion of two scFv fragments resulting in tandem scFv molecules. Here, the two scFv moieties form independent folding units connected in a flexible manner through a peptide linker [24]. An alternative approach is based on the diabody format. Here, the variable domains from two antibodies, A and B, are expressed as two polypeptide chains, VHA–VLB and VHB–VLA, with the domains connected by a short peptide linker, forcing heterodimerization of the two chains [25]. The bivalent diabody format was further improved by the

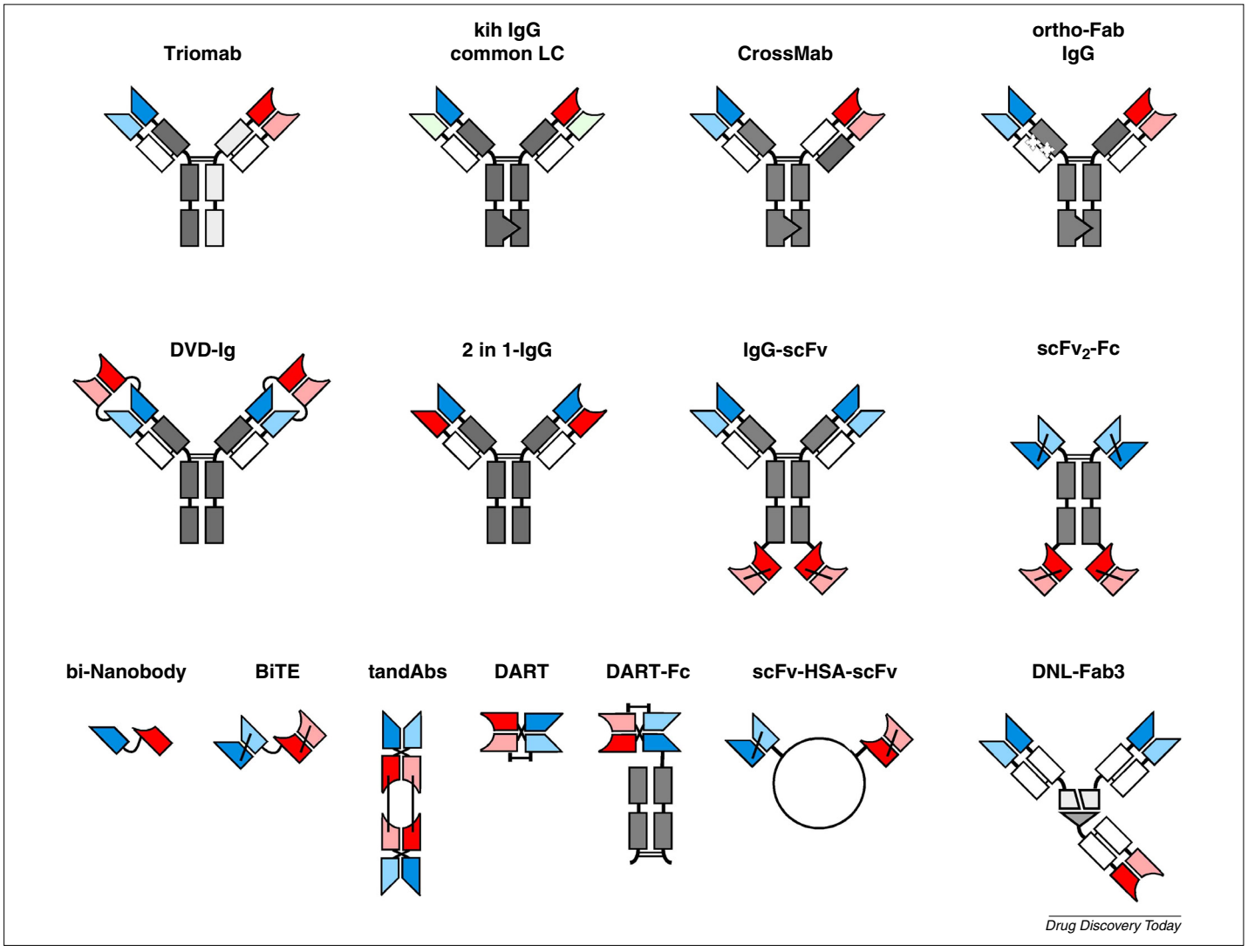

FIG. 2

Various bispecific antibodies (bsAbs) are currently in clinical development or are already approved for cancer therapy. The upper two lines depict immunoglobulin (Ig)-like bsAbs comprising an IgG Fc region, either as bivalent or tetravalent molecules. Furthermore, several small bsAb and bsAb fusion proteins have entered clinical trials. *Abbreviations*: BiTE, bispecific T cell engager; DART, Dual affinity retargeting; DNL, dock-and-lock; DVD-Ig, dual variable domain immunoglobulins; HSA, human serum albumin; kih, knobs into holes.

conversion into a single-chain version (scDb) [26] and dimeric tetravalent derivatives thereof, so-called 'tandAb' molecules with two binding sites for each antigen [27], as well as disulfide-stabilized variants, such as the dual-affinity retargeting molecules (DART) [28]. The small size of these molecules, as well as the lack of the Fc region, leads to rather rapid renal elimination *in vivo*. Although the small size might be advantageous regarding tissue penetration (e.g., in tumor therapy), the short plasma half-lives affects dosing (i.e., frequent injections or infusions are required). The implementation of half-life extension moieties, including conjugation of polyethylene glycol (PEG), fusion of PEG-mimetic polypeptides, or albumin-binding moieties, might be useful [29].

BsAbs can also be generated by fusing different antigen-binding moieties (e.g., scFv or Fab) to other protein domains, which enables further functionalities to be included. For example, two scFv fragments have been fused to albumin, which endows the antibody fragments with the long circulation time of serum albumin [30,31]. Another example is the 'dock-and-lock' approach based on heterodimerization of cAMP-dependent protein kinase A and A kinase-anchoring protein [32]. These domains can be linked to Fab fragments and entire antibodies to form multivalent bsAb [33].

Figure 1 shows schematically an overview of the different strategies used to obtain bsAb formats by different groups in academia, and the biotech and pharma industries. Most of these formats are still in preclinical evaluation and many might remain at this stage. However, some formats have made it already into clinical development and are detailed in Table 1 and discussed below. The composition and features of bsAb formats that are in clinical development are depicted in Fig. 2.

Recruitment and activation of immune cells

Quadroma bsAbs for T cell recruitment: Trion Pharma

The anti-epithelial cell adhesion molecule (EpCAM)/anti-CD3 bsAb catumaxomab (Removab®) was the first bsAb to receive market approval [34]. Trion Pharma developed catumaxomab as

TABLE 1

bsAbs in clinical development

Molecule	Targets	Format	MOA	Indication	Status[a]	Developed by
Catumaxomab	EPCAM + CD3	TrioMab	T cell recruitment	Malignant ascites Ovary cancer Gastric cancer Epithelial cancer	Market 2 2 1–2	Fresenius Biotech (Trion)
Lymphomun FBTA05	CD20 + CD3	TrioMab	T cell recruitment	BCL	1–2	Fresenius Biotech (Trion)
Ertumaxomab	Her2-CD3	TrioMab	T cell recruitment	Metastatic breast cancer	1	Fresenius Biotech (Trion)
Blinatumomab AMG103 MT103	CD19 + CD3	BiTE	T cell recruitment	B cell ALL ALL relapsed refractory ALL pediatric	Market 2 1–2	Amgen (Micromet)
MT111	CEA + CD3	BiTE	T cell recruitment	Gastric cancer advanced adenocarcinoma	1b	Amgen (Micromet)
MT112 BAY2010112	PSMA + CD3	BiTE	T cell recruitment	Prostate cancer	1	Bayer (Micromet)
MT110 AMG 110	EPCAM + CD3	BiTE	T cell recruitment	Colorectal cancer Lung and gastrointestinal cancer	1 1	Amgen (Micromet)
RG7221	Angiopoietin 2 + VEGF	CrossMab	Two-ligand inactivation	Colorectal cancer	2	Roche
RG6013	FXI + FX	CLC-IgG	Two-factor dimerization	Hemophilia A	2	Chugai (Roche group)
RG7597	Her1 + Her3	DAF-IgG	Two-RTK inactivation	Head and neck cancer, colorectal cancer	2	Genentech (Roche group)
RG7716	Angiopoietin 2 + VEGF	CrossMab	Two-ligand inactivation	Wet AMD	1	Roche
MM111	Her2 + Her3	scFv2-HSA	Two-RTK inactivation	Advanced gastric and esophageal cancer	2	Merrimack
MM141	IGF1R + Her3	IgG-scFv	Two-RTK inactivation	Advanced solid tumors	1	Merrimack
ABT122	TNFalpha + IL17	DVD-IgG	Two-ligand inactivation	RA and inflammation	1–2	Abbvie
ABT981	IL1a + IL1b	DVD-IgG	Two-ligand inactivation	Osteoarthritis	1–2	Abbott
MGD006	CD123 + CD3	DART	T cell recruitment	AML	1	Macrogenics and Servier
MGD007	GPA33 + CD3	DART-Fc	T cell recruitment	Colorectal cancer	1	MacroGenics and Servier
BI1034020	beta amyloid two epitopes	Bi-nanobody	Double epitope binding	Alzheimer's disease	1	Ablynx (Boehringer Ingelheim)
ALX0761	IL17A + IL17F	Bi-nanobody	Two-ligand inactivation	Inflammatory disease	1	Ablynx (Merck Serono)
SAR156597	IL4 + IL13	TBTI (DVD)-IgG	Two-ligand inactivation	IPF	1	Serono
TF2	CEA + hapten	D&L Fab3	Payload delivery	Colorectal cancer	1	Immunomedics
IL-17/IL-34 biAb	IL23 + IL17	scFv-Fc	Two-ligand inactivation	Inflammatory and autoimmune disease	1	BMS (Zymogenetics)
AFM13	CD30 + CD16	TandAb	NK cell recruitment	Hodgkin's disease	1	Affimed
AFM11	CD19 + CD3	TandAb	T cell recruitment	NonHodgkin's lymphoma	1	Affimed
LY3164530	Her1 + cMET	orthoFab-IgG	Two-RTK inactivation	Solid tumors	1	Eli Lilly

[a] 1, phase I clinical trials; 2, phase 2 clinical trials.

a trifunctional bsAb, a tumor-antigen and CD3-binding hybrid of murine IgG2a and rat IgG2b. It targets the tumor, recruits T effector cells via binding to the CD3ε subunit of the T cell receptor complex, and also activates monocytes, macrophages, dendritic cells, and NK cells by Fcγ-receptor binding [35]. This induces the killing of, and thus reduction in, tumor cells in patients with ovarian carcinoma (Fig. 3A), preventing or reducing the accumulation of ascites. As a 'first-generation' bsAb, catumaxomab is a nonhuman IgG-like rat–mouse hybrid antibody and binds a different antigen with each arm. It is produced by mouse–rat quadromas. Preparations of correct heterodimeric antibodies are obtained by differentiating the desired molecule from undesired monospecific rat- or mouse-only antibodies, and from contaminants with wrongly associated L-chains. L-chain mispairing can also be reduced given that rat L-chains favor association with the rat H-chain and vice versa; mouse H-and L-chains also associate preferentially [36].

Catumaxomab is potent with acceptable safety. It requires administration of only small doses (10–150 μg) four to five times by intraperitoneal infusions over 9–13 days. In addition to being approved for the treatment of malignant ascites, catumaxomab is currently also in clinical trials for application in ovarian cancer

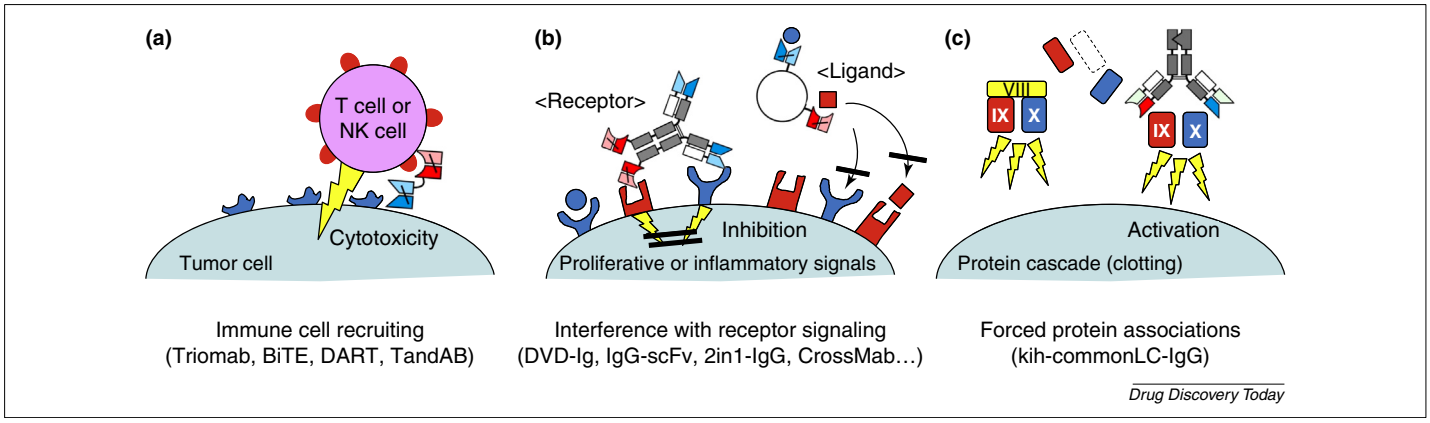

FIG. 3

Mode of action of therapeutic bispecific antibodies (bsAbs). **(a)** Recruiting of T cells or natural killer (NK) cells to tumors is achieved by entities that bind to tumor cell surface antigens as well as to immune cells. Examples are TrioMabs (catumaxomab), BiTEs (blinatumomab), DARTs, and TandAbs. **(b)** Interference with receptor signaling is achieved by binding cell surface receptors or to their cognate ligands. BsAbs in various formats have been developed for this mode of action, such as DVD-Igs, DAFs, 2-in1-IgG, Tv-IgGs, and CrossMabs. **(c)** One exciting 'unusual' application of bsAbs is antibody-mediated forced assembly of the coagulation Xase complex. A heterodimeric common light chain IgG connects FXIa and FX and thereby overcomes FVIII deficiency. Abbreviations: BiTE, bispecific T cell engager; DAF, dual-action Fab; DART, Dual affinity retargeting; DNL, dock-and-lock; DVD-Ig, dual variable domain immunoglobulins; FX, Factor X; HSA, human serum albumin; Ig, immunoglobulin; kih, knobs into holes; Tv, tetravalent.

(phase 2), gastric cancer (phase 2), and epithelial cancer (phase 1/2, Table 1). Given that catumaxomab is a rat–mouse hybrid, some anti-rat IgG or anti-mouse IgG responses are observed in most patients; however, efficacy of the treatment appears not to be significantly affected.

A series of additional bsAbs have been generated using the quadroma technology, including bsAbs that target CD3 as well as CD20 or human epidermal growth factor receptor 2 (HER2), and other cancer antigens, such as the gangliosides GD2 and GD3 [34–42]. All bsAbs were active in preclinical evaluations and two are currently in clinical trials: ertumaxomab (Rexomun®) targets tumor cells by binding to HER2, recruits T cells via its anti-CD3 arm and is currently in a phase 1 trial in patients with metastatic breast cancer [42]. Lymphomun targets the CD20 antigen on B cells and recruits T cells to those cells via its anti-CD3 arm. It is currently in a phase 1–2 trial in patients with B cell lymphoma.

BiTEs for T cell recruitment: Amgen (Micromet)

Whereas Trion Pharma approached immune cell recruitment with large IgG-like bsAbs that contain Fc regions and, hence, have a rather long serum half-life, Micromet (now Amgen) applied a conceptually different format to achieve effector cell recruitment using BiTEs [43,44]. These are small entities comprising only the variable regions of antibodies in the form of scFvs that are connected by flexible linker peptides [44–46]. They are produced as recombinant proteins in CHO cells and contain only two antigen-binding sites. One is directed at a tumor-associated cell surface antigen (with higher affinity than to CD3) and the other binds CD3 with lower affinity [44]. Given their small size and lack of Fc region, BiTEs have a short serum half-life. However, they are potent and can induce specific antitumoral cytotoxicity (target lysis of cultured cells) at concentrations as low as 10 pg/ml [47,48] in cell culture. Thereby, BiTEs are not 'consumed' but, as recruiter molecules, enable repeated rounds of target cell lysis by T cells at low effector:target cell ratios [49,50].

The BiTE that is most advanced in clinical development is blinatumomab (AMG103, MT103) [45], which binds the CD19 antigen on acute lymphoblastic leukemia (ALL) cells and demonstrated high potency (tumor eradication) in many preclinical models [51,52]. Given its potency and to ameliorate risks, blinatumomab is applied to patients at a very low dose (as low as 0.005–0.06 mg/m^2). The pharmacokinetics (PK; short half-life) also mandates an appropriate dosing schedule of continuous infusion over several weeks to ensure continuous activation of T cells against target cells. Blinatumomab is currently in phase 3 trials (NCT02013167) for minimal residual disease ALL, and additionally in phase 2 for ALL, in phase 1–2 in relapsed ALL, and in phase 1 for pediatric ALL [53]. Recently (December 2014), blinatumomab (Blincyto™) was approved by the US Food and Drug Administration (FDA) for the treatment of patients with Philadelphia chromosome-negative precursor B cell ALL (B cell ALL). Chronic lymphocytic leukemia (CLL) B cells can be depleted by blinatumomab in the presence of autologous T cells, and also in patients pretreated with different chemotherapies [54]. Patients with non-Hodgkin's lymphoma also showed tumor regression with blinatumomab [55].

Other BiTEs that have been developed by Micromet bind tumor-associated antigens EpCAM, HER2, carcinoembryonic antigen (CEA), ephrin A2 (EphA2), melanoma-associated chondroitin sulfate proteoglycan (MCSP), or CD33. Clinical studies are ongoing for EpCAM-CD3 binders (MT110, AMG110) in colorectal cancer, lung cancer and gastrointestinal cancer (all phase 1 trials). CEA-CD3 binders (MT111) are in phase 1b trials in advanced gastric adenocarcinoma, and another phase 1 trial is investigating a prostate-specific membrane antigen (PSMA)-CD3 binder in prostate cancer (MT112, Bay2010112, Bayer).

DARTs for T cell recruitment: Servier and Macrogenics

Macrogenics and its partner Servier develop T cell recruiting bispecific molecules based on the dual affinity re-targeting (DART) format. DARTs are diabody-like entities that have the VH of the

first variable region linked to the VL of the second binder, and the VH of the second variable region linked to the VL of the first (Fig. 2). Additional disulfide stabilization generates DARTs [56]. MGD006 is a DART that binds the cell surface protein CD123 as well as CD3. It does not contain an Fc region and, therefore, has a rather short serum half-life. It recruits T cells to hematological cancers, has shown promising activity in preclinical evaluation, and is currently in phase 1 trials in patients with hematological cancer. Another CD3-binding DART (MGD007) binds to the GPA33 protein, which is present in gastrointestinal cancers. In contrast to MGD006, this DART is fused to an Fc region (Fig. 2) and, therefore, has a prolonged serum half-life. Having completed preclinical development, investigational new drug (IND) for MGD007 was filed late 2014 and recruiting for a phase 1 trial in gastrointestinal cancer is ongoing (ClinicalTrials.gov NCT02248805)

TandAbs for NK and T cell recruitment: Affimed

AFM13 is a TandAb developed by Affimed to recruit immune cells to tumors [57]. In contrast to the above-described BiTEs (Micromet–Amgen), DARTs (Macrogenics–Servier) and bsAbs (Trion), immune cell recruiting by AFM13 is not aimed at T cell binding but instead at NK cells. To achieve this, the first binding entity of AFM13 binds CD30 and the second binding specificity is directed at CD16A on NK cells and macrophages. TandAbs are bispecific fusion proteins with four binding sites, two of which bind to tumor cell surface antigens and the other two to the immune cells. TandAbs do not carry Fc domains, are smaller than whole IgGs or IgG-derived bsAbs, but larger than BiTEs. Therefore, they have a shorter serum half-life than IgGs but remain longer in the circulation compared with BiTEs (because they exceed the glomerular filtration cut-off size). AFM13 is currently in clinical phase 1 in patients with Hodgkin's disease.

Another TandAb molecule is AFM11, which also recruits immune cells to tumors. In contrast to AFM13, it binds to CD3 on T cells and mediates simultaneous binding to CD19 on lymphomas [58]. Thus, its mode of action is similar to the BiTE blinatumomab. AFM11 is currently in a phase 1 trial in patients with nonHodgkin's lymphoma.

Interference with receptor signaling and inactivation of signaling ligands

Receptor tyrosine kinase (RTKs), such as members of the Her family or insulin-like growth factor (IGF) receptors, stimulate or modulate the growth of tumor cells. Thus, they are preferred targets in cancer therapy; monospecific RTK-targeting IgGs, such as cetuximab (Erbitux™) and panitumumab (Vectibix™), which are directed against HER1, or trastuzumab (Herceptin™) and pertuzumab (Perjeta™) directed against HER2, are already established in cancer therapy. However, cancer cells can escape growth inhibition caused by the blockage of one signaling pathway by switching to another. Therefore, bsAb-mediated simultaneous interference with two (or more) RTK signaling pathways, by inactivating either the RTKs or their ligand, should reduce the possibility of such escape mechanisms and, hence, improve therapeutic efficacy [59,60]. In a similar manner, receptors that are involved in inflammatory pathways or their ligands can be used to interrupt pro-inflammatory signals and treat inflammatory or autoimmune diseases.

$(scFv)_2$-HSA and Tetravalent-IgG derivatives (Tv-IgGs) against Her2 + Her3 or IGF1R+ Her3: Merrimack

MM111 (Merrimack) is a bsAb that targets two HER family members. Given the four members of this family (HER1/EGFR, HER2, HER3 and HER4), different RTK heterodimers containing HER1, 2, or 3 bind epidermal growth factor (EGF)-related growth factors and trigger intracellular signaling cascades to promote tumor growth [59]. One example is HER2, the elevated expression of which in up to 30% of breast carcinomas correlates with shorter overall survival and reduced time to relapse [60]. Phosphorylation of HER3 was also observed in HER2-amplified breast cancer [61], and HER2–HER3 heterodimerization contributes to breast cancer development. An improved growth inhibitory effect of blocking two HER family members has been demonstrated by simultaneously blocking HER2 as well as HER3. This can interfere with the growth of cancer cell lines with *HER2* gene amplifications [62], even where HER2 binders by themselves (e.g., trastuzumab and pertuzumab) inhibit ligand-induced HER3 activation rather weakly [31]. The bsAb MM111 (Merrimack) binds HER2 as well as HER3 and comprises two antagonistic scFv fragments, each with one specificity but linked to each other (Fig. 2). MM111 does not contain a Fc region but instead has the two scFvs additionally fused to modified human serum albumin to extend the PK [31]. By binding to HER2 and HER3, MM111 is a potent inhibitor of the proliferation of HER2-expressing tumor cells *in vitro* and *in vivo*. MM111 is currently in a phase 2 trial in patients with advanced gastric and/or esophageal cancer.

Another bsAb that has been generated by Merrimack is MM141 [63]. It binds HER3 as well as the non-HER RTK IGF-1R. The rationale for combining HER3 and IGF1 inhibitors is that IGF1R signaling is increased in many cancers [63–66] and could provide a way for tumor cells to escape from other signaling blockades. Therefore, bsAbs that inhibit HER3- and IGF1R signaling could overcome or reduce therapy escape of tumor cells. The format of MM141 is based upon an improved 'Morrison-Prototype' format (Fig. 2). It has stable scFvs added to the constant region of an IgG so that the bsAb contains four binding regions, two for each specificity. MM141 is a potent inhibitor of the proliferation of IGF1R and HER3-expressing tumor cells *in vitro* and *in vivo* and is currently undergoing a phase 1 trial in patients with advanced solid tumors.

Dual-action Fab (DAF)-IgG directed at HER1 + HER3: Genentech

RG7597 (Genentech) is a bsAb that binds to HER1 and HER3. The combination of HER1 and HER3 binding specificities into one bsAb could improve cancer therapy because complete inhibition of mitogen-activated protein kinase (MAPK) and AKT signaling (and subsequent growth arrest of pathway addicted cells) can be achieved upon simultaneous blockade of HER1 and HER3 signaling [10]. In contrast to most other current bsAb formats, this antibody is not composed of two different variable regions with different specificities. Instead, it is a human IgG that has dual specificity built into one 'special' variable region. Smart genetic engineering, display, and selection technologies were combined to evolve and isolate binding entities that bind HER1 and HER3 with good affinities (1.9 nM and 0.4 nM, respectively) [10]. The presence of a functional Fc region enables this bsAb to behave like a normal human IgG and to also exert immune effector functions, such as ADCC. RG7597 is currently being evaluated in a phase 2 trials in patients with head and neck cancer [67].

H-chain heterodimers with orthogonal Fab interfaces directed at HER1 and c-Met: Eli Lilly

LY3164530 (Lilly) is a bsAb in phase 1 clinical trials that binds and inhibits the receptors HER1 and c-MET [23]. This bsAb has a general IgG shape containing H-chain heterodimers (by forced heterodimerization) and two different Fab versions (mutated in V and C domains). This generates an 'orthogonal interface' that causes preferential alignment of the different Fab domains in the correct assembly. HER1 and c-MET have a role in tumor growth and metastasis, and simultaneous interference with their receptor signaling could prove beneficial for therapy compared with the application of monospecific HER1- or c-MET binders. In addition, binding HER1 and c-MET by normal bivalent IgGs can by itself induce receptor signaling, which is triggered by IgG-mediated dimerization. However, this potential issue is not applicable to the IgG-like format, which contains only one binding arm for each antigen.

DVD-IgG directed at receptor ligands TNF+IL-17 and IL1α + IL1β: Abbott and Abbvie

Interference with receptor–ligand systems is not only applied in cancer therapy, but also serves as a therapeutic principle in inflammatory diseases, such as rheumatoid arthritis (RA). Therefore, bsAbs that interfere with multiple receptors or ligands are applied in clinical studies for these indications. ABT122 is a bsAb developed by Abbvie that binds to the receptor ligand tumor necrosis factor (TNF) and to interleukin 17 (IL-17). Both factors contribute to inflammatory diseases, such as RA, and monospecific antibodies that target these ligands are in the clinic and under development (e.g., the TNF inhibitors infliximab and adalimumab and the IL-17 inhibitor secukinumab). Given that simultaneous inhibition of TNF and IL-17 was more efficacious in murine arthritis models, Abbvie (Abbott) generated bsAbs that bind to and, thus, inactivate both factors. The DVD-Ig format [19] that was used comprised a whole IL-17-binding 'IgG' (with Fc region) to which additional variable regions that bind TNF were fused 'on top' of the existing variable regions (Fig. 2). Optimized for binding affinities and ligand access and being able to bind both targets, the DVD molecule also has a constant region (human IgG1/κ) to provide a long serum half-life. ABT122 is currently in a phase 1 study in patients with RA [68].

Another DVD-Ig that has been constructed following the same design principles is ABT981 (Abbott). This molecule interferes with the functionality of the receptor ligands IL-1α as well as IL-1β [69]. Both factors are validated targets in RA and interference with their functionality can interrupt pro-inflammatory signaling via their cognate receptor. Thus, bsAb-mediated simultaneous inhibition is expected to generate enhanced efficacy. ABT981 is currently also undergoing a phase 1 trial in patients with RA.

Bi-nanobodies directed at receptor ligands IL-17A + IL-17F or at two Aβ epitopes: Ablynx

ALX0761 (Merck Serono–Ablynx) is a bispecific nanobody that binds the IL-17 members IL-17F and IL-17A, and is currently in phase 1 evaluation in healthy volunteers [70]. IL-17 and Th17 cells are associated with inflammatory and autoimmune diseases. Given that IL17A and IL-17F are important ligands for this signaling, bsAbs that interfere with the activity of both ligands might be more efficacious blockers of inflammatory responses compared with monospecific entities alone. Bispecific nanobodies comprise two monomeric VH-like binding entities of different specificities that are fused to each other in a bivalent-bispecific manner. Rather than Fc, these entities contain a half-life-extending addition to overcome potential PK issues (prevents otherwise extremely short serum half-lives).

The anti-Aβ bsAb BI1034020 (Boehringer Ingelheim–Ablynx) is another bispecific nanobody derivative with a similar format to ALX0761 and has been evaluated in a phase 1 study. It comprises two monodomains with different specificities and contains a PK-modulating moiety. Generated originally also by Ablynx, this biparatopic half-life-extended nanobody binds two paratopes and/or epitopes on the amyloid-beta peptide Aβ, rather than binding two ligands.

Crossmabs directed at the receptor ligands VEGFA and Angiopoetin 2: Roche

RG7221 (Roche) is a bsAb that inhibits the receptor ligands vascular endothelial growth factor A (VEGFA) and angiopoietin 2 (Ang2), which have a role in angiogenesis. This bsAb has been generated by applying the Crossmab format to generate an IgG-shaped entity that binds each ligand with one arm (Fig. 2). As a IgG-like molecule that contains a functional Fc region, it has the PK properties of normal IgGs [16,17]. Tumor vascularization is necessary for tumor growth and is controlled and modulated by multiple angiogenic factors; therefore, depletion of angiogenesis factors, such as VEGF (e.g., by the normal IgG Avastin) can be applied for tumor therapy. However, tumors can escape antiangiogenic treatment that inactivates just one factor (e.g., VEGF) by utilizing other pathways [71,72]. Thus, hitting two or more pathways simultaneously (by depleting essential ligands) should improve antiangiogenic therapy and reduce escape mechanisms. The target of the IgG bevacizumab (Avastin) is VEGF; bevacizumab binds to VEGF receptor 1 (VEGFR1) and VEGFR2 and thereby modulates angiogenesis. Ang2 is a ligand for Tie-2 kinase and, by binding to its receptor, also contributes to tumor angiogenesis. Preclinical studies provided evidence that hitting both angiogenesis pathways with the bsAb generated better activity in comparison to the monospecific Ang2- or VEGFA-binding antibodies [73]. The Crossmab RG7221 is currently being evaluated in phase 2 trials in patients with colorectal cancer [74].

A related Crossmab molecule that also contains binding regions that recognize VEGFA and Ang2 is RG7716. It is similar in size, composition, and specificity to the above-described RG7221 Crossmab, but has different PK properties. Ligand (VEGFA and Ang2)-driven angiogenesis poses a problem not only in oncology, but also in wet age-related macula degeneration (AMD). Therefore, the RG7716 Crossmab was developed for use in ophthalmology [75], and is currently in a phase 1 trial in patients with wet AMD.

TBTI (DVD)-IgG directed at receptor ligands IL4 and IL13: Serono

SAR156597 (Serono) is a bsAb that simultaneously binds the signaling ligands IL4 and IL13, thereby inhibiting signaling of their cognate receptors. This can reduce IL4- and IL13-dependent fibroblast activation because a combined blockade of IL4 and IL13 has a greater inhibitory efficacy compared with inhibition of either factor alone [76]. Therefore, bsAbs that simultaneously inhibit IL4 or IL13 might be suitable for treating fibrotic diseases, in particular idiopathic pulmonary fibrosis (IPF), a severe disease with currently

limited treatment options and low survival time. SAR156597 is a bsAb in IgG-like format that contains additional V-regions attached to its H- and L-chain N termini. This N-terminal tandem configuration of V-regions (termed 'TBTI' for tetravalent bispecific tandem Ig) is similar in composition to DVD-Igs. SAR156597 is currently being evaluated in a phase 1 clinical study in patients with IPF.

ScFv-IgGs directed at receptor ligands IL17 and IL23: BMS–Zymogenics

The IL17/IL23-binding bi-Mab is a bsAb that can bind both signaling ligands simultaneously and, hence, interfere with their signaling toward their receptors [77]. Simultaneous reduction of IL17 and IL23 signaling is expected to reduce inflammatory reactions and cytokine releases, and, therefore, might be beneficial for the treatment of inflammatory or autoimmune diseases. The bsAb format (from Zymogenetics) contains an Fc region (for benign PK behavior) to which scFvs with two specificities are fused. IL17/IL23 bi-Mab is currently in a phase 1 trial in patients with inflammatory and autoimmune diseases.

BsAbs for targeted or pretargeted payload delivery

DNL(Fab3) for radiotherapy of CEA-expressing cells: Immunomedics

BsAbs can not only be applied to crosslink proteins, cells, or to simultaneously interfere with two targets, but can also serve as vehicles for targeted or pretargeted delivery of payloads to tumor cells [32,33,78]. TF2 (Immunomedics) is one bsAb that has been designed for targeted payload delivery, and is currently being evaluated in phase 1 studies. It binds to CEA, which is present on the surface of many solid tumors, including colorectal cancer. The bsAb can simultaneously bind to and thereby capture a peptide-'hapten', a small entity that by itself can be labeled with radioactive substances [78]. The bsAb comprises three Fab modules that are stably coupled to each other in a triangular fashion by the 'dock-and-lock' technology (Fig. 2) [32,33]. Without a Fc region, these molecules have a rather short serum half-life, which is an advantage for pretargeting approaches. TF2 is currently being evaluated in a pretargeting setting (cold bsAb first followed separately by a Lutetium-177-labeled or Indium-111 payload for imaging) in phase 1 trials in patients with advanced colorectal cancer.

Forced association of protein complexes

Common LC-IgG for protein–protein (FXIII) complexation: Chugai

RG6013 (Chugai) is an IgG derivative with H-chain heterodimerization motifs. This was combined with the common light chain approach to prevent L-chain mispairing issues [79,80]. The therapeutic field of application of RG6013 is differs from those of the molecules described so far. Bivalent in composition with one arm each binding to one protein, RG6013 was designed and selected to bring together two protein antigens into one complex. The cognate antigens that are bound by RG6013 are Factor IXa and Factor X in the coagulation cascade. In healthy subjects, these factors are brought together by coagulation factor VIIIa, which is missing in the bleeding disorder hemophilia A. Current treatment of this severe disorder is a supplementation of FVIII, which can effectively reduce bleeding complications. However, absence of this protein (because of gene defects) in patients can lead to provided FVIII being recognized as a foreign protein, resulting in an immune response against it. This occurs in as many as 30% of such patients, who cannot continue treatment with FVIII. In addition, FVIII is rapidly cleared (half-life of less than 15 h) and is poorly bioavailable upon subcutaneous application. The humanized bsAb that links factor IXa and factor X contains an Fc region that provides a long serum half-life, which could overcome both limitations (expected lower immunogenicity and long serum half-life). Therefore, RG6013 could provide a superior treatment for hemophilia A compared with existing FVIII supplementation [81,82]. RG6013 is currently being evaluated in phase 2 trials in patients with hemophilia A.

Concluding remarks

Clinical development of bsAb is currently focusing on two main areas, cancer therapy and inflammatory diseases. The major goal is to address simultaneously different targets involved in pathophysiological processes and thereby increase therapeutic efficacy. Production processes are available that enable the generation of bsAbs in prokaryotic and eukaryotic cells, and even via *in vitro* translation without involving living organisms [83]. Other important aspects to be addressed include identifying optimal application modes and potentially modulating the PK properties, especially of small-sized bsAb molecules. In the case of BiTEs, the drug is applied via continuous intravenous infusion (enabling tight control of drug levels). Alternatives to that include half-life extension strategies (e.g., through PEGylation or albumin-binding entities [19,29]). In cancer therapy, bsAb are being developed for either the retargeting of immune effector cells for tumor cell destruction (cancer immunotherapy) or the neutralization of two different signaling cascades through inactivation either on the level of the receptor or the ligands. With a growing number of bsAb entering clinical trials, it is likely that new bsAb will be approved in the near future. Importantly, novel strategies are also emerging, as shown for the substitution of coagulation factor VIII activity by a bsAb, further supporting the immense potentials of these molecules for disease therapy.

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