



Review

Alternative molecular formats and therapeutic applications for bispecific antibodies[☆]Christoph Spiess, Qianting Zhai, Paul J. Carter^{*}

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ABSTRACT

Bispecific antibodies are on the cusp of coming of age as therapeutics more than half a century after they were first described. Two bispecific antibodies, catumaxomab (Removab[®], anti-EpCAM × anti-CD3) and blinatumomab (Blinicyto[®], anti-CD19 × anti-CD3) are approved for therapy, and >30 additional bispecific antibodies are currently in clinical development. Many of these investigational bispecific antibody drugs are designed to retarget T cells to kill tumor cells, whereas most others are intended to interact with two different disease mediators such as cell surface receptors, soluble ligands and other proteins. The modular architecture of antibodies has been exploited to create more than 60 different bispecific antibody formats. These formats vary in many ways including their molecular weight, number of antigen-binding sites, spatial relationship between different binding sites, valency for each antigen, ability to support secondary immune functions and pharmacokinetic half-life. These diverse formats provide great opportunity to tailor the design of bispecific antibodies to match the proposed mechanisms of action and the intended clinical application.

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1. Introduction

Antibodies are a well established and rapidly growing drug class with at least 45 antibody-based products currently marketed for imaging or therapy in the USA and/or Europe with ~63 billion USD in total worldwide sales in 2013 (Ecker et al., 2014; Walsh, 2014) (antibodysociety.org). This major clinical and commercial success with antibody therapeutics has fueled much interest in developing next generation antibody drugs including bispecific antibodies (Chan and Carter, 2010; Kontermann, 2012; Byrne et al., 2013). As their name implies, bispecific antibodies (BsAb) bind to two different antigens, or two different epitopes on the same antigen, as

first demonstrated more than 50 years ago (Nisonoff and Rivers, 1961). Engineering monospecific antibodies for bispecificity opens up many new potential therapeutic applications as evidenced by >30 BsAb in clinical development (Table 1). Here we review the rationale for making BsAb, criteria for selection of molecular format from a plethora of alternatives and potential therapeutic applications.

2. Alternative formats for BsAb

The modular architecture of immunoglobulins has been exploited to create a growing number (>60) of alternative BsAb formats (Chan and Carter, 2010; Kontermann, 2012; Byrne et al., 2013; Jost and Plückthun, 2014). BsAb are classified here into five distinct structural groups: (i) bispecific IgG (BsIgG) (ii) IgG appended with an additional antigen-binding moiety (iii) BsAb fragments (iv) bispecific fusion proteins and (v) BsAb conjugates (Fig. 1). Each of these different BsAb formats brings different properties in binding valency for each antigen, geometry of antigen-binding sites, pharmacokinetic half-life and in some cases effector functions.

2.1. Bispecific antibodies with IgG-like structure

Bispecific IgG (BsIgG) is a commonly used BsAb format that is monovalent for each antigen (1 + 1 antigen-binding valency).

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; ALL, acute lymphoblastic leukemia; AMD, age-related macular degeneration; AML, acute myeloid leukemia; BsAb, bispecific antibody; BsIgG, bispecific IgG; CDC, complement-dependent cytotoxicity; CEA, carcinoembryonic antigen; CLL, chronic lymphocytic leukemia; DAF, dual action Fab; DART, dual-affinity retargeting; DLBCL, diffuse large B-cell lymphoma; FDA, Food and Drug Administration; ImmTACs, immune-mobilizing monoclonal T cell receptors against cancer; MAPG, melanoma-associated proteoglycan; NHL, non-Hodgkin's lymphoma.

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Table 1
Bispecific antibodies and other bispecific immunotherapeutics in clinical development.

BsAb (other names, sponsoring organizations)	BsAb format	Targets	Proposed mechanisms of action	Development stages	Diseases (or healthy volunteers)
Catumaxomab (Removab®; Fresenius Biotech, Trion Pharma, Neopharm)	BsIgG; Triomab	CD3, EpCAM	Retargeting of T cells to tumor. Fc mediated effector functions	Approved in EU	Malignant ascites in EpCAM positive tumors
Ertumaxomab (Neovii Biotech, Fresenius Biotech)	BsIgG; Triomab	CD3, HER2	Retargeting of T cells to tumor	Phase I/II	Advanced solid tumors
Blinatumomab (Blinicyto®, AMG 103, MT 103, MEDI 538, Amgen)	BITE	CD3, CD19	Retargeting of T cells to tumor	Approved in USA Phase II and III Phase II Phase I	Precursor B-cell ALL ALL DLBCL NHL
Solitomab (AMG 110, MT110, Amgen)	BITE	CD3, EpCAM	Retargeting of T cells to tumor	Phase I	Solid tumors
MEDI 565 (AMG 211, MedImmune, Amgen)	BITE	CD3, CEA	Retargeting of T cells to tumor	Phase I	Gastrointestinal adenocarcinoma
BAY2010112 (AMG 212, Bayer; Amgen)	BITE	CD3, PSMA	Retargeting of T cells to tumor	Phase I	Prostate cancer
MCD006 (Macrogenics)	DART	CD3, CD123	Retargeting of T cells to tumor	Phase I	AML
MCD007 (Macrogenics)	DART	CD3, gpA33	Retargeting of T cells to tumor	Phase I	Colorectal cancer
AFM11 (Affimed Therapeutics)	TandAb	CD3, CD19	Retargeting of T cells to tumor	Phase I	NHL and ALL
AFM13 (Affimed Therapeutics)	TandAb	CD30, CD16A	Retargeting of NK cells to tumor cells	Phase II	Hodgkin's Lymphoma
GD2 (Barbara Ann Karmanos Cancer Institute)	T cells preloaded with BsAb	CD3, GD2	Retargeting of T cells to tumor	Phase I/II	Neuroblastoma and osteosarcoma
pGD2 (Barbara Ann Karmanos Cancer Institute)	T cells preloaded with BsAb	CD3, Her2	Retargeting of T cells to tumor	Phase II	Metastatic breast cancer
EGFRB1-armed autologous activated T cells (Roger Williams Medical Center)	T cells preloaded with BsAb	CD3, EGFR	Autologous activated T cells to EGFR-positive tumor	Phase I	Lung and other solid tumors
Anti-EGFR-armed activated T-cells (Barbara Ann Karmanos Cancer Institute)	T cells preloaded with BsAb	CD3, EGFR	Autologous activated T cells to EGFR-positive tumor	Phase I	Colon and pancreatic cancers
rM28 (University Hospital Tübingen)	Tandem scFv	CD28, MAPG	Retargeting of T cells to tumor	Phase II	Metastatic melanoma
IMCgp100 (Immunocore)	ImmTAC	CD3, peptide MHC	Retargeting of T cells to tumor	Phase I/II	Metastatic melanoma
DT2219ARL (NCI, University of Minnesota)	2 scFv linked to diphtheria toxin	CD19, CD22	Targeting of protein toxin to tumor	Phase I	B cell leukemia or lymphoma
Duligotuzumab (MEHD7945A, Genentech, Roche)	DAF	EGFR, HER3	Blockade of 2 receptors, ADCC	Phase I and II Phase II	Head and neck cancer Colorectal cancer
LY3164530 (Eli Lilly)	Not disclosed	EGFR, MET	Blockade of 2 receptors	Phase I	Advanced or metastatic cancer
MM-111 (Merrimack Pharmaceuticals)	HSA body	HER2, HER3	Blockade of 2 receptors	Phase II Phase I	Gastric and esophageal cancers Breast cancer
MM-141, (Merrimack Pharmaceuticals)	IgG-scFv	IGF-1R, HER3	Blockade of 2 receptors	Phase I	Advanced solid tumors
RG7221 (RO5520985, Roche)	CrossMab	Ang2, VEGF A	Blockade of 2 proangiogenics	Phase I	Solid tumors
RG7716 (Roche)	CrossMab	Ang2, VEGF A	Blockade of 2 proangiogenics	Phase I	Wet AMD
TF2 (Immunomedics)	Dock and lock	CEA, HSG	Pretargeting tumor for PET or radioimaging	Phase II	Colorectal, breast and lung cancers
ABT-981 (AbbVie)	DVD-Ig	IL-1 α , IL-1 β	Blockade of 2 proinflammatory cytokines	Phase II	Osteoarthritis
ABT-122 (AbbVie)	DVD-Ig	TNF, IL-17A	Blockade of 2 proinflammatory cytokines	Phase II	Rheumatoid arthritis
COVA322	IgG-fynomer	TNF, IL17A	Blockade of 2 proinflammatory cytokines	Phase I/II	Plaque psoriasis

SAR156597 (Sanofi)	Tetavalent bispecific tandem IgG	IL-13, IL-4	Blockade of 2 proinflammatory cytokines	Phase I	Idiopathic pulmonary fibrosis
GSK2434735 (GSK)	Dual-targeting domain	IL-13, IL-4	Blockade of 2 proinflammatory cytokines	Phase I	(Healthy volunteers)
Ozoralizumab (ATN103, Ablynx)	Nanobody	TNF, HSA	Blockade of proinflammatory cytokine, binds to HSA to increase half-life	Phase II	Rheumatoid arthritis
ALX-0761 (Merck Serono, Ablynx)	Nanobody	IL-17A/F, HSA	Blockade of 2 proinflammatory cytokines, binds to HSA to increase half-life	Phase I	(Healthy volunteers)
ALX-0061 (AbbVie, Ablynx)	Nanobody	IL-6R, HSA	Blockade of proinflammatory cytokine, binds to HSA to increase half-life	Phase I/II	Rheumatoid arthritis
ALX-0141 (Ablynx, Eddingpharm)	Nanobody	RANKL, HSA	Blockade of bone resorption, binds to HSA to increase half-life	Phase I	Postmenopausal bone loss
RG6013/ACE910 (Chugai, Roche)	ART-Ig	Factor IXa, factor X	Plasma coagulation	Phase II	Hemophilia

ALL, acute lymphoblastic leukemia; AMD, age-related macular degeneration; AML, acute myeloid leukemia; ART-Ig, asymmetric reengineering technology immunoglobulin; CLL, chronic lymphocytic leukemia; DAF, dual action Fab; DLBCL, diffuse large B-cell lymphoma; HSA, human serum albumin; ImmtACs, immune-mobilizing monoclonal T cell receptors against cancer; MAPC, melanoma-associated proteoglycan; NHL, non-Hodgkin's lymphoma. BsAb that are no longer in clinical trials are not included in this table. Data sources: clinicaltrials.gov, Roots Analysis and web sites for sponsoring companies.

Production of BsIgG by co-expression of the two light and two heavy chains in a single host cell can be highly challenging because of the low yield of desired BsIgG and the difficulty in removing closely related mispaired IgG contaminants (Suresh et al., 1986). This reflects that heavy chains form homodimers as well as the desired heterodimers – the heavy chain-pairing problem. Additionally, light chains can mispair with non-cognate heavy chains – the light chain pairing problem. Consequently, coexpression of two antibodies can give rise to up to nine unwanted IgG species in addition to the desired BsIgG.

Nevertheless, a few BsIgG have been purified from such complex mixtures and advanced into clinical development (Weiner et al., 1995; Renner et al., 1997). Moreover, one such BsIgG, catumaxomab (anti-CD3 × anti-EpCAM), has reached clinical approval (Table 1 and Section 3). Catumaxomab production in a single cell line (quadroma) was facilitated by preferential species restricted pairing of heavy and light chains of the component rat IgG2b and mouse IgG2a antibodies (Chelius et al., 2010). In addition, purification of catumaxomab using protein A was possible as only the desired BsIgG and the mouse IgG bind to protein A and are readily separated by sequential pH elution. Much more efficient methods for BsIgG production have been developed over the last two decades, as discussed below, facilitating clinical development of BsIgG.

2.1.1. Overcoming the heavy chain-pairing problem

Engineering antibody heavy chains for heterodimerization has emerged as a successful strategy to overcome the BsIgG heavy chain-pairing problem. The homodimerization of the two heavy chains in an IgG is mediated by the interaction between the C_H3 domains alone. Heavy chains were first engineered for heterodimerization in the 1990s using a “knobs-into-holes” strategy. Starting from a “knob” mutation (T366W) (Ridgway et al., 1996) that disfavors C_H3 homodimerization, compensating “hole” mutations (T366S, L368A, and Y407V) (Atwell et al., 1997) were identified by phage display providing efficient pairing with the “knob” while disfavoring homodimerization. The promiscuity in the IgG domain interface led in recent years to several other successful strategies for heavy chain heterodimerization (Table 2). Solutions were identified by rational design of electrostatic steering mutations (Gunasekaran et al., 2010; Strop et al., 2012) and by computational design (Moore et al., 2011; Von Kreudenstein et al., 2013), as well as derived from natural processes. Inspired by the Fab arm exchange of human IgG4 isotype in human serum (van der Neut Kofschoten et al., 2007; Labrijn et al., 2009) two solutions were developed that exploit either the destabilized hinge-region or C_H3/C_H3 dimer interface of IgG4 (Strop et al., 2012; Labrijn et al., 2013).

The structural similarity but sequence divergence between immunoglobulins from different classes or species was used to create additional routes to heterodimeric heavy chains. For example, the SEED platform exploits the sequence divergence but structural similarity of the C_H3 domains of IgG and IgA (Davis et al., 2010) and the inability of human IgG3 isotype to bind to protein A was exploited for differential tagging of the Fc region to enable efficient purification of heterodimers (Davis et al., 2013). Many BsAb generated are of the human IgG1 isotype. However, some BsAb technology platforms have been extended to other immunoglobulin isotypes including IgG2 (Strop et al., 2012) and IgG4 (Spiess et al., 2013a).

An alternative way to overcome the heavy chain-pairing problem is to use a common heavy chain. For example, κλ-bodies contain a common heavy chain plus κ and λ light chains to confer the two different antigen specificities. Two sequential affinity purification steps are used to purify κλ-bodies with their κ and

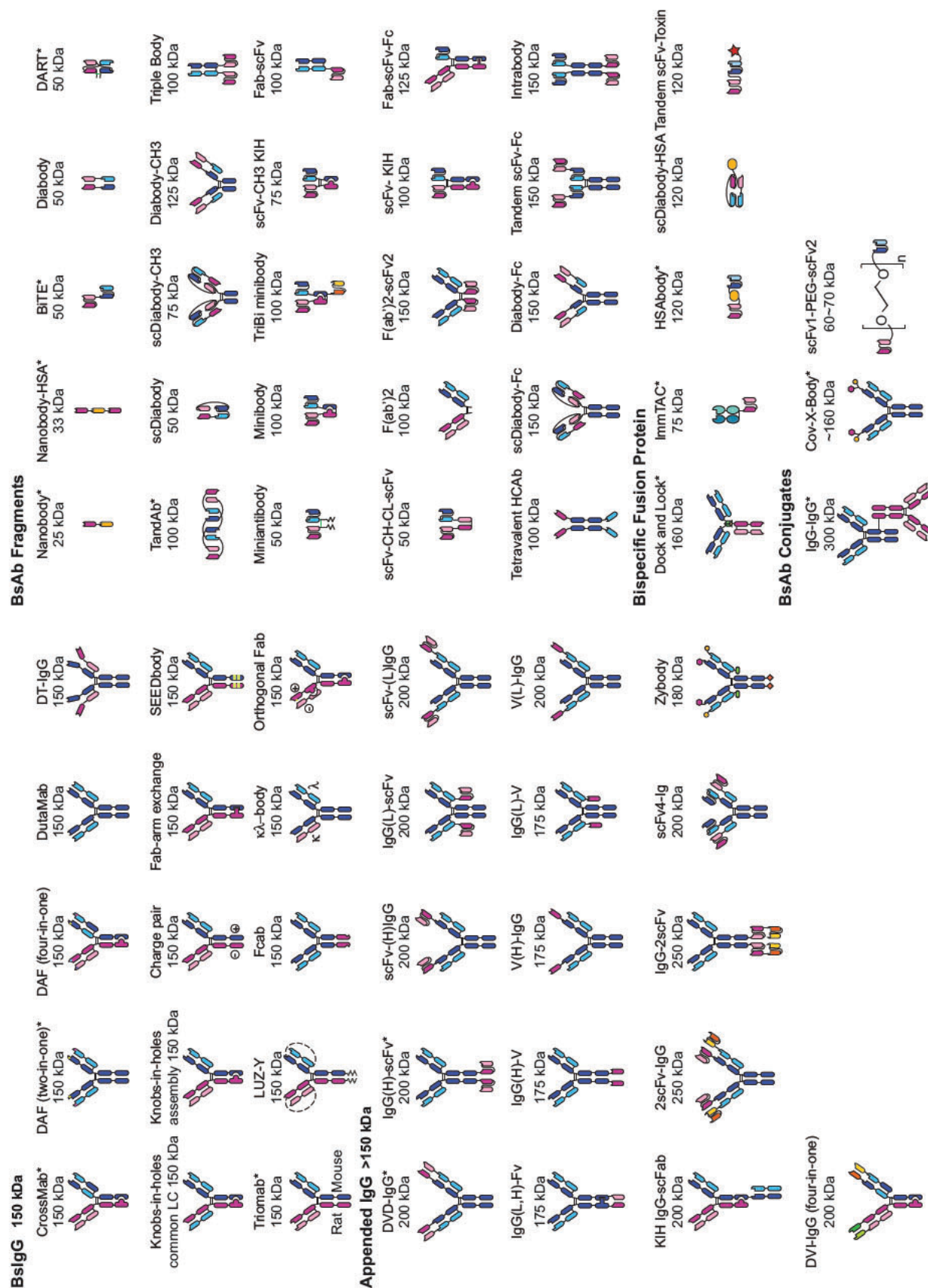


Fig. 1. Alternative formats for bispecific antibodies and other bispecific immunotherapeutics subdivided into five major classes: BsIgG, appended IgG, BsAb fragments, bispecific fusion proteins and BsAb conjugates. Heavy chains are shown in dark blue, dark pink and dark green and corresponding light chains are in lighter shades of the same colors. Connecting peptide linkers are shown by thin black lines and engineered disulfide bonds by thin green lines. Approximate molecular weights are shown assuming ~12.5 kDa per immunoglobulin domain. BsAb formats that have advanced into clinical testing are highlighted (*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2

Strategies and mutations to overcome the BslgG heavy chain-pairing problem.

Company	Technology Name	Mutations in first heavy chain	Mutations in second heavy chain	Reference
Genentech	Knobs-into-holes	T366W	T366S, L368A, Y407V	Ridgway et al. (1996), Atwell et al. (1997)
Genmab	DuoBody	F405L	K409R	Labrijn et al. (2013)
Zymeworks	Azymetric	T350V, L351Y, F405A, Y407V	T350V, T366L, K392L, T394W	Von Kreudenstein et al. (2013)
Amgen	Charge pair	K409D, K392D	D399K, E356K	Gunasekaran et al. (2010)
Rinat-Pfizer	Charge pair	D221E, P228E, L368E	D221R, P228R, K409R	Strop et al. (2012)
Xencor	HA-TF	S364H, F405A	Y349T, T394F	Moore et al. (2011))
EMD Serono	SEEDbody	IgG/A chimera	IgA/G chimera	Davis et al. (2010)
Regeneron	Differential protein A affinity	H435R	None	Davis et al. (2013)

λ light chains away from monospecific antibodies that contain a single type of light chain (www.novimmune.com).

Additional applications of heterodimeric Fc regions include the construction of one-armed antibodies, e.g., onartuzumab, a one-armed anti-MET antibody (Merchant et al., 2013). Onartuzumab comprises humanized anti-MET antibody heavy and light chains and an Fc chain and incorporates knobs-into-holes mutations for preferential assembly of the anti-MET heavy and Fc chains. Onartuzumab in combination with erlotinib (Tarceva®) has reached phase III clinical trials in non-small cell lung cancer (www.clinicaltrials.gov). Heterodimeric Fc regions have also been used for exquisite tailoring of antibody specificity for FcγR to enhance effector functions without impairing Fc stability (Mimoto et al., 2013).

2.1.2. Overcoming the light chain-pairing problem

Several robust strategies have been developed to overcome the BslgG heavy chain (Section 2.1.1) and light chain (this section) pairing problems. Light chain mispairing can be obviated by using antibodies with a common light chain identified from phage display libraries with limited light chain diversity (Merchant et al., 1998). More recently antibodies with a common light chain have been identified from other technologies including transgenic mice with a single light chain (McWhirter et al., 2011; Dhimolea and Reichert, 2012).

Currently the most widely used route to BslgG is by separate expression of the component antibodies in two different host cells followed by purification and assembly into BslgG *in vitro* (Jackman et al., 2010; Strop et al., 2012; Labrijn et al., 2013; Spiess et al., 2013b). A major advantage of these two host cell strategies over the common light chain approach is that they are much more broadly applicable to pre-existing antibodies. Moreover, the two different light chains typically contribute to antigen-binding affinity and specificity. Disadvantages of the two-host cell strategies compared to the common light chain are that they are associated with significantly greater expense and complexity in manufacturing.

Efficient production of BslgG in a single host cell has been accomplished by simultaneously overcoming both light and heavy chain pairing problems. In the CrossMab technology (Schaefer et al., 2011b) light chain mispairing was overcome using domain crossovers and heavy chains heterodimerized using knobs-into-holes (Merchant et al., 1998). For the domain crossovers either the variable domains or the constant domains are swapped between light and heavy chains to create two asymmetric Fab arms that drive cognate light chain pairing while preserving the structural and functional integrity of the variable domain (Fenn et al., 2013). An alternative approach for overcoming light chain mispairing was designed by heavy and light chains with orthogonal Fab interfaces (Lewis et al., 2014). This was accomplished by computational modeling with Rosetta (Das and Baker, 2008) in combination with X-ray crystallography to identify mutations at the V_H/V_L and C_H1/C_L interfaces. For the antibodies tested it was necessary to engineer mutations into both V_H/V_L and C_H1/C_L interfaces to minimize heavy/light chain mispairing. The designed orthogonal Fab

interface was used in conjunction with a heavy chain heterodimerization strategy to facilitate efficient IgG production in a single host cell (Lewis et al., 2014). Electrostatic steering has also recently been used to generate orthogonal Fab interfaces to facilitate the construction of BslgG (Liu et al., 2015). Peptide linkers have also been used to ensure cognate pairing of light and heavy chains in a format known as “LUZ-Y” (Wranik et al., 2012). Heavy chain heterodimerization was accomplished using leucine zippers that were subsequently removed by proteolysis *in vitro*.

2.1.3. Bispecific IgG that avoid the heavy and light chain pairing problems

The BslgG chain pairing problems can be obviated by using a single kind of heavy and light chain and engineering the variable domains to recognize two unrelated antigens via two different binding sites on the antibody (paratopes). For example, a monospecific anti-HER2 antibody was engineered by phage display to bind a second and unrelated antigen, VEGF, whilst maintaining high affinity binding for the first antigen (Bostrom et al., 2009). Such so called “two-in-one” antibodies, also known as Dual Action Fab (DAF), make differential but overlapping use of the light and heavy chain complementarity determining regions (CDRs) as main contacts for each antigen (Bostrom et al., 2009; Schaefer et al., 2011a). A tetra-specific “four-in-one” antibody (FL518) was recently generated (Hu et al., 2015) by combining two different two-in-one antibodies, anti-HER2 × anti-VEGF (Bostrom et al., 2009) and anti-EGFR × anti-HER3 (Schaefer et al., 2011a) in CrossMabs format (Schaefer et al., 2011b). The four-in-one antibody (FL518) inhibited signaling mediated by these receptors *in vitro* and *in vivo* and also disrupted HER-MET crosstalk. Additionally, the four-in-one antibody showed superior *in vivo* anti-tumor activity to the component two-in-one antibodies.

An alternative format known as a DutaMab utilizes the diversity of only three CDRs for each antigen thus creating two independent paratopes (Beckmann, 2012). Attractive features of “two-in-one” antibodies and DutaMabs are that they have a single kind of heavy and light chain and are amenable to standard production processes developed for monospecific antibodies. A downside is that sophisticated upfront engineering is required to create these formats that is not invariably successful. Two-in-one antibodies have overlapping antigen-binding sites that results in variable valency. They can potentially bind monovalently to both antigens or alternatively they can bind bivalently to a single antigen. These binding attributes of “two-in-one” antibodies may be advantageous for some applications but a drawback where monovalency is preferred, or required.

2.2. Bispecific and multispecific antibodies by appending IgG

Monospecific IgG can be engineered for bispecificity by appending either the amino or carboxy termini of either light or heavy chains with additional antigen-binding units (Fig. 1). Alternatives for these additional antigen-binding units include single domain antibodies (unpaired V_L or V_H), paired antibody variable domains

(e.g., Fv or scFv) or engineered protein scaffolds (LaFleur et al., 2013).

One such appended IgG format that has reached clinical development is the dual variable domain IgG (DVD-Ig) (Wu et al., 2007). DVD-Ig have been generated by appending the V_L and V_H domains of an IgG with similar domains from a second antibody via short peptide linkers. In constructing DVD-Ig linker optimization can be required to provide the desired antigen-binding affinity (Jakob et al., 2013). DVD-Ig are bispecific and bivalent for each antigen specificity (2 + 2 antigen-binding valency). Combining DVD-Ig with other technologies can be used to vary the valency and extend the range of specificities. For example, a tetravalent and tetraspecific antibody (1 + 1 + 1 + 1 antigen-binding valency) known as CRTB6 (Hu et al., 2015) that binds to EGFR, HER2, HER3 and VEGF was generated by combining DVD-Ig (Wu et al., 2007), with knobs-into-holes (Ridgway et al., 1996; Atwell et al., 1997) and CrossMab (Schaefer et al., 2011b) technologies (see also Section 2.1.3).

One potential advantage of appended BslgG is that they can enable the simultaneous binding of antigen to all variable domains and hence provide a higher specific binding capacity (Jakob et al., 2013). This may be useful in targeting low abundance proteins such as cytokines and it may enable longer dosing intervals. In addition, the simultaneous binding of antigen (Correia et al., 2013) preserves the natural antibody avidity to cell surface receptors or dimeric antigens and it provides an extended distance between the bound epitopes by placing the additional binding domains at the distal end of the heavy chain (Croasdale et al., 2012). In addition, the flexibility to choose between the 2 + 2 and 2 + 1 antigen-binding valencies provides the opportunity to eliminate avidity by placing a single binding unit at the carboxy-terminus to create monovalent binding while conserving the binding capacity to the second antigen (Niewoehner et al., 2014).

2.3. Bispecific antibody fragments

A growing repertoire of different BsAb fragment formats have been described that lack some or all of the antibody constant domains (Fig. 1). For many BsAb fragments heavy and light chains are connected with short peptide linker sequences that can allow efficient expression of the BsAb in a single host cell. Further engineering may be necessary to minimize the formation of unwanted antibody species during production (Tan et al., 1998; Igawa et al., 2010) or to improve the stability of BsAb fragments (Perchiacca and Tessier, 2012).

ScFv fragments (Bird et al., 1988; Huston et al., 1988) are a commonly used building block in generating BsAb. ScFv can be constructed in either V_H – V_L or V_L – V_H orientations. However, the V domain orientation can sometimes impact antigen binding (Albrecht et al., 2006). The construction of scFv fragments with short (1–10 amino acid) linkers permits inter-chain but not intra-chain pairing of V_H and V_L domains. Co-expression of two such scFv fragments can be used to form a bispecific fragment known as a diabody (Holliger et al., 1993) that has 1 + 1 antigen-binding valency. Mutations in the V_L / V_H interface for diabodies can be used to favor heterodimeric over homodimeric chain pairing (Zhu et al., 1997; Tan et al., 1998; Igawa et al., 2010). Additional refinements of the diabody format include the introduction of a connecting linker between chains to create a single-chain diabody (Alt et al., 1999; Kipriyanov et al., 1999). A disulfide bond engineered into the diabody can improve stability as in the dual-affinity-retargeting (DART) format (Johnson et al., 2010). Two pairs of V_L and V_H domains can be connected in a single polypeptide chain as in the case of the tetravalent tandem diabody (TandAb) (Arndt et al., 1999; Kipriyanov et al., 1999) that is bispecific and bivalent for each antigen (2 + 2 antigen-binding valency). One popular BsAb fragment format is the tandem scFv fragment that has 1 + 1 antigen-binding

valency. The Bispecific T cell Engager (BiTE) (Mack et al., 1995) is a type of tandem scFv in which the component scFv fragments are designed to bind CD3 on T cells and a surface antigen on tumor cells to redirect T cells to kill tumor cells. Single domain antibody fragments (dAbs) can be used to reduce molecular size further. Phage display libraries have been used to isolate murine dAbs (Ward et al., 1989) and later human dAbs (Davies and Riechmann, 1995; van den Beucken et al., 2001). Subsequently human dAbs were used in the construction of BsAb fragments (De Bernardis et al., 2007). DAbs known as nanobodies have been obtained from llama and camel heavy-chain only antibodies. Nanobodies are readily combined by short linkers providing a facile way to vary their antigen-binding valency and introduce bispecificity (Els Conrath et al., 2001) or multispecificity. The small size of dAbs can allow efficient tissue penetration – a potential advantage over IgG (Coppeters et al., 2006). Another proposed advantage of dAbs is that they can provide access to epitopes on some targets that may be sterically inaccessible to antibodies in IgG format (Desmyter et al., 1996). However this is not a compelling advantage of dAbs since IgG with long CDR H3 loops can also penetrate crevices in proteins, as shown for some anti-HIV antibodies (Saphire et al., 2001).

Many BsAb fragments lack an Fc region resulting in short serum half-life that may necessitate more frequent dosing than an IgG or administration by continuous infusion (Nagorsen et al., 2012). Alternatively BsAb fragments can be engineered to extend half-life (Section 3).

2.4. Bispecific fusion proteins

Antibody fragments can also be linked to other proteins to add additional functionality or specificity. E.g., ImmTACs comprise an anti-CD3 scFv linked to an affinity-matured T-cell receptor that recognizes target HLA-presented peptides (Oates and Jakobsen, 2013). ImmTACs provide the opportunity to expand the retargeting of T cells beyond cell surface antigens accessible by antibodies to intracellular proteins as peptides on MHC complexes.

Strong and specific natural protein-protein interactions have been exploited in the Dock-and-Lock method (DNL) (Rossi et al., 2006; Chang et al., 2007) to create bispecific antibodies with higher valency. Initially a bispecific and trivalent antibody was created by dimerizing a monospecific Fab dimer with a second Fab molecule via the DNL. An anti-CEACAM5 × anti-hapten DNL BsAb has been explored for a pretargeting strategy for imaging and radioimmunotherapy (Sharkey et al., 2010). The format has been further expanded by placing the DNL domains at the C-terminus of an antibody to create a hexavalent BsAb (Rossi et al., 2009). Fusion to human serum albumin or albumin binding proteins has been exploited to extend the serum half-life of several antibody fragments (Müller et al., 2007; Stork et al., 2007).

2.5. Bispecific antibody conjugates

Chemical conjugation of antibodies or antibody fragments to form BsAb was more commonly practiced in the past prior to the advent of recombinant methods described in this review. Indeed, the first reported BsAbs were BsF(ab')₂ obtained by pepsin digestion of two rabbit IgG followed by reduction and then reoxidation of the resulting Fab' fragments. More efficient production of BsF(ab')₂ fragments was achieved using cysteine-reactive homo- and heterobifunctional crosslinking reagents (Brennan et al., 1985; Glennie et al., 1987) then later in conjunction with recombinantly expressed Fab' fragments (Shalaby et al., 1992).

The CovX-Body represents a more recent innovation in the use of conjugation to generate BsAb. In the CovX-Body format

(Doppalapudi et al., 2007) the serum half-life of low molecular weight drugs is extended by site-specific conjugation to a single reactive lysine in each Fab arm. A bispecific CovX-Body (CVX-241) was created by first joining two short peptides inhibiting either VEGF or Ang2 with a branched linker and subsequent site-specifically conjugating to the antibody (Doppalapudi et al., 2010). A phase I study with CVX-241 was prematurely discontinued for lack of significant pharmacologic effects (safety, pharmacodynamics or efficacy; trial identifier NCT01004822). The catalytic antibody scaffold of CovX-Body is readily programmed with different specificities by conjugation to different small molecules.

3. Selection of bispecific antibody format

A major challenge in developing BsAb drugs is the selection of the molecular format from >60 structurally diverse alternatives (Fig. 1 and Section 2) that can support a wide range of different biologic and pharmacologic properties. The BsAb format is best chosen to match the proposed mechanisms of action and the specific clinical application. Ideally BsAb in several alternative formats are constructed and the final format is chosen after *in vitro* and *in vivo* functional characterization. The format also needs to be compatible with available antibodies or antibody discovery technologies. Intellectual property is an additional important consideration, albeit beyond the scope of this review, that may restrict the choice of BsAb format for clinical and commercial development.

3.1. Bispecific antibody developability characteristics

BsAb are complex molecules that commonly comprise 1–4 polypeptide chains (Fig. 1). BsAb are ideally constructed from component antibodies that themselves have drug-like properties as the behavior of the BsAb may be limited by the least stable component (Mabry et al., 2009). Desirable attributes include high thermal stability, high solubility, low propensity to aggregate, low viscosity and high chemical stability (Demarest and Glaser, 2008). Additionally, high-level expression (grams per liter titers) of a BsAb is desirable, if not necessary, to support production for clinical development, as for monospecific antibodies (Kelley, 2009). CHO cells or *E. coli* are often chosen as host systems for BsAb production as they are widely used for other biopharmaceuticals including monospecific antibodies (Kelley, 2009; Chon and Zarbis-Papastoitis, 2011). The design of BsAb is sometimes used to limit the number and quantity of unwanted antibody side products and/or facilitate their removal (see Section 2).

An ideal BsAb format for therapeutic applications would be broadly applicable to a broad variety of parental antibodies (Strop et al., 2012; Labrijn et al., 2013; Spiess et al., 2013b) without requiring extensive customization for individual antibody pairs. However, BsAb formats that do require extensive customization for individual antibodies can also be useful in patients as evidenced by DAF (Bostrom et al., 2009) and DVD-Ig (Wu et al., 2007) that have advanced into clinical development (Table 1). An ideal BsAb format should also preserve the antigen-binding affinity and beneficial biological activities of their component monospecific antibodies. Simultaneous binding of both antigens to the BsAb may be essential depending on the application. Selection of the BsAb format to match the valency of each component antibody to the biology of the target antigens and the proposed mechanism of action may be necessary. Fortunately, this is readily achievable by selection from the many alternative BsAb formats (Fig. 1). The spatial separation and orientation of the antigen-binding sites varies between different BsAb formats. This is likely to be important for some applications including biepitopic antibodies (Section 5).

3.2. Effector functions

Some BsAb are designed to deplete target cells as part of their mechanism of action. Designing BsAb to include an Fc region is the method of choice to support target depletion using secondary functions such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP). For example, one of the biologic activities of the anti-EGFR $\times$ anti-HER3 DAF, MEHD7945A, is cytotoxicity (ADCC) against target cells *in vitro* in the presence of human effector cells (Schaefer et al., 2011a). BsAb fragments lacking an Fc region can also be endowed with the ability to support Fc-mediated effector functions as shown for a bispecific diabody binding to carcinoembryonic antigen (CEA) and human IgG (Holliger et al., 1997). However, this strategy has been not been widely used.

In appending monospecific IgG with additional antigen-binding units the location of the appendage can sometimes impact function. In one case fusing a scFv fragment to the carboxy-terminus of the light or heavy chain resulted in potent or minimal ADCC activity, respectively (Croasdale et al., 2012). In another example, fusing a scFv fragment to the amino-terminus of either light or heavy chains gave similar ADCC to the parent antibodies (Dimasi et al., 2009). Thus for appended BsgG formats systematic evaluation of the alternative locations for the second antigen-binding unit can be helpful in identifying BsAb with the desired activities, including the ability to support ADCC.

3.3. Pharmacokinetic half-life

Long pharmacokinetic half-life is a desired property of many BsAb and is commonly accomplished by including an Fc region in the design (Fig. 1). The Fc region of immunoglobulins binds to the salvage receptor (FcRn) thereby providing the potential for long pharmacokinetic half-life *in vivo* (Roopenian and Akilesh, 2007). BsAb fragments lacking an Fc region typically have a short pharmacokinetic half-life that is suitable and desirable for some applications while not others. The half-life of BsAb fragments is readily extended, if needed, using technologies developed for other protein pharmaceuticals (Kontermann, 2011). For example, bispecific nanobodies with long serum half-life have been created by incorporating one domain antibody that binds to the long-lived serum protein, albumin (Tijink et al., 2008). Alternatively, BsAb fragments have been fused directly to albumin to achieve long pharmacokinetic half-life (Müller et al., 2007).

4. Clinical applications of bispecific antibodies

At least 30 different BsAb and related molecules are currently in clinical development (Table 1) mainly for oncology, autoimmune or chronic inflammatory indications. These clinical stage BsAb represent many different technologies for BsAb generation including: Triomab (Chelius et al., 2010), BiTEs (Frankel and Baeuerle, 2013), TandAbs (Cochlovius et al., 2000), ImmTac (Oates and Jakobsen, 2013), DAF (Bostrom et al., 2009), HSA-body (Müller et al., 2007), IgG-scFv (Orcutt et al., 2009), CrossMab (Schaefer et al., 2011b), dock-and-lock (Rossi et al., 2006), DVD-Ig (Wu et al., 2007), and nanobodies (Els Conrath et al., 2001).

4.1. Retargeting effector cells to tumor cells using bispecific antibodies

Currently the most common application of BsAb in the clinic is in oncology where anti-CD3 $\times$ anti-tumor BsAb are being used to retarget T cells to kill tumor cells (Table 1), a concept first demonstrated *in vitro* ~30 years ago (Staerz et al., 1985). Early clinical

trials with anti-CD3 $\times$ anti-tumor BsAb in the 1990s met with only limited anti-tumor responses and in some cases significant toxicities (Riethmüller, 2012).

However, in 2009 one such T cell retargeting BsAb, catumaxomab (anti-EpCAM $\times$ anti-CD3), was approved for the treatment of EpCAM-positive cancers in Europe based on anti-tumor activity in an open label phase II/III clinical trial in the treatment of malignant ascites (Heiss et al., 2010). Catumaxomab is a BslgG known as a Triomab in which rat IgG2b and mouse IgG2a antibodies (Chelius et al., 2010) are coexpressed in a single host cell (Section 2.1). As expected, most patients developed a human anti-mouse (or rat) antibody response to catumaxomab. Surprisingly, this anti-drug antibody response correlated with *more* favorable clinical outcome including an increase in median overall survival likely involving immune mechanisms that are not yet well understood (Ott et al., 2012).

Currently the most common format for retargeting T cells to tumor cells in clinical development is the BiTE (Section 2.3, Mack et al., 1995). At least 4 BiTE molecules are undergoing clinical investigation (Frankel and Baeuerle, 2013) (Table 1). The anti-CD3 $\times$ anti-CD19 BiTE, blinatumomab, gave partial and complete responses in early clinical trials in non-Hodgkin's lymphoma (Bargou et al., 2008) and is currently in multiple phase II and phase III trials (Nagorsen et al., 2012). Late in 2014 blinatumomab was approved by the US Food and Drug Administration (FDA) for the treatment of a rare form of B cell acute lymphoblastic leukemia (ALL).

Other BsAb formats being used to retarget T cells to tumors include the TandAb (Kipriyanov et al., 1999) and DARTs (Johnson et al., 2010). BsAb are also being used to retarget other types of cytotoxic effector cells such as NK cell and macrophage via CD16 (Fc γ RIIIA) (Weiner et al., 1995) to eliminate unwanted target cells. The anti-CD16a $\times$ anti-CD30 TandAb, AFM13, is currently in a phase I clinical trial for Hodgkin's lymphoma. The advent of ImmTACs extends the opportunity for retargeting T cells, beyond the conventional cell surface antigens of antibodies, to intracellular proteins as peptides in MHC complexes (Section 2.4, Oates and Jakobsen, 2013). The ImmTAC, IMCgp100 (Bossi et al., 2014), has advanced into clinical development.

4.2. Dual blockade of two disease mediators using bispecific antibodies

Blockade of individual disease mediators with antibodies (e.g., TNF, VEGF, IL-6, BlyS and RANKL) can lead to significant therapeutic benefit to patients (Chan and Carter, 2010). Dual blockade of two disease mediators with BsAb may overcome biologic redundancy and increase potency or efficacy (Marvin and Zhu, 2005). However, targeting two different disease mediators can also sometimes give rise to additional toxicities as shown by the increase in infection complications in patients on combining blockade of TNF by etanercept and CD80 and CD86 by abatacept (Weinblatt et al., 2007). This dual targeting idea was first explored in oncology with BsAb targeting VEGFR1 and VEGFR2 (Lu et al., 2001) or EGFR and IGF1R (Lu et al., 2004, 2005). Many more dual blockers have been generated including several that have advanced into clinical development (Table 1). For example, DVD-Ig targeting mouse IL-1 α and IL-1 β was found to have similar *in vivo* potency to a combination of the two parent monospecific antibodies from which it was derived (Wu et al., 2007). A DVD-Ig binding human IL-1 α and IL-1 β known as ABT-981 was then created (Wu et al., 2009) and has advanced to phase II clinical trials for osteoarthritis. The dual engagement of receptors can also be leveraged to mimic natural ligands engaging two receptors to induce signaling as demonstrated by an anti-factor IXa $\times$ anti-factor $\times$ BsAb that mimics binding of the natural ligand factor VIII (Kitazawa et al., 2012).

5. Preclinical stage bispecific antibodies

Beyond effector cell retargeting BsAb have been firmly established in clinical and pre-clinical experiments as a means to silence or activate signaling pathways by targeting two different receptors, soluble ligands or a combination thereof. While usually two different antigens are targeted, it has recently been demonstrated that biepitopic targeting of a single protein such as HER2 or EGFR can provide superior antagonistic properties (Boersma et al., 2011; Lewis et al., 2014).

BsAb also provide excellent tools for antibody delivery to immunoprivileged organs. A major difficulty in the development of therapeutics, including antibodies, for brain diseases is the very inefficient uptake imposed by tightly packed vascular endothelial cells—the so-called blood-brain barrier. BsAbs against transferrin receptor and BACE-1 can “hijack” the transferrin receptor-mediated transcytosis mechanism and thereby increase antibody uptake into the brain by at least a few fold (Watts and Dennis, 2013). BsAb binding to BACE-1 and transferrin receptor can reduce A β levels in the brain by inhibiting BACE-1 (Yu et al., 2011; Niewoehner et al., 2014). In a related strategy, a dAb (FC5) was functionally selected for transmigration across the blood-brain barrier (Farrington et al., 2014). The FC5 dAb was fused to an Fc fragment to extend its pharmacokinetic half-life and then chemically conjugated to the neuropeptides, dalargin and neuropeptide Y, for delivery into the brain (Farrington et al., 2014).

BsAb have also been used successfully in preclinical models to target cytokines such as IFN α 2b (Rossi et al., 2010) and TNF α (Larbouret et al., 2007) to tumors. In the latter example, the BsAb can bind a cytokine in addition to the tumor specific antigen. After delivery of the cytokine to the tumor, the cytokine can then ‘jump’ to the receptor based on the higher receptor-cytokine affinity. Possible advantages of delivering the cytokine with a BsAb to the tumor are reduction of systemic toxicity of the co-administered cytokine by scavenging of the free form plus increasing the pharmacokinetic half-life of the cytokine. BsAb have also been explored for targeted delivery of drug payloads. In one example the first antigen specificity is utilized to target the antibody to a cell surface receptor such as HER2, while the second antigen specificity is utilized to bind the hapten digoxigenin (Metz et al., 2011). Different payloads such as small molecules and proteins can then be attached to digoxigenin for targeted cell delivery without the need to further engineer the antibody.

Beyond therapeutic applications, BsAb enable the design of highly sensitive and specific tools for the diagnosis or imaging of infectious diseases and cancer. In using BsAb in diagnostic assays the first antigen specificity is often directed towards the pathogen, virus or tumor cell, while the second specificity can bind a detection enzyme such as horseradish peroxidase or alkaline phosphatase (Kreutz et al., 1998; Bhatnagar et al., 2008). This design provides flexibility and one-step detection without the need to directly label the antibody. In tumor imaging a BsAb enables a two-step process by pre-targeting of the BsAb to tumor cells and subsequent administration of the imaging radionuclide. Because the unbound radionuclide clears rapidly from the body, it enables enhanced imaging of the tumor compared to radiolabeled antibodies with superior specificity, sensitivity and reduced background (Sharkey et al., 2005).

6. Alternative strategies to BsAb

Some applications of BsAb require both antigen-binding activities be included in a single molecule. Examples of these “obligatory BsAb” are co-engagement of tumor and cytotoxic T cells (Frankel and Baeuerle, 2013), dimerization of receptors to mimic a natural

ligand (Kitazawa et al., 2012) and hijacking a transcytosis mechanism for the delivery of antibodies to the brain (Yu et al., 2011). By contrast, the other applications of BsAb do not require both binding activities to be present in the same molecule. Examples of these “optional BsAb” include the blocking and neutralization of independent targets or the decoration of a single target with multiple antibodies. Alternative therapeutic strategies include antibody combinations, mixtures or the co-administration of two or multiple antibodies (see below) or engineered protein scaffolds as recently reviewed (Jost and Plückthun, 2014).

Antibody mixtures offer the advantage that multiple antibodies can be combined to decorate the target mimicking a natural polyclonal immune response. For example, a mixture of three different antibodies to ebola virus known as ZMAb provided sustained protection against ebola virus infection following treatment of infected non-human primates (Qiu et al., 2013). Antibody mixtures can be efficacious for other infectious disease as reviewed elsewhere (Bregenholt et al., 2006) and for natural toxins such as botulinum toxin (Nowakowski et al., 2002). Antibody mixtures can also provide superior blockade of receptor signaling (Koefoed et al., 2011) and may provide a mechanism to prevent drug resistance in oncology.

One limitation of BsAb is that they fix the ratio (i.e., relative dose) of the two component antibodies. By contrast, the use of antibody mixtures and co-administration of antibodies provides great flexibility in the ratio and number of antibodies. Separate dosing of two different antibodies provides even more flexibility by allowing the timing of dosing to be varied. For clinical studies mixtures of antibodies can be created by co-formulating individually purified antibodies (Nayak et al., 2014), or streamlining the purification process by co-culturing the individual antibody expressing cells (Frandsen et al., 2011). Proceeding with individual antibodies instead of a BsAb has the advantage that the target engagement is uncoupled, and unfavorable properties such as short serum half-life due to receptor internalization are restricted to a single target. Furthermore, differential species cross-reactivity can complicate the interpretation of *in vivo* studies of BsAb. Antibody mixtures also have several major drawbacks for drug development including introducing much complexity into formulation, analytical characterization, clinical development and the regulatory pathway.

Beyond antibodies, engineered protein scaffolds are also being explored for bispecific or multi-specific targeting (Jost and Plückthun, 2014). Like antibodies alternative protein scaffolds can recognize other proteins with high affinity and selectivity. The small size and high stability of many engineered scaffolds lend themselves to expression in prokaryotic hosts or even peptide synthesis. Since many alternative scaffolds such as the Affibody, DARPins, and Adnectins are derived from natural repeat domains, it was natural to engineer them into bispecific (Eggel et al., 2009; Friedman et al., 2009; Emanuel et al., 2011) or biepitopic molecules (Boersma et al., 2011) for preclinical studies. Engineered protein scaffolds can have a short serum half-life due to their small size and inability to bind FcRn. This is not a significant limitation since many successful strategies have been developed to extend the half-life of proteins, including engineered protein scaffolds (Section 3).

7. Future perspectives

Recent years have witnessed much progress with BsAb technologies and therapeutics as reviewed here and elsewhere (Chan and Carter, 2010; Kontermann, 2012; Byrne et al., 2013). For some applications of BsAb, e.g., neutralization of two different soluble disease mediators, it may be sufficient to pick the best candidate antibody for each arm separately and then simply combine. By contrast, for other BsAb such as biepitopics or ligand mimetics (Sampei

et al., 2013), it is desirable if not necessary to permute panels of monospecific antibodies to find optimal antibody pairs. The BsAb field could benefit from the advent of higher throughput methods, such as BsAb libraries, to construct and screen BsAb to find such optimal antibody pairs.

Beyond BsAb we anticipate more extensive exploration of multi-specific and multivalent antibodies and appending antibodies with engineered protein scaffolds. Indeed, so-called Zybodies with up to 5 different antigen specificities and a total antigen-binding valency of 10 have already been demonstrated by appending a monospecific IgG with different molecular recognition domains at both the amino and carboxy termini of both heavy and light chains (LaFleur et al., 2013). Such multivalent and multispecific antibodies open broad new therapeutic opportunities albeit with the challenge of much greater complexity in drug development starting with the selection of target antigens. BsAb will likely be combined with other advances in antibody engineering such as half-life extension, effector function enhancement (Schanzer et al., 2014) or attenuation (Couch et al., 2013) and antibody-drug conjugates.

A large number of potential clinical applications of BsAb have been described. Additional therapeutic applications of BsAb are anticipated given the widespread interest in BsAb, the expanding repertoire of alternative formats (Fig. 1) and the growing body of clinical experience with BsAb (Table 1). BsAb seem likely to emerge as an important class of therapeutics over the next decade or so. Indeed, two BsAbs, catumaxomab and blinatumomab, are already approved for human therapy and >30 BsAb in clinical development, including >13 BsAb in phase II clinical trials or beyond.

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