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REVIEW

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Ten years in the making: application of CrossMab technology for the development of therapeutic bispecific antibodies and antibody fusion proteins

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ABSTRACT

Bispecific antibodies have recently attracted intense interest. CrossMab technology was described in 2011 as novel approach enabling correct antibody light-chain association with their respective heavy chain in bispecific antibodies, together with methods enabling correct heavy-chain association using existing pairs of antibodies. Since the original description, CrossMab technology has evolved in the past decade into one of the most mature, versatile, and broadly applied technologies in the field, and nearly 20 bispecific antibodies based on CrossMab technology developed by Roche and others have entered clinical trials. The most advanced of these are the Ang-2/VEGF bispecific antibody faricimab, currently undergoing regulatory review, and the CD20/CD3 T cell bispecific antibody glofitamab, currently in pivotal Phase 3 trials. In this review, we introduce the principles of CrossMab technology, including its application for the generation of bi-/multispecific antibodies with different geometries and mechanisms of action, and provide an overview of CrossMab-based therapeutics in clinical trials.

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Introduction into bispecific antibodies

Antibodies, or so-called immunoglobulins, are Y-shaped proteins of ca. 150 kDa generated by B and plasma cells of the immune system as response to infection. They consist of two identical heavy and two identical light chains forming: 1) two variable antigen-binding sites within the antigen-binding fragments (Fabs) that serve for the specific recognition of (foreign) antigens; and 2) a constant Fc domain that serves for the recruitment of the human immune system.

Recombinant antibodies have been used therapeutically for over 30 years, and today more than 120 therapeutic antibodies are approved or under regulatory review by health authorities for use in humans (source: https://www.antibodysociety.org/resources/approved-antibodies/). Since the advent of recombinant antibody technologies, there has been substantial interest in the generation of engineered and bispecific antibodies that are characterized by having two independent specificities in the Fabs, resulting in novel mechanisms of action that typically cannot be achieved with conventional monospecific antibodies. More than 100 bispecific antibodies are currently being tested in clinical trials.[1–6]

While uncountable approaches for the generation of bispecific antibodies have been described, [1–6] some of the most broadly applied technologies for the generation of bispecific antibodies include ART-Ig,[7–10] BEAT,[11] BiTE,[12,13] common light chains,[9,10,14–16] DAF,[17] DART,[18] DuoBody,[19] DutaFab,[20] DVD-Ig,[21] Fab arm exchange,[22] Fcab,[23–25] FORCE,[26] half antibody assembly,[27] Hetero-Ig,[28,29] IgG-scFv,[3031,131] κλ-bodies,[32] Multiclonics,[14] orthogonal Fab interface,[33] Tandab,[34] XmAb,[35] VELOCI-Bi,[15] and WuxiBODY.[36]

As of August 2021, three bispecific antibodies have been approved, the tandem single-chain variable fragment (Fv)-based CD19/CD3 Bispecific T-cell Engager (BiTE) blinatumomab developed by Amgen for the treatment of acute lymphocytic leukemia (ALL),[37] the heterodimeric ART-Ig-based coagulation factor IX/X bispecific IgG antibody emicizumab developed by Chugai and Roche for the treatment of hemophilia A,[7,8,10] and the heterodimeric DuoBody-based EGFR/c-Met bispecific IgG antibody amivantamab developed by Janssen for treatment of non-small cell lung cancer harboring EGFR exon 20 insertion mutations.[38–40]

CrossMab technology for the generation of bispecific antibodies

We have developed an alternative technology, known as CrossMab technology, which together with methods enabling correct heavy-chain association such as the so-called knobs-into-holes technology (KiH),[16] that enables the correct association of the different antibody light chains with their respective counterparts. This is achieved in different antibody formats and geometries by the exchange or crossover of antibody domains.[41–43] Here, we give a brief overview of the basic principles of CrossMab technology and its application for the generation of various CrossMabs with different molecular formats and mechanisms of action.[42,44,45] In fact, back in 2011, this approach was the first technology described allowing the conversion of two pre-existing antibodies into heterodimeric bispecific antibodies of the bivalent

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IgG format without the need to rely on so-called common light-chain antibodies that have identical light chains in each Fab.[42]

Since in bispecific antibodies the two heavy chains as well as the two light chains are different and can randomly associate, expression of these four chains leads to the formation of ten different antibody variants.[46] Correct heavy-chain association resulting in a heterodimeric Fc can be enforced using KiH technology by introducing a bulky tryptophan (Trp) residue in one Fc fragment and forming a corresponding cavity on the other Fc fragment that can accommodate the Trp residue.[16,47,48] More recently, multiple alternative approaches to enable correct heavy-chain association have been described, such as relying on charge interactions.[7–11,14,29,49]

Although KiH technology was developed in the late 1990s,[16] enabling correct light-chain association remained a major problem, and the only approach to achieve this at the time relied on the use of common light chains for both specificities.[9,10,14–16] However, the use of a common light chain requires the *de novo* identification of the corresponding antibody pairs, which can be challenging and/or time-consuming depending on the desired target, and restricts the availability and diversity of antibodies that can be used; thus, methods allowing the generation of bispecific antibodies from pre-existing antibody pairs were highly desired.

Figure 1 shows the basic principle of the domain crossover applied in CrossMab technology to enable correct light-chain association in bispecific antibodies.[41] By incorporating the original heavy chain VH-CH1 domains in the Fab of the second specificity of the bispecific antibody as the novel "light chain" and the original light chain VL-CL domains for the novel "heavy chain" by fusing them to the hinge region of the Fc fragment, correct light-chain association can be enforced in the CrossMab^{Fab} format. This format has recently also been described as Fabs-in-Tandem Ig (FIT-Ig).[50,51]

Alternatively, only the VH-VL or only the CH1-CL domains can be exchanged in the CrossMab$^{VH\text{-}VL}$ and CrossMab^{CH1-CL} formats (Figure 1). In the case of the CH1-CL crossover, no theoretical side product due to domain crossover is expected and crystal structure analysis confirmed the structural integrity of the crossed Fab domain in the CrossMab$^{CH11-CL}$ format.[52] In the case of the Fab crossover in the CrossMab^{Fab}, two heavy and light chain-based monovalent side products can be observed. However, the correct preferential formation of the CrossMab^{Fab} can be fostered by relative over-expression of the respective light chains so that the respective undesired monovalent and binding inactive side products do not form in significant amounts. Similarly, in the case of VH-VL crossover, a Bence-Jones-like side product based on VL-VL together with the CH1-CL interaction can be observed. In order to avoid formation of this side product, natural charge pairs in the Fab were identified and the respective orthogonal charge interactions were introduced into the non-exchanged antibody CH1-CL domains.[53] As a consequence, the undesired Bence-Jones-like side product does not form due to repulsive charge interactions, whereas the desired light-chain pairs correctly in the non-crossed Fab due to attractive charge interactions so that the corresponding CrossMab$^{VH\text{-}VL\pm}$ constructs can subsequently be obtained in high yields and purity without major side products.

Notably, these design principles can be applied not only to heterodimeric antibodies where one arm is directed to the first antigen, the other arm to the second antigen (1 + 1 format), but the CrossMab technology also allows generation of so-called MonoMabs, monovalent antibodies with one Fc portion, and DuoMabs, bivalent antibodies with two Fc portions (Figure 1).[54] Furthermore, it can be applied to enable the correct light-chain association in hetero-/homodimeric bi-/multispecific antibody appended or tandem-Fab formats with, for example, 2 + 1, 2 + 2, 3 + 1, 4 + 1 or 4 + 2 valencies and in antibody fusion proteins (Figure 2).[44,45] In line with this, Wu and colleagues from Lilly applied Fab crossover to generate orthogonal Fab-based trispecific antibody formats termed "OrthoTsAbs".[55,56] Interestingly, domain crossover has also been described as a means to prevent mispairing of T-cell receptor (TCR) domains in adoptive T-cell therapy.[57]

Because the CrossMab approach showed advantages in terms of production, stability, developability, and versatility over analogous formats based on either single-chain Fv[58–60] or single-chain Fab[61–67] building blocks, it was ultimately cho-

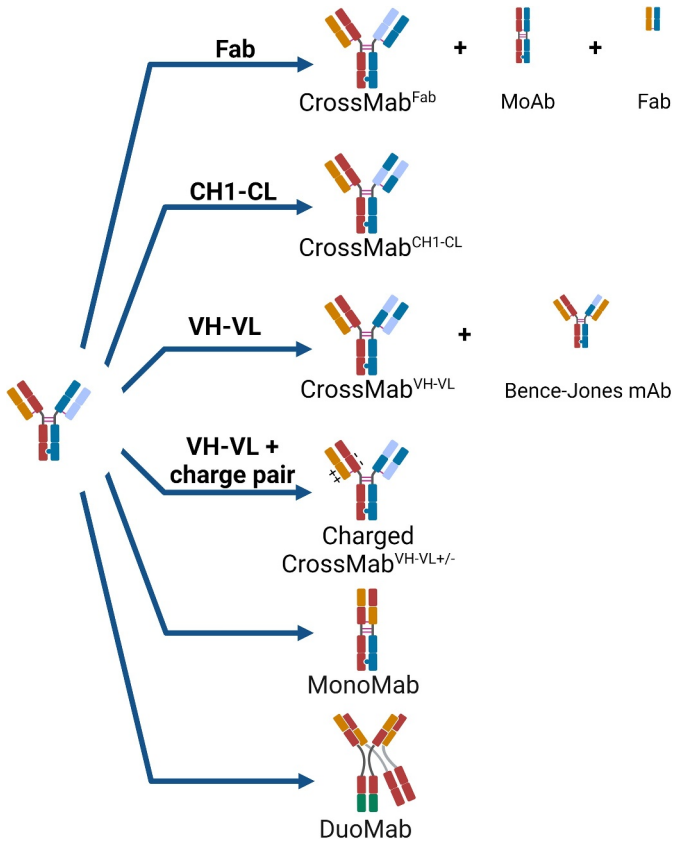

Figure 1. Principles of CrossMab technology: The four major CrossMab formats as applied to 1 + 1 heterodimeric bispecific antibodies are depicted as well as potential side products. On the bottom, the structure of mono- and duomabs is indicated. Heavy-chain domains are depicted in dark colors and respective light-chain domains are depicted with corresponding bright colors. Created with BioRender.com.

sen as the antibody engineering approach of choice for the generation of various clinical development candidates. Obviously, in order to develop CrossMabs for therapeutic use, the establishment of various methods covering CMC (Chemistry, Manufacturing, and Controls) aspects, including upstream and downstream processing (USP, DSP) and the establishment of the respective bioassay and (bio-) analytical methods was and is essential.[68–8081] When considering the formation of (undesired) side products, it has to be taken into account that, independent of CrossMab technology, other unrelated side products can occur, such as half- or ¾-antibodies missing two or one light chains, respectively, or hole-hole/knob-knob heavy-chain homodimers. In order to avoid the formation of these side products, achieving equal expression levels for the four heavy and light chains during transient expression and/or stable cell line generation by selecting suitable clones is advantageous. Based on the general advancement in the field of therapeutic antibody manufacturing, as well as considering these specific learnings, bispecific antibodies of different formats based on CrossMab technology can generally be manufactured in a consistent and reproducible fashion with volumetric yields in the several g/L range and in quality comparable to conventional therapeutic antibodies using established USP and DSP platforms.

Consequently, since the original description of this concept, the technology has evolved in the past decade into one of the most mature, versatile, and broadly applied technologies in the field for the generation of various bispecific antibody formats. As of mid-2021, at least 19 bispecific antibodies and fusion proteins based on CrossMab technology developed by Roche and others have entered clinical trials, of which 16 continue to be evaluated in active clinical trials (Table 1 and Figure 2). In the following sections, an overview of therapeutic bispecific antibodies and fusion proteins based on CrossMab technology is provided, with a focus on those in clinical trials.

Applications in targeted cancer therapy: Angiogenesis, receptor tyrosine kinases, and death receptor signaling

For many years, anti-angiogenesis approaches blocking the vascular endothelial growth factor-A (VEGF-A) have been a major area of targeted cancer therapy.[95–96] One of the first IgG-based antibodies and the first bispecific CrossMab to enter clinical trials, in 2012, was the heterodimeric 1 + 1 VEGF/Ang-2 CrossMab^{CH1-CL} vanucizumab (RG7221) (Figure 2a) targeting the pro-angiogenic ligands VEGF-A and angiopoietin-2 (Ang-2), which are involved in (tumor) angiogenesis.[95,96,97] Vanucizumab, as well as a mouse-specific surrogate bispecific, mediated potent anti-tumoral and anti-angiogenic efficacy in various preclinical models as monotherapy and in combination with chemotherapy,[81,98–101] as well as combined with PD-1 checkpoint inhibition[102–104] and CD40 agonism.[105,106] Vanucizumab was generally well tolerated as a monotherapy in a Phase 1 clinical trial and demonstrated promising anti-tumor efficacy, IgG-like pharmacokinetics and low immunogenicity,[107] as well as the anticipated pharmacodynamic mechanism of action.[108] Based on the negative outcome of the randomized McCAVE Phase 2 study, where it was compared to bevacizumab in combination with FOLFOX-6 chemotherapy in patients with untreated metastatic colorectal carcinoma, clinical development was discontinued.[109] Similarly, in spite of promising preclinical data, Phase 1b studies of vanucizumab in combination with the PD-L1 antibody atezolizumab (NCT01688206) and the CD40 antibody selicrelumab (NCT02665416) were ultimately discontinued. Recently, preclinical data demonstrated that dual inhibition of VEGF and Ang-2 by the vanucizumab mouse-specific surrogate bispecific in murine sepsis models improved the outcomes, making it a potential therapeutic against vascular barrier breakdown.[110] Similarly, Zhou and colleagues reported on an alternative anti-angiogenic approach for cancer therapy using a heterodimeric 1 + 1 VEGF/DLL4 CrossMab^{CH1-CL} called HB-32 that mediated potent anti-angiogenic activity *in vitro*, as well as *in vivo* anti-tumor activity in breast cancer xenograft models.[111]

In addition to anti-angiogenesis, targeting receptor tyrosine kinases (RTKs) like EGFR, HER2 or c-Met has been a major area for cancer therapy during the past decades.[112] Accordingly, various preclinical-stage bispecific CrossMabs targeting RTKs have been developed during the past years, but none of these have advanced to clinical trials so far. Zhang and colleagues created a biparatopic HER2/HER2 1 + 1 CrossMab^{Fab} based on trastuzumab and an avidity-improved variant L56TY derived from pertuzumab called Tras-Permut CrossMab. Tras-Permut CrossMab mediated improved activity against trastuzumab-resistant breast cancer and enhanced calreticulin exposure, which may contribute to the induction of tumor-specific T-cell responses.[113] Lu and colleagues, in turn, generated a bispecific HER2/EGFR 1 + 1 CrossMab^{CH1-CL} based on the trastuzumab and cetuximab.[72] Interestingly, Hu and colleagues went a step further and generated so-called four-in-one antibodies that exhibited four different specificities against EGFR, HER2, HER3, and VEGF by generating a 1 + 1 CrossMAb^{CH1-CL} using dual-acting Fabs (DAF) as building blocks in the FL518 bispecific or by combining CrossMab and DVD-Ig technology in the tetraspecific, tetravalent antibody CRTB6 to enable correct light-chain association in the DVD format.[114] Not surprisingly, these tetraspecific antibodies showed superior efficacy as compared to the respective bispecific antibodies.[114] Finally, different bispecific EGFR/Notch CrossMabs were described to block EGFR signaling together with Notch signaling. The first of these antibodies, termed CT16, combined the EGFR antibody cetuximab and the Notch 2/3 antibody tarextumab using the prototypical heterodimeric 1 + 1 CrossMab$^{CH11-CL}$ format, which served as a radiosensitizer and prevented acquisition of resistance to EGFR inhibitors and radiation in cell line models of non–small cell lung cancer and patient-derived xenograft tumors.[115] In a second publication from the same group, three heterodimeric bispecific 1 + 1 CrossMab^{CH1-CL} antibodies (PTG12, RTB3, MTJ16) were generated from panitumumab/tarextumab, RG7116/tarextumab, and MEHD7945A/tarextumab and were shown to increase the response to PI3K inhibition with GDC-0941 by inhibiting stem cell–like subpopulation, reducing tumor-initiating cell frequency, and downregulating mesenchymal gene expression.[116]

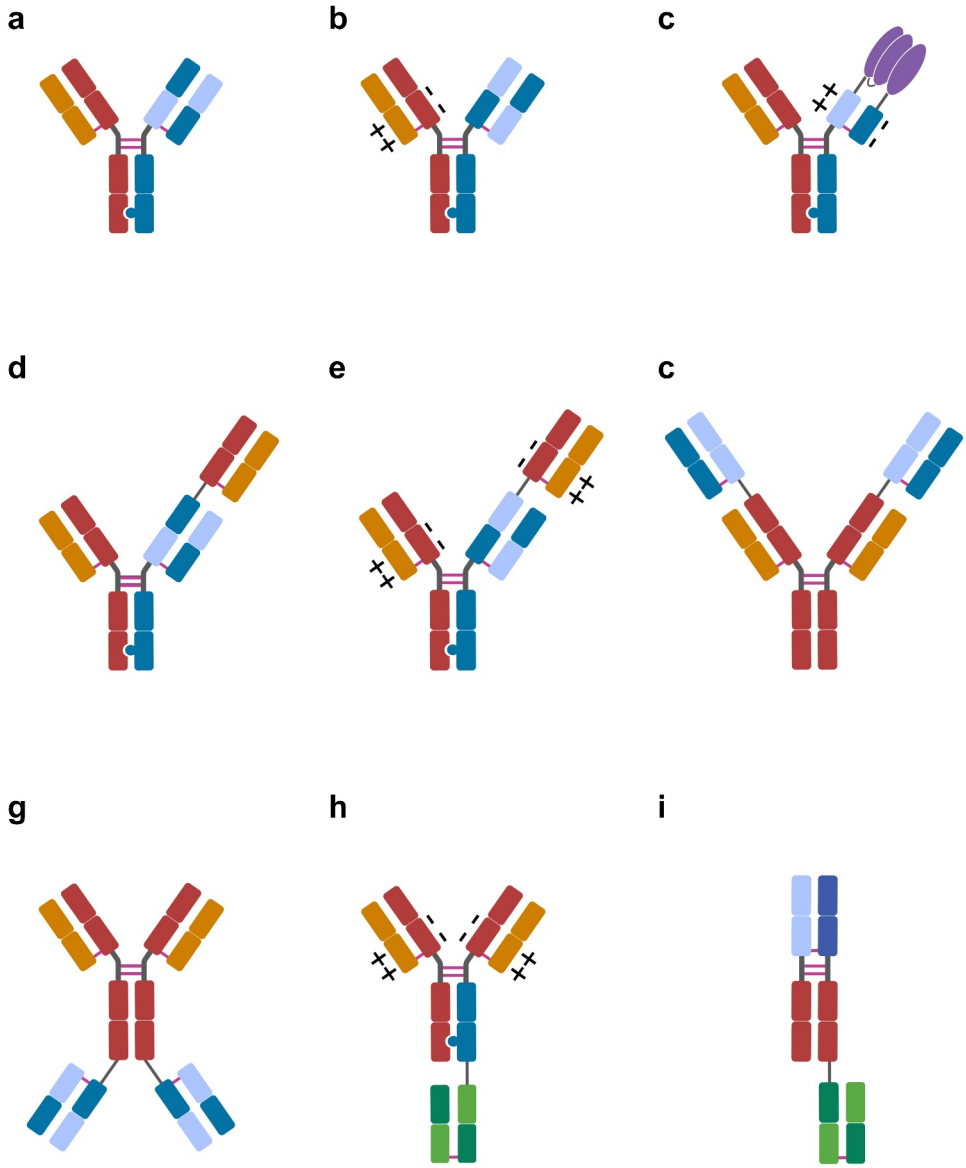

Figure 2. Major CrossMab formats: A) 1 + 1 CrossMab:$^{CH1-CL}$ vanucizumab, faricimab, 10E8.4/iMab 1 + 1; **B)** 1 + 1 CrossMab$^{VH-VL\pm}$: PD1-TIM3, PD1-LAG3; **C)** CrossMab$^{CH1-CL+/-}$based FAP-4-1BBL, CD19-4-1BBL fusion proteins; **D)** 2 + 1 CrossMab:$^{CH1-CL}$ cibisatamab; **E)** 2 + 1 CrossMab$^{VH-VL\pm}$: glofitamab, CC-93269, TYRP1-TCB, WT1-TCB, RG6123; **F)** 2 + 2 CrossMab^{CH1-CL}-based FIT-Ig EMB-01, EMB-02, EMB-06; **G)** 2 + 2 CrossMab:$^{CH1-CL}$ FAP-DR5; **H)** 2 + 1 CrossMab $^{VH-VL\pm}$: BS-GANT, FAP-CD40; **I)** 1 + 1 CrossMab^{CH1-CL}-based Nkp46-based NK cell engager (NKCE). Heavy-chain domains are depicted in dark colors and respective light-chain domains are depicted with corresponding bright colors. Fusion protein depicted in purple. Note: Differences in variable regions and/or isotype and Fc engineering are not depicted. Created with BioRender.com.

Another major field in targeted cancer therapy has been and continues to be apoptosis induction through death receptor (DR) signaling.[117,118] As conventional DR5 antibodies have not been successful in clinical trials, approaches for tumor-targeted DR5 agonism have been pursued. Expression of the fibroblast activation protein (FAP) on tumor fibroblasts is found in the majority of solid tumors, making FAP an attractive antigen for tumor targeting.[119,120] Based on this rationale, FAP-targeted bispecific antibodies and fusion proteins have been created using CrossMab technology that rely on FAP binding with one moiety to induce, with their second moiety, hyper-clustering of tumor necrosis factor (TNF) receptor superfamily members[121] like DR5 for apoptosis induction,[85] 4–1BB/CD137 for T cell activation,[89] or CD40 for activation of antigen-presenting cells,[94,122] as described below. The first of these conditional FAP-targeted TNFR agonistic antibodies entering Phase 1 clinical trials was the symmetric tetravalent C-terminally fused FAP/DR5 targeted 2 + 2 CrossMab^{CH1-CL} RG7386 (Figure 2g). Preclinical data demonstrated that RG7386 effectively triggered FAP-dependent, avidity-driven DR5 hyper-clustering and subsequent tumor cell apoptosis,[85] but ultimately, clinical development of RG7383 was not further continued after the completed Phase 1 study (NCT02558140) due to portfolio reprioritization.

Finally, Tung and colleagues described novel HER2 or CD19 tumor-targeted heterodimeric 1 + 1 CrossMab^{CH1-CL} antibodies that recognize with their second specificity PEGylated proteins, liposomes, and nanoparticles. Using these bispecific antibodies, cytotoxic cargo such as PEGylated liposomal doxorubicin can be delivered to tumor cells.[123]

Table 1. CrossMabs in clinical trials (status July 2021), FP: Fusion protein, FIT-Ig: Fabs-in-tandem Ig, EIH: Entry into human date.

	Name	Target A/B	Format	Indication	Stage	Company	EIH	Clinical trial	Reference
1	Vanucizumab (RG7221)	Ang-2/VEGF-A	1 + 1 CrossMab^{CH1-CL}	Oncology	Terminated Ph 2	Roche	2012	NCT02141295, NCT01688206, NCT02665416	81
2	Faricimab (RG7716)	Ang-2/VEGF-A	1 + 1 CrossMab^{CH1-CL}	DME, wAMD	Ph 3	Roche	2013	NCT03823287, NCT03823300, NCT03622580, NCT03622593	82,83
3	Cibisatamab (RG7802)	CEA/CD3ε	2 + 1 CrossMab$^{CH11-CL}$	Oncology	Ph 1b	Roche	2014	NCT03866239, NCT04826003	84
4	FAP-DR5 (RG7386)	FAP/DR5	2 + 2 CrossMab$^{CH11-CL}$	Oncology	Terminated Ph 1	Roche	2015	NCT02558140	85
5	Glofitamab, RG6026)	CD20/CD3ε	2 + 1 CrossMab$^{VH-VL\pm}$	NHL	Ph 2/3	Roche	2017	NCT04703686, NCT04914741 NCT04077723, NCT04408638	86
6	PD1-TIM3 (RG7769)	PD-1/TIM-3	1 + 1 CrossMab$^{VH-VL\pm}$	Oncology	Ph 1/2	Roche	2018	NCT03708328, NCT04785820	87
7	RG6123	CEACAM5/CD3ε	2 + 1 CrossMab$^{VH-VL\pm}$	Oncology	Terminated Ph 1	Roche	2018	NCT03539484	-
8	BCMA TCE (CC-93269)	BCMA/CD3ε	2 + 1 CrossMab$^{VH-VL\pm}$	Multiple Myeloma	Ph 1	BMS	2018	NCT03486067	88
9	FAP-4-1BBL (RG7827)	FAP/4-1BB	1 + 3 CrossMab$^{CH1-CL\pm}$ 4-1BBL FP	Oncology	Ph 1b	Roche	2018	NCT03869190, NCT04826003	89
10	10E8.4/iMab, TMB-370	HIV-1 Env/CD4	1 + 1 CrossMab^{CH1-CL}	HIV-1	Ph 1	TaiMed	2019	NCT03875209	90
11	EMB-01	EGFR/c-Met	2 + 2 CrossMab^{Fab} /FIT-Ig	Oncology	Ph 1	EpimAb	2019	NCT03797391	50,151
12	BS-GANT (RG6102)	Abeta/TfR	2 + 1 CrossMab$^{VH-VL\pm}$	Alzheimer's	Ph 2	Roche	2019	NCT04639050	
13	CD19-4-1BBL (RG6076)	CD19/4-1BB	1 + 3 CrossMab$^{CH1-CL\pm}$ 4-1BBL FP	NHL	Ph 1b	Roche	2019	NCT04077723	89
14	PD1-LAG3 (RG6139)	PD-1/LAG-3	1 + 1 CrossMab$^{VH-VL\pm}$	Oncology	Ph 1/2	Roche	2019	NCT04140500, NCT04785820	91
15	TYRP1-TCB (RG6232)	TYRP1/CD3ε	2 + 1 CrossMab$^{VH-VL\pm}$	Melanoma	Ph 1	Roche	2020	NCT04551352	92
16	WT1-TCB (RG6007)	WT1/CD3ε	2 + 1 CrossMab$^{VH-VL\pm}$	AML	Ph 1	Roche	2020	NCT04580121	93
17	EMB-02	PD-1/LAG-3	2 + 2 CrossMab^{Fab} /FIT-Ig	Oncology	Ph 1	EpimAb	2020	NCT04618393	-
18	EMB-06	BCMA/CD3ε	2 + 2 CrossMab^{Fab} /FIT-Ig	Multiple Myeloma	Ph 1	EpimAb	2021	NCT04735575	-
19	FAP-CD40	FAP/CD40	2 + 1 CrossMab$^{VH-VL\pm}$	Oncology	Ph 1	Roche	2021	NCT04857138	94

Applications in cancer immunotherapy: Dual checkpoint inhibitors, T and innate cell engaging bispecifics and tumor-targeted co-stimulation

With the advent of cancer immunotherapy and checkpoint inhibitor antibodies during the past decade, the development of bispecific antibodies for immunotherapy has attracted substantial attention in industry and academia, whereas the interest in anti-angiogenic and pro-apoptotic therapies has declined. In this context, bispecific monovalent dual checkpoint inhibitory PD-1 antibodies co-targeting the checkpoint inhibitory receptors TIM-3 or LAG-3 have been designed based on a bispecific 1 + 1 CrossMab$^{VH-VL\pm}$ format (Figure 2b), allowing avidity-mediated selectivity gain and thus enhanced selectivity for PD-1^{+} and PD-1^{+} TIM-3^{+}/LAG-3^{+} double-positive T cells. Both of these bispecific dual checkpoint inhibitory antibodies, PD1-TIM3 (RG7769) [87] and PD1-LAG3 (RG6139),[91] are currently in Phase 1 and 2 clinical trials (NCT03708328, NCT04140500, NCT04785820).[124,125] Preclinically, a heterodimeric 1 + 1 PD-1/RANKL CrossMab^{CH1-CL} was shown to demonstrate potent tumor growth inhibition as a monotherapy and combined with CTLA-4 antibodies, particularly in models showing checkpoint inhibitor resistance to PD-1 antibodies.[126]

Many of bispecific antibodies currently being developed are bispecific T-cell engagers.[22,127–131] One of the first IgG-based, and Roche's first, T-cell bispecific antibody (TCB) to enter clinical trials was the heterodimeric and trivalent CEA/CD3ε 2 + 1 TCB cibisatamab (RG7802). It is a heterodimeric CEA/CD3ε bispecific antibody in the 1 + 1 CrossMab^{CH1-CL} format to which a single additional Fab targeting CEA is fused to the N-terminus of the knob-containing heavy chain (Figure 2d).[84,132] FcγR and C1q binding are abolished by introduction of P329G LALA mutations.[133] This so-called 2 + 1 TCB format provides advantages over conventional heterodimeric 1 + 1 TCB formats through the highly flexible head-to-tail fusion in the tandem Fab arm and by being bivalent for the tumor antigen, allowing a better differentiation between tumor and normal cells due to avidity-mediated affinity tuning.[132] Cibisatamab demonstrated tumor targeting and *in vitro* and *in vivo* anti-tumor efficacy dependent on CEA over-expression due to the bivalent binding mode in models of colorectal and gastric cancer,[84,134–137] which was further enhanced when combined with PD-L1 inhibition.[138] Based on these data and using a MABEL approach due to the lack of cross-reactive toxicology species,[139,140] clinical studies were initiated in relapsed/refractory CEA-positive colorectal cancer patients. Cibisatamab is currently in Phase 1b clinical trials in combination with the PD-L1 antibody atezolizumab (NCT03866239) and with FAP-4-1-BBL (NCT04826003) (see below). Pre-treatment with obinutuzumab is being clinically explored to mitigate the potential development and impact of anti-drug antibodies that could be observed in patients treated with cibisatamab.

The most advanced 2 + 1 T cell bispecific antibody is glofitamab (RG6026), which, in contrast to cibisatamab, is based on a 2 + 1 CrossMab$^{VH\text{-}VL}$ format with charge interactions using variable regions derived from obinutuzumab (Figure 2e). Glofitamab showed potent tumor cell killing and antitumor efficacy in preclinical *in vitro, ex vivo* and *in vivo* lymphoma models, as well as superiority over the respective conventional heterodimeric 1 + 1 TCB formats as a consequence of its head-to-tail orientation and bivalent binding to CD20, allowing pre-treatment with obinutuzumab, in this case as a strategy to reduce the incidence of cytokine-release syndrome by glofitamab.[86,141,142] Based on the clinical efficacy and safety in the Phase 1 clinical trial in relapsed/refractory non-Hodgkin lymphoma (NHL) patients and particularly the high rate of durable complete responses,[143,144] glofitamab is currently being evaluated in multiple clinical trials in lymphoma patients, including trials in patients relapsed after CAR-T cell therapy (NCT04703686) and in Phase 3 clinical trials in relapsed/refractory diffuse large B cell lymphoma patients (NCT04077723, NCT04408638).[145] No anti-drug antibodies recognizing glofitamab were detected in the Phase 1 clinical study.[143]

Additional analogous 2 + 1 T cell bispecific antibodies using this technology have entered early clinical Phase 1 trials, including the BCMA-TCE CC-93269 for the treatment of multiple myeloma (NCT03486067)[88] and the TYRP1-TCB (RG6232) for the treatment of TYRP1-expressing melanoma (NCT04551352).[92] Recently, the WT1-peptide-MHC-specific TCR-like WT1-TCB (RG6007) for the treatment of acute myeloid leukemia (AML) became the first TCR-like bispecific antibody to enter a clinical trial (NCT04580121).[93] While Immunocore pioneered the field of targeting peptide-MHC complexes with recombinant TCR-based bispecific T-cell engagers, the so-called ImmTACs,[146] WT1-TCB is based on a TCR-like antibody fragment recognizing the RMF WT1 peptide-HLA-A*02 complex. WT1-TCB can mediate specific killing of AML cell lines and primary AML cells, and it has antitumor activity in humanized mice bearing SKM-1 tumors.[93]

Additional preclinical stage 2 + 1 TCBs based on this format have been described, including ones that target HER2[147,148] or the p95 HER2 fragment.[149] The p95 HER2 fragment is only found on a portion of ~ 30–40% of HER2^{+++} tumor cells, and as such can be considered a highly tumor-specific neoantigen. Thus, 2 + 1 TCBs targeting specifically p95 HER2 are of particular interest as they do not mediate T cell killing of normal cells that express HER2, such as cardiomyocytes or breast epithelial cells, as opposed to conventional HER2-TCBs.[149] An alternative approach to overcome the on-target off-tumor killing of normal cells is tumor-specific activation of TCBs by protease expressed in the tumor. For this purpose, a protease-activated mesothelin-TCB using CrossMab technology has been described that is blocked by an anti-CD3 anti-idiotypic mask that is cleaved in the tumor-microenvironment.[150] Alternatively, to counteract and manage any undesired T cell activation, it was shown that the Src/lck inhibitor dasatinib is able to reversibly switch off cytokine release and T-cell cytotoxicity following stimulation with different 2 + 1 TCBs targeting CEA, CD19 and WT1.[151] Finally, related to the TCB approach, 2 + 1 bispecific antibodies designed based on CrossMab technology have been developed specifically for the recruitment of synthetic agonistic receptor transduced T-cells (SAR-T) in adoptive T-cell therapy together with Kobold and colleagues.[152–154]

In order to further boost the potency of T-cell bispecific antibodies, the tumor (stroma)-targeted FAP-4-1BBL (RG7827), CD19-4-1BBL (RG6076) and CEA-4-1BBL fusion proteins have been developed for solid tumors and NHL. These molecules are used to provide the co-stimulatory TNF receptor superfamily-mediated signal 2 to T cells in combination with the T-cell bispecific antibodies cibisatamab or glofitamab, which provide the signal 1.[89,155,156] These 4–1BBL fusion proteins contain a split trimeric 4–1BB ligand fused to the CH1 and CL domains, and constant chain mispairing is abolished by CH1-CL domain crossover in conjunction with the respective charge pairs (Figure 2c).[89] FAP-4-1BBL and CD19-4-1BBL have been designed to trigger 4–1BB/CD137 hyper-clustering specifically in the tumor microenvironment, but not in circulation or in the liver, with the goal to overcome typical 4–1BB antibody-mediated toxicities.[89] Tumor-targeted 4–1BBL fusion proteins were shown to mediate improved T-cell activation, superior tumor control in combination with TCBs and checkpoint inhibitors, and strong T-cell infiltration in preclinical models.[89,155,157] Clinical Phase 1b studies combining cibisatamab with FAP-4-1-BBL (NCT04826003) and glofitamab with CD19-4-1BBL (NCT04077723) are currently ongoing.

A similar rationale was applied to trigger the TNF receptor superfamily member CD40 on antigen-presenting cells (APCs) through a trivalent C-terminally fused FAP/CD40 2 + 1 bispecific antibody in a 2 + 1 CrossMab$^{VH\text{-}VL\pm}$ format with charges (Figure 2h). This design was chosen to make FAP-CD40 (RG6189), a FAP-targeted CD40 agonistic bispecific antibody, with the goal of abrogating systemic toxicity and enabling administration of doses sufficiently high to result in highly tumor- and lymph node-specific activation of APCs with subsequent induction of antitumor immunity.[94,122] Phase 1 clinical trials have been initiated to validate this approach in the clinic (NCT04857138).

Notably, the domain crossover/CrossMab technology has also been used by researchers outside of Roche for the development of bispecific antibodies for cancer immunotherapy. This includes the so-called Fabs-in-tandem Ig (FIT-Ig) approach developed by Gong and colleagues from EpimAb, which relies on Fab crossover to enable correct light-chain association for the generation of symmetric tetravalent N-terminally fused bispecific antibodies in the 2 + 2 CrossMab^{Fab} format (Figure 2f).[50,51] Three different bispecific FIT-Igs have reached clinical Phase 1 trials to date co-targeting: 1) EGFR/c-Met for receptor tyrosine kinase inhibition (EMB-01) (NCT03797391), 2) PD-1/LAG-3 for dual checkpoint inhibition (EMB-02) (NCT04618393), and 3) BCMA/CD3ε for T cell engagement in multiple myeloma (EMB-06) (NCT04735575).

In order to recruit innate immune cells for cancer cell killing, Gauthier and colleagues from Innate Pharma recently described an advanced preclinical approach to generate multifunctional natural killer cell engagers (NKCE) targeting a tumor antigen and the NK cell ligand NKp46 in a FcγRIII-binding competent monovalent C-terminally fused 1 + 1 antibody format using CH1-CL crossover to ensure correct light-

chain association (Figure 2i).[158] Trifunctional NKCEs targeting CD19, CD20, or EGFR as tumor antigens triggered tumor killing by human primary NK cells *in vitro* and induced NK cell infiltration and anti-tumor efficacy, as well as protective tumor immunity *in vivo*.[158]

Zhao and colleagues demonstrated that a bispecific heterodimeric CD20/HLA-DR 1 + 1 CrossMab^{CH1-CL} termed CD20–243 CrossMab for the treatment of NHL patients co-expressing CD20 and HLA-DR mediated strong complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity and anti-proliferative activity.[159] Similarly, Rajendran and colleagues generated a bispecific heterodimeric CD30/CD137 1 + 1 CrossMab^{CH1-CL} to target specifically these two co-expressed antigens on Hodgkin and Reed-Sternberg cells without inducing CD137 signaling.[160]

In an alternative approach to activate innate immunity, Du and colleagues devised a bispecific heterodimeric GPC3/CD47 1 + 1 CrossMab^{CH1-CL} to bind to GPC3 and CD47 on hepatocellular cancer cells, and at the same time inhibit the CD47 interaction with SIRP1α responsible for the "do-not-eat-me signal" to recruit myeloid cells for phagocytosis.[161] The GPC3/CD47 CrossMab induced enhanced Fc-mediated effector functions by both macrophages and neutrophils toward dual antigen-expressing hepatocellular carcinoma (HCC) cells *in vitro*, and strong *in vivo* efficacy against xenograft HCC tumors in a fashion superior to the respective monotherapies and combination thereof.[161]

In order to further boost antigen presentation and foster the generation of a secondary anti-tumor immune response, again Zhao and colleagues created a novel CD20/Flt3 ligand antibody fusion protein, termed CD20-Flex BiFP using CrossMab technology.[162] CD20-Flex BiFP not only eliminated lymphoma temporarily but also potentiated tumor-specific T-cell immunity by expanding and fostering infiltration of antigen-presenting dendritic cells into the tumor tissue.[162]

Most recently, Panina and colleagues described a novel bispecific heterodimeric HER2/IFNα-1 + 1 CrossMab^{CH1-CL} with the ultimate goal to deliver IFNα into HER2 expressing tumors.[163]

Applications in therapy of viral infections and autoimmune diseases

The application of CrossMab technology has become quite popular for the generation of bispecific and multispecific antibodies targeting various viruses. During the past years, multiple highly potent bispecific antibodies targeting HIV-1 have been generated using CrossMab technology for the prevention and treatment of HIV-1.[164,165] Examples of these approaches are: 1) four different 1 + 1 CrossMab^{CH1-CL}-based bispecific antibodies, of which the one based on VRC07 and PG9-16 displayed the most favorable neutralization profile and IgG-like pharmacokinetic properties in monkeys;[166] 2) 1 + 1 CrossMab^{CH1-CL}-based bispecific antibodies that, however, did not allow intra-spike binding;[167] 3) unique bispecific antibodies based on the broadly neutralizing antibodies (bNAbs) 3BNC117 and 10–1074 with a modified hinge region of human IgG3 isotype for increased Fab flexibility and improved neutralization potency based on a 1 + 1 CrossMab^{CH1-CL} format;[168] 4) a 1 + 1 CrossMab^{CH1-CL}-based bispecific antibody targeting two non-competing epitopes on the HIV-1 co-receptor CCR5 based on RoAb13 and PRO 140 to increase avidity;[169] 5) the 1 + 1 CrossMab^{CH1-CL}-based bispecific antibody iMab-CAP256 comprising the highly potent CAP256.VRC26.25 bNAb and the host-directed CD4 antibody, ibalizumab (iMab);[170] and 6) the 1 + 1 CrossMab^{CH1-CL}-based bispecific antibody BICM-1A for simultaneous recognition of two critical V2- and V3-glycan epitopes of the single HIV-1 envelope glycoprotein.[171] Of all these approaches, the heterodimeric bispecific 1 + 1 CrossMab^{CH1-CL} antibody 10E8.4/iMab showed exquisite potency and breadth against various HIV-1 strains, including activity in HIV-1 *in vivo* treatment and prevention models,[90,172] and compared very favorably to conventional antibodies and other bispecific bNAbs.[173] Based on these data, 10E8.4/iMab is currently being evaluated in a Phase 1 clinical trial (NCT03875209).[174]

Wang and colleagues generated a symmetric and tetravalent FIT-Ig-based bispecific antibody against Zika virus that showed high *in vitro* and *in vivo* potency, and prevented viral escape, supporting its potential use for the therapy of Zika virus prevention or infections.[175]

Interestingly, and most recently, De Gasparo and colleagues described the first bispecific antibody targeting SARS-CoV-2 based on a 1 + 1 CrossMab^{CH1-CL} format targeting two non-overlapping sites on the receptor binding domain of SARS-CoV-2 and blocking binding to angiotensin-converting enzyme 2 (ACE2).[176] The respective bispecific antibody CoV-X2 was designed using C121 and C135, two antibodies derived from donors who had recovered from COVID-19. Most notably, CoV-X2 neutralized wild-type SARS-CoV-2 and variants of concern and escape, protected mice from disease and suppressed viral escape.[176] Along these lines, Jette and colleagues described a subset of donor-derived neutralizing bispecific CrossMabs with broad cross-reactivity to sarbecoviruses.[177]

Bispecific CrossMab-based antibodies have also been generated with the goal of treating autoimmune diseases.[178,179] Fischer and colleagues showed that combined inhibition of TNFα and IL-17 was more effective in inhibiting the development of inflammation and bone and cartilage destruction in arthritic mice compared to the respective monotherapies. For this purpose, bispecific TNFα/IL-17 1 + 1 and 2 + 2 CrossMab^{CH1-CL} antibodies were prepared that showed superior efficacy in blocking cytokine and chemokine responses *in vitro*.[180] Similarly, Xu and colleagues showed that a tetravalent bispecific TNFα/IL-17 1 + 1 CrossMab^{VH-VL} together with electrostatic steering for heavy-chain heterodimerization significantly decreased the expression level of neutrophil and Th17 chemokines, and the secretion of IL-6/IL-8 on fibroblast-like synoviocytes. Moreover, combined inhibition of both cytokines by the bispecific antibody was superior to inhibition of either cytokine alone.[181] Based on these data,

dual-targeting bispecific antibodies neutralizing pro-inflammatory cytokines may provide novel treatment options for autoimmune diseases. However, as they are not necessarily differentiated from the combination of the respective monotherapies, the benefit of using a bispecific antibody over a combination therapy needs to be assessed on a case-by-case basis.

Applications in ophthalmology and therapy of central nervous system diseases

The heterodimeric 1 + 1 VEGF/Ang-2 CrossMab^{CH1-CL} vanucizumab (RG7221) was the first anti-angiogenic bispecific antibody to enter clinical trials with the goal of suppressing tumor angiogenesis via simultaneous blockade of the pro-angiogenic ligands VEGF-A and Ang-2. VEGF and Ang-2 have also been shown to play an important role in ocular angiogenesis in diseases like wet age-related macular degeneration (wAMD) and diabetic macular edema (DME).96[182–184] However, until now only the VEGF blocking antibody fragments ranibizumab and brolucizumab and the VEGFR1/2-ECD-Fc fusion protein aflibercept are approved for use in ophthalmology.[185]

Faricimab (RG7716) is a heterodimeric 1 + 1 VEGF/Ang-2 CrossMab^{CH1-CL} specifically optimized for intraocular use and high concentration formulation in ophthalmology indications by use of optimized anti-VEGF and anti-Ang-2 Fabs, as compared to vanucizumab, and by the introduction of P329G LALA and Triple A mutations in the KiH-containing IgG1 Fc portion to abolish FcγR-mediated effector functions and FcRn recycling for low systemic exposure.[82,83,186–189] While faricimab neutralizes two soluble ligands, particularly in the field of ophthalmology the use of such a bispecific antibody provides advantages in terms of intraocular administration via a single injection due to the simultaneous inhibition of two different angiogenic pathways with a single agent. Importantly, as compared to VEGF inhibition alone, faricimab mediated improved anti-angiogenic activity in various preclinical models to limit pathological angiogenesis in the eye.[82,83,190,191] Based on these data, faricimab was the first bispecific antibody worldwide entering Phase 1 clinical trials in ophthalmology, where it was well tolerated and exhibited a favorable safety profile with evidence of improvements in best-corrected visual acuity (BCVA) and anatomic parameters supporting further clinical investigation.[192] Subsequently, faricimab was compared head-to-head to ranibizumab in the BOULEVARD Phase 2 randomized clinical trial in patients with DME, where it met the primary end point and demonstrated statistically superior visual acuity gains versus ranibizumab, suggesting a benefit of simultaneous inhibition of angiopoietin-2 and VEGF-A.[193] In the AVENUE Phase 2 randomized clinical trial in patients with AMD, it did not meet the primary end point of superiority over ranibizumab in BCVA at week 36, but visual and anatomical gains observed with faricimab supported pursuing Phase 3 trials for an alternative to monthly anti-VEGF therapy.[194] This was taken into account together with the data from the STAIRWAY Phase 2 randomized clinical trial in AMD where faricimab dosed every 16 weeks or 12 weeks resulted in maintenance of initial vision and anatomic improvements comparable with monthly ranibizumab.[195,196] Recently, positive outcomes were reported from four independent pivotal Phase 3 trials in wAMD and DME patients where faricimab was compared to aflibercept and met the primary endpoints (NCT03823287, NCT03823300, NCT03622580, NCT03622593). Based on these data, marketing applications for faricimab have been filed with health authorities for approval in DME and wAMD, with FDA granting it a priority review.[197]

The treatment of central nervous system (CNS) diseases with monoclonal antibodies is hampered by the low penetration of antibodies through the blood-brain barrier, and the field still is in its infancy.[198] To overcome this limitation, Niewoehner and colleagues have generated transferrin receptor-targeted bispecific antibodies that allowed delivery of these antibodies through the blood-brain barrier and showed improved brain exposure and prevented plaque formation.[66,67] Using this approach, BS-GANT (RG6102) was generated based on the amyloid-beta antibody gantenerumab[199] as a trivalent C-terminally fused amyloid-beta/TfR 2 + 1 bispecific antibody in a 2 + 1 CrossMab$^{VH-VL\pm}$ format with charges (Figure 2h). BS-GANT (RG6102) recently entered Phase 2 clinical trials in patients with prodromal or mild-to-moderate Alzheimer's disease (NCT04639050).

Conclusions

During the past 20 years, numerous technologies have been developed to generate bispecific antibodies, and these molecules represent a rapidly growing class of biopharmaceuticals in clinical trials and on the market. CrossMab technology was first described in 2011 as a novel approach enabling correct antibody light-chain association with their respective heavy chain in bi-/multispecific antibodies, together with methods enabling correct heavy-chain association.

As briefly mentioned in the introduction, alternative technologies to achieve correct heavy-light-chain pairing are currently being applied for the generation of prototypical (heterodimeric) IgG-like bispecific antibodies. These include *in vitro* assembly approaches, where the two bispecific antibodies are produced separately and subsequently assembled *in vitro* like DuoBody,[19] Fab arm exchange,[22] FORCE[26] or half antibody assembly,[27] as well as approaches allowing the production of bispecific antibodies in one cell line, for example via the use of common light chains or orthogonal Fab interfaces. Recently, several groups have also reported that the specific pairing preferences of selected heavy and light chain pairs can be used to drive the assembly of correct bispecific antibodies.[200,201] In the field of common light chains, much progress has been made in the selection of suitable common light-chain antibodies from common light-chain-bearing animals or use of *in vitro* display technologies.[9,10,14–16,202–205] Based

on this progress, several bispecific common light-chain-based IgG antibodies are currently in clinical trials, including odronextamab, REGN4018, REGN5678, REGN7075, MCLA-145, MCLA-158, and others.[7,8,10,15,206–213] Alternatively, the correct light-chain-heavy-chain association can be enforced using orthogonal Fab interfaces by introduction of (several) mutations in the Fab interface.[28,29,33,55,56,214,215]

CrossMab technology continues to represent a simple, straightforward and clinically validated antibody engineering solution to achieve correct light-chain association with minimal engineering using existing pairs of antibodies. In fact, since its original description, it has evolved into one of the most mature, versatile, and broadly applied technologies in industry and academia, in conjunction with the KiH technology. Until now ~20 bispecific antibodies and fusion proteins based on CrossMab technology developed by Roche and others have entered clinical trials. Based on the available clinical data, CrossMabs show favorable IgG-like properties in terms of pharmacokinetics and immunogenicity similar to conventional therapeutic monoclonal antibodies. The most advanced of these bispecific antibodies are: 1) the 1 + 1 heterodimeric Ang-2/VEGF bispecific antibody faricimab for the treatment of DME and wAMD, which is currently undergoing regulatory review, and 2) the 2 + 1 heterodimeric CD20/CD3 T-cell bispecific antibody glofitamab for the treatment of relapsed/refractory DLBCL or follicular NHL, which is currently in pivotal Phase 3 clinical trials.

Based on the progress in making bi- and multispecific antibodies, we anticipate that this class of therapeutics with novel mechanisms of actions as compared to conventional therapeutic antibodies will have a major impact on the treatment of various diseases, including oncology, infectious diseases, autoimmunity, CNS, and metabolic diseases. Taken together, CrossMab technology has proven to be very useful for the fast and straightforward generation of bispecific antibody formats to tackle novel biological challenges and help to develop novel therapeutic concepts for patients in need.

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Disclosure statement

MS declares employment with Roche, WS declares patents/royalties with Roche and CK declares employment, stock ownership, and patents/royalties with Roche. CROSSMAB® is a registered trademark by Genentech/Roche.

Abbreviations

ACE2	Angiotensin-Converting Enzyme 2
ALL	Acute Lymphocytic Leukemia
AML	acute myeloid leukemia
Ang-2	Angiopoietin-2
BCVA	Best-Corrected Visual Acuity
BiTE	Bispecific T cell Engager
bNAbs	broadly Neutralizing Antibodies
CEA	Carcinoembryonic antigen
CMC	Chemistry, Manufacturing, and Controls
CNS	Central Nervous System
DAF	Dual Acting Fab
DME	Diabetic Macular Edema
DSP	Downstream Processing
DVD-Ig	Dual Variable Domain-Ig
FDA	Federal Drug Administration
FAP	Fibroblast Activation Protein
FIT-Ig	Fabs-In-Tandem-Ig
Ig	Immunoglobulin
KiH	Knobs-into-Holes
NKCE	NK Cell Engager
SAR	Synthetic Agonistic Receptor
TCB	T-Cell Bispecific Antibody
TCR	T-Cell Receptor
TfR	Transferrin Receptor
TNFR	TNF Receptor
USP	Upstream Processing
VEGF-A	Vascular Endothelial Growth Factor-A
wAMD	wet Age-Related Macular Degeneration

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