The use of CrossMAb technology for the generation of bi- and multispecific antibodies

Christian Klein\textsuperscript{a}, Wolfgang Schaefer\textsuperscript{b}, and Jörg T. Regula\textsuperscript{b}

\textsuperscript{a}Roche Innovation Center Zurich, Roche Pharmaceutical Research & Early Development, Wagistrasse, Schlieren, Switzerland; \textsuperscript{b}Roche Innovation Center Munich, Roche Pharmaceutical Research & Early Development, Nonnenwald, Penzberg, Germany

**ABSTRACT**

The major challenge in the generation of bispecific IgG antibodies is enforcement of the correct heavy and light chain association. The correct association of generic light chains can be enabled using immunoglobulin domain crossover, known as CrossMAb technology, which can be combined with approaches enabling correct heavy chain association such as knobs-into-holes (KiH) technology or electrostatic steering. Since its development, this technology has proven to be very versatile, allowing the generation of various bispecific antibody formats, not only heterodimeric/asymmetric bivalent 1+1 CrossMAbs, but also tri- (2+1), tetravalent (2+2) bispecific and multispecific antibodies. Numerous CrossMAbs have been evaluated in preclinical studies, and, so far, 4 different tailor-made bispecific antibodies based on the CrossMAb technology have entered clinical studies. Here, we review the properties and activities of bispecific CrossMAbs and give an overview of the variety of CrossMAb-enabled antibody formats that differ from heterodimeric 1+1 bispecific IgG antibodies.

**INTRODUCTION TO CROSSMAB TECHNOLOGY**

Historically, the fundamental issue in the generation of bispecific heterodimeric/asymmetric IgG antibodies has been the random association of heavy and light chains.\textsuperscript{1,2} While the correct heavy chain heterodimerization was enabled early on using the knobs-into-holes (KiH) approach,\textsuperscript{3} the correct association of generic light chains has remained a problem for decades.\textsuperscript{2} Since 2011, when we described the CrossMAb technology as a method to enforce correct light chain association in bispecific heterodimeric IgG antibodies,\textsuperscript{4} this technology has proven to be one of the most versatile antibody engineering technologies, allowing the generation of various bispecific antibody formats, including bi- (1+1), tri- (2+1) and tetra- (2+2) valent bispecific antibodies, as well as non-Fc tandem antigen-binding fragment (Fab)-based antibodies. These formats may be derived from any existing antibody pair using domain crossover, without the need for the identification of common light chains, post-translational processing or in vitro chemical assembly or the introduction of a set of mutations enforcing correct light chain association. The technology has also successfully and independently been validated by a number of academic investigators, as described below. Four different tailor-made bispecific antibodies based on the CrossMAB technology are currently in active Phase 1/2 clinical trials. These CrossMAbs can be produced using the well-established IgG production workflow based on one single standard Chinese hamster ovary cell line and typical upstream and downstream processing. The product is comparable in scale, yield, glycosylation, stability\textsuperscript{5} and quality to conventional IgG antibodies.

In this review, we briefly outline the basic concept, describe the properties and activities of bispecific CrossMAbs developed by Roche and others, and give an overview of CrossMAb-enabled antibody formats that are different from heterodimeric 1+1 bispecific IgG antibodies. Since the description of the CrossMAb technology, alternative technologies have been further developed that allow the generation of bispecific IgG-based antibodies from any antibody pair, and address different aspects of antibody engineering enabled by CrossMAb technology. These include DVD-IgG\textsuperscript{6–9} and CODV-Ig\textsuperscript{10} technologies, common light chain approaches,\textsuperscript{11–15} assembly of bispecific antibodies, e.g., Duobodies,\textsuperscript{16–19} dual-action Fabs (DAFs)\textsuperscript{20} or Dutafabs,	extsuperscript{21} the introduction of guiding disulfide bridges in Duetmab\textsuperscript{22} or the introduction of different guiding mutations in re-designed Fab moieties to enforce correct light chain association. The latter comes conceptually closest to the CrossMAb concept.\textsuperscript{23–25} Several of these approaches are being applied in clinical stage bispecific antibodies as well; they are, however, not within the scope of this review, and we refer the reader to the original publications and to recent reviews on the topic of bispecific antibodies.\textsuperscript{2,26,27}

The CrossMAb technology is based on the crossover of the antibody domain within one Fab-arm of a bispecific IgG antibody in order to enable correct chain association.\textsuperscript{2,4,28} whereas
the correct association of heavy chains can be enforced by the KiH\textsuperscript{3},\textsuperscript{4,29} electrostatic steering\textsuperscript{14,30,31} or alternative technologies.\textsuperscript{30,31} As shown in Fig. 1, the complete Fab domain can be exchanged in the CrossMAb\textsuperscript{Fab}, or either only the variable domains in the CrossMAb\textsuperscript{VH-VL} or the constant domain of the Fab arm in the CrossMAb\textsuperscript{CH1-CL}. In the case of the CrossMAb\textsuperscript{CH1-CL}, no theoretical side products are formed, whereas in the case of the CrossMAb\textsuperscript{Fab} design a non-functional monovalent antibody (MoAb/MonoMAb) is formed by the VH-CH1 and VL-CL domains, as well as a non-functional Fab from the 2 different parental antibodies. In the case of the CrossMAb\textsuperscript{VH-VL} design, an antibody that carries an associated VL-CL chain in the crossed Fab-arm (VL-CH1), as known from Bence-Jones proteins, can occur as a side product. The introduction of repulsive charges into the constant CH1 and CL domains of the Fab can be done to avoid the formation of the Bence-Jones-like side product (unpublished data). In the following, we will describe different applications of the CrossMAb technology to design multispecific and multivalent antibodies of various formats.

**Heterodimeric/asymmetric bivalent 1+1 IgG CrossMAbs**

The CrossMAb technology was first described for bispecific heterodimeric IgG antibodies recognizing vascular endothelial growth factor (VEGF)-A and angiopoietin-2 (Ang-2),\textsuperscript{[4]} with the Fab carrying the crossover based on bevacizumab and the wildtype Fab based on an Ang-2 antibody termed LC06.\textsuperscript{32} Based on this experience, an optimized version of the original bispecific CrossMAb was generated for clinical development. The respective bispecific anti-Ang-2/VEGF CrossMAb vanucizumab (RG7221) is of IgG1 isotype, and carries the original unmodified bevacizumab Fab on the knob arm, whereas the LC06-bearing arm is a Fab carrying a CH1-CL domain crossover.\textsuperscript{33} Correct heavy chain association is enabled by use of disulfide-stabilized KiH mutations (Fig. 2A). The potency of vanucizumab was determined using a surface plasmon resonance-based assay principle for bispecific antibodies.\textsuperscript{34} Vanucizumab or a mouse specific surrogate bispecific CrossMAb showed potent antitumoral efficacy using several orthotopic/syngeneic mouse tumors, as well as patient/cell line-derived human tumor xenografts. Overall, vanucizumab or the mouse specific surrogate bispecific CrossMAb strongly inhibited angiogenesis, and resulted in an enhanced vessel maturation phenotype. Notably, and in contrast to Ang-1 inhibition, inhibition of Ang-2 did not increase the adverse effect of anti-VEGF treatment on physiologic vessels, but was able to reduce micrometastatic growth in an adjuvant setting,\textsuperscript{33} in line with a study demonstrating anti-metastatic

![Figure 1. Schematic overview about the 3 major CrossMAb formats: CrossMAb\textsuperscript{Fab}, CrossMAb\textsuperscript{VH-VL}, and CrossMAb\textsuperscript{CH1-CL}, and predicted side products that can be formed. Mass spectrometry following transient expression and purification via protein A confirmed the presence of the predicted antibody species. In the case of CrossMAb\textsuperscript{Fab}, a non-functional monovalent heavy chain dimer is formed; in the case CrossMAb\textsuperscript{VH-VL}, an antibody with a VL-CL Bence-Jones-like associated chain can occur. KiH technology or alternative heavy chain heterodimerization technologies applied where appropriate and indicated by colors only. Constant heavy chain domains are colored in gray, constant light chain domains in white, variable heavy chains are colored uniformly, light chain domains are colored with a line pattern.](image-url)
activity of Ang-2 inhibition. In models of renal cell cancer (RCC), it significantly reduced tumor volume, vessel density, and interstitial fluid pressure compared to either monotherapy alone.

The crystal structure analysis of the isolated LC06-based anti-Ang-2 CrossFab fragment compared to the parental Fab demonstrated the structural and functional integrity of the variable domain, supporting the notion that the CrossMAb approach allows correct light chain association with unperturbed structure compared to the parental Fab structure.

Recently, Kloepper and colleagues showed that an anti-Ang-2/VEGF mouse specific surrogate bispecific CrossMAb could overcome resistance to anti-VEGF treatment in mice bearing orthotopic syngeneic or human glioblastoma models. In both models, the bispecific CrossMAb reprogrammed protumor M2 macrophages toward the antitumor M1 phenotype.

In 2012, vanucizumab became one of the first human heterodimeric bispecific IgG antibodies to enter clinical trials in cancer patients (NCT01688206). Based on the acceptable safety profile with favorable pharmacokinetic (PK) and pharmacodynamic effects in the Phase 1 study and an extension in ovarian cancer patients, the Phase 2 McCAVE study (NCT02141295) was initiated in 2014. In this clinical trial, vanucizumab is being evaluated in direct comparison to bevacizumab (Avastin®), both in combination with mFOLFOX-6 followed by maintenance, in patients with first line metastatic colorectal cancer. In addition, vanucizumab has been studied in a Phase 1b study in platinum-resistant ovarian cancer and is being studied in two Phase 1b studies in combination with atezolizumab or the anti-CD40 antibody RO7009789 respectively.

We and others have shown that Ang-2 plays an important role in ocular diseases characterized by angiogenesis and inflammation, and that inhibition of Ang-2 alone can have an anti-angiogenic effect in the eye comparable to VEGF-A inhibition. Based on this rationale, a tailor-made and optimized VEGF-Ang-2 CrossMAb, RG7716, was designed specifically for ophthalmology (unpublished data). RG7716 is differentiated from vanucizumab by the following features: 1) the bevacizumab Fab is replaced by ranibizumab, which exhibits higher affinity for VEGF-A in order to strongly block VEGF-A; 2) the anti-Ang-2 LC06 Fab is replaced by LC10, which is specific for Ang-2 and does not bind to Ang-1; 3) the Fc of the antibody carries P329G, L235A, L234A (P329G, LALA) mutations (unpublished data) in order to abolish any undesired immune effector function of the antibody, e.g., antibody-dependent cell-mediated cytotoxicity (ADCC),
phagocytosis (ADCP) and complement dependent cytotoxicity (CDC), thus reducing the potential for inflammatory events; and 4) in order to abolish FcRn recycling and reduce peripheral half-life after diffusion out of the eye into circulation, the Triple A mutations I253A, H310A, H435A have been introduced into the Fc with the goal of minimizing any potential for systemic anti-angiogenic side effects (Fig. 2A). The introduction of these Triple A mutations also led to reduced viscosity, which is beneficial for intravitreal application through a thin needle. In pre-clinical models, RG7716 demonstrated potent anti-angiogenic activity superior to VEGF-A inhibition alone in a laser-induced choroidal neovascularization (CNV) model (unpublished data).

In 2013, RG7716 became the first bispecific antibody to be evaluated in clinical Phase 1 studies in ophthalmology. Based on RG7716s safety and efficacy profile, the Phase 2 AVENUE study (NCT02484690) in wet age-related macular degeneration patients in which it is directly compared to ranibizumab (Lucentis\textsuperscript{\textregistered}) as current standard of care was initiated in 2015. RG7716 is also being tested in the BOULEVARD Phase 2 study in patients with diabetic macular edema (CI-DME) in direct comparison to ranibizumab (NCT02699450).

Several academic investigators have used the CrossMAb technology to successfully generate bispecific antibodies. Zhang and colleagues created a bispecific heterodimeric/asymmetric CrossMAb\textsuperscript{CH1-CL} using KiH technology based on an optimized variant of pertuzumab (L56TY) and trastuzumab. The resulting molecule, termed Tras-Permut CrossMAb, was shown to inhibit the progression of trastuzumab-resistant breast cancer in a mouse model.\textsuperscript{44}

Similarly, Zhao and colleagues used KiH technology to generate a bispecific heterodimeric/asymmetric CrossMAb\textsuperscript{CH1-CL}, termed CD20-243 CrossMAb, that targeted both CD20 and HLA-DR.\textsuperscript{45} The CD20-243 CrossMAb induced high levels of CDC, ADCC and anti-proliferative activity. Notably, although HLA-DR is expressed on normal and malignant cells, the CrossMAb exhibited highly anti-tumor specificity, showing efficient eradication of hematological malignancies both in vitro and in vivo.\textsuperscript{45} Zhao and colleagues also constructed an analogous CrossMAb\textsuperscript{CH1-CL} based bispecific fusion protein (BiFP), termed CD20-Flex, that targeted both CD20 and Flt3.\textsuperscript{46} Notably, CD20-Flex BiFP not only eliminated lymphoma, but also potentiated tumor-specific T-cell immunity, likely through the expansion and infiltration of dendritic cells into the tumor tissue.\textsuperscript{46}

Likewise, Asokan and colleagues generated several bispecific heterodimeric/asymmetric CrossMAbs\textsuperscript{CH1-CL} using KiH technology using different broadly neutralizing antibodies (bNAbs) against the HIV-1 envelope (Env) with the aim to target different antigenically diverse HIV strains and improve coverage against the majority of these viruses.\textsuperscript{47} The bispecific CrossMAbs displayed features of both antibody specificities, and, in some cases, displayed improved coverage over the individual parental antibodies. All four bispecific CrossMAbs neutralized 94% to 97% of antigenically diverse viruses in a panel of 206 HIV-1 strains. Among the bispecific antibodies tested, VRC07 × PG9-16 displayed the most favorable neutralization profile, neutralizing 97% of viruses with a median 50% inhibitory concentration (IC50) of 0.055 μg/ml. VRC07 × PG9-16 demonstrated in vivo PK parameters comparable to those of the parental bNAbs in rhesus macaques, and may qualify as a promising candidate for the prevention and treatment of HIV-1 infection.\textsuperscript{47}

Huang, Ho and colleagues used asymmetric CrossMAbs\textsuperscript{CH1-CL} with KiH technology to generate the most potent and broad HIV neutralizing antibodies to date. One bispecific antibody, 10E8V2.0/iMab, neutralized >100 HIV-1 pseudotyped viruses and potentially neutralized 99% of viruses in a second panel of 200 HIV-1 isolates belonging to clade C, the dominant subtype accounting for ~50% of new infections worldwide. 10E8V2.0/iMab also reduced virus load substantially in HIV-1-infected humanized mice, and provided complete protection when administered prior to virus challenge. These bispecific antibodies hold promise as prophylactic or therapeutic agents for therapy of HIV-1.\textsuperscript{48} In independent work, Bournazos, Nussenzweig Ravetch and colleagues created IgG3 hinge engineered bispecific anti-Env neutralizing antibodies (biNAbs) using a CH1-CL crossover based on broadly neutralizing antibodies (bNAbs) in order to improve neutralization breadth/potency. In these biNAbs the engineered hinge domain of IgG3 enabled Fab domain flexibility required for simultaneous binding to the Env trimer while retaining the functional properties of the Fc. These bispecific antibodies exhibited synergistically enhanced viral neutralization and superior control of viremia.\textsuperscript{49}

Finally, in a novel application of bispecific antibodies, Tung and colleagues used CrossMAb technology to generate bispecific antibodies against PEG and HER2 or CD19 for the specific delivery of PEGylated compounds, including PEGylated proteins, liposomes, and nanoparticles, to HER2+/CD19+ tumor cells.\textsuperscript{50}

In general, in our hands as well as those of external investigators (see below), the generic CrossMAb approach works very well. In exceptional cases, however, the yield or purity of the bispecific CrossMAb antibody may not fulfill all requirements and require further optimization. For example, adjusting the expression rate of the 4 different antibody chains of the CrossMabs can minimize the formation of hole-hole- or knob-knob-containing antibody species or 1/2 (one HC and one LC) or 3/4 (lacking one LC) antibodies (unpublished data).

The CrossMAb technology can also be applied to generate the respective mouse surrogate bispecific antibodies based on murine isotypes, e.g., mlgG1, using charged residues for heterodimerization (K392D, K409D and E356K, D399K) or mlgG2a using homologous KiH mutations.\textsuperscript{38}

**Heterodimeric/asymmetric trivalent 2+1 IgG CrossMAbs**

By fusing a Fab to the N-terminus of the VH domain of a CrossMAb\textsuperscript{CH1-CL}, a novel trivalent 2+1 IgG antibody can be generated (Fig. 3A). Further trivalent formats can be generated by fusing the Fab to the N-terminus of the VL domain, the C-terminus of the light chain or the C-terminus of the Fc domain. Alternatively, a CrossFab can be used to fuse to the N- or C-termini of a conventional IgG antibody to generate trivalent bispecific antibodies via a flexible (G2)\textsubscript{2}-linker. Such antibodies can be advantageous, for example, in cases where one wants to bind with avidity to an antigen A, but only in a monovalent fashion to a second antigen.
In the case of T cell bispecific antibodies (TCBs), the goal is to bind only monovalently to the CD3ε chain of the T-cell receptor (TCR), so that the TCR is only cross-linked and activated upon concomitant binding of the 2 tumor antigen binding domains of the antibody to its target on the tumor cell, resulting in crosslinking, T cell activation, and subsequent strictly tumor antigen-dependent T cell killing of the targeted cell. In 2014, CEA TCB (RG7802), a 2+1 TCB recognizing carcinoembryonic antigen (CEA), became the first T cell bispecific antibody to enter clinical Phase 1 study (NCT02324257) where

Figure 3. The CrossMAb zoo: Schematic overview about different mono-, bi- and multispecific antibody formats enabled by CrossMAb technology: (A) Heterodimeric/asymmetric trivalent 2+1 IgG CrossMAbs; (B) Symmetric tetravalent 2+2 IgG CrossMAbs; (C) MoAb (MonoMAb) and MoAb-Dimer (DuoMAb; (D) Trispecific Pan-HER family DAF-CrossMAb antibody; (E) Trispecific CrossMAb-VH-VL; (F) Tri-, tetraspecific CrossMAb-scFAB fusions; (G) DVD-CrossMAb; (H) Heterodimeric/asymmetric Kappa-Lambda-CrossMAb; (I) Fc-free Tandem Fab-CrossFab antibody; (J) Fc-free bispecific Fab fusion proteins using alternative fusion partners (green, yellow) based on Fab crossover. KiH technology or alternative heavy chain heterodimerization technologies applied where appropriate and indicated by colors only. The drawings given represent only examples, since in many cases crossed and uncrossed Fabs can be assembled in various ways. Constant heavy chain domains are colored in gray, constant light chain domains in white, variable heavy chains are colored uniformly, light chain domains are colored with a line pattern.
such a 2+1 head-to-tail design principle was implemented using the CrossMAb technology, and it is one of the first IgG-based T cell bispecific antibodies in clinical trials (Fig. 2C). In this case, a CH1-CL crossover was introduced into the anti-CD3ε CrossFab to enforce correct chain pairing. Notably, the CD3ε binding region was introduced in an “inside” position because it allows fusion of a Fab to the N-terminus of its VH domain without loss of activity (unpublished data). Furthermore, CEA TCB has been engineered using the P329G LALA mutation to exclude any FcyR co-activation upon TCR or target cell binding. CEA TCB binds simultaneously to CEA-expressing tumor and T cells, resulting in T-cell activation, secretion of cytotoxic granules and tumor cell lysis. As a consequence of bivalent CEA binding, CEA TCB activity correlates with CEA expression, which allows cells with high versus low CEA expression to be distinguished. In xenograft models, CEA TCB mediates anti-tumoral efficacy, and leads to a strong increase in frequency of tumor-infiltrating T cells. Recently, Vu and colleagues described a novel B cell maturation antigen (BCMA) specific 2+1 TCB based on this platform.53,54

### Symmetric tetravalent 2+2 IgG CrossMABS

As we have described, symmetric tetravalent bispecific 2+2 antibodies can be generated by fusing either a CrossFab fragment to the C-terminus of the Fc domain of an IgG antibody. Analogous tetravalent formats can be generated by fusing the Fab to the N-terminus of the VH or VL domain or to the C-terminus of the VL domain via flexible (G,S) x-linkers. Alternatively, a Fab can be fused to the N- and C-termini of a CrossMAbCH1-CL (Fig. 3B). These antibodies are made using one heavy chain and 2 light chains. By introduction of KiH mutations into the Fc of such tetravalent antibodies, it is also possible to generate an asymmetric tetravalent 2+2 IgG CrossMAb antibody based on 2 heavy chains and 2 light chains that carries its 2 different specificities on its 2 opposed sides (Fig. 3B, top right). In addition, 2+1 and 2+2 IgG CrossMABS can also be designed using flexible connectors in the heavy and light chains.

This design principle was applied to generate RG7386, a symmetric tetravalent bispecific 2+2 CrossMAb antibody recognizing fibroblast activation protein-α (FAP) on activated tumor fibroblasts and death receptor 5 (DR5) on tumor cells (Fig. 2D). Activation of apoptosis in tumor cells can be triggered through DR5 hyperclustering on the cell surface; however, conventional DR5 antibodies that rely on FcyRIIB-mediated hyperclustering have failed in clinical trials. RG7386 was designed as a bispecific antibody using a low affinity DR5 antibody to activate the extrinsic apoptotic pathway in tumor cells only by avidity-driven DR5 hyperclustering in a strictly FAP expression-dependent fashion. Due to the introduction of the P329G LALA mutation, FcyR-mediated crosslinking cannot contribute to the mechanism of action of RG7386. RG7386 potently triggers tumor cell apoptosis in vitro and in vivo in preclinical tumor models with FAP-positive stroma or FAP-expressing malignant cells in a fashion superior to conventional DR5 antibodies. RG7386 is being tested in a clinical Phase 1 study (NCT02558140).

Similarly, a symmetric tetravalent bispecific 2+2 CrossMAb antibody targeting the inflammatory tumor necrosis factor (TNF) and interleukin (IL)-17 was generated and compared to a classical heterodimeric/asymmetric bivalent 1+1 IgG CrossMAbCH1-CL. Blocking TNF and IL-17 showed additive/synergistic effects in promoting production of IL-6, IL-8, and granulocyte colony-stimulating factor in human fibroblast-like synoviocytes. The bispecific TNF/IL-17 CrossMAb showed superior efficacy, both in blocking cytokine and chemokine responses, in vitro. Furthermore, dual vs. single inhibition of both cytokines was more effective in inhibiting the development of inflammation and bone and cartilage destruction in arthritic mice. Importantly, in these studies the tetravalent 2+2 TNF/IL-17 CrossMAb was more potent than the bivalent 1+1 TNF/IL-17 CrossMAb due to avidity, supporting the importance of testing different antibodies formats to identify the most suitable one.

### Additional applications of CrossMAb technology

Apart from the applications of the CrossMAb technology to the IgG-based bispecific antibody formats described above, the CrossMAb technology can also be applied for the engineering of other antibody formats.

A functional monovalent antibody (MoAb/MonoMAb, Fig. 3C) can be generated by combining 2 crossover heavy chains with either CH1-CL or the analogous VH-VL crossover (unpublished data, ref 60). Surprisingly, when we expressed the corresponding 2 heavy chains without enforcing heterodimerization by KiH, in addition to the correct MoAb antibody, a dimeric MoAb (MoAb-dimer, DuoMAb) comprising 2 Fab and 2 Fc regions was formed (Fig. 3C) (unpublished data, ref. 61).

Fuh and colleagues have described dual-action Fabs (DAFs) that can bind 2 different antigens with the same variable region, such as HER2 and VEGF-A or EGFR/HER1 and HER3. Using DAfs, tri- or tetrascpecific heterodimeric/asymmetric bivalent 1+1 DAF-CrossMABS can be generated, e.g., a trispecific pertuzumab and EGFR/HER1-HER3 DAF-based pan-HER family CrossDAF antibody (Fig. 3D).

Trispecific antibodies may also be generated by fusing a VH domain to the C-terminus of the Fc of a heterodimeric CrossMAb, and a VL domain to the other C-terminus of the Fc as previously described (trispecific CrossMAb-VH-VL, Fig. 3E). Alternatively, tri- or tetrascpecific antibodies can be generated by fusing scFv or single chain Fab (scFab) moieties to the Fc of a heterodimeric CrossMAb (Fig. 3F).

Hu and colleagues used CrossMAb technology to generate 2 different tetrascpecific, tetravalent antibodies targeting EGFR/HER1, HER2, HER3, and VEGF to therapeutically overcome crosstalk among the HER family receptors. They generated FL518, a 4-in-one CrossMAbCH1-CL based on the EGFR/HER1-HER3 and HER2-VEGF DAF antibodies and CRTB6, a tetrascpecific, tetravalent DVD-CrossMAb antibody based on a bevacizumab-cetuximab CrossMAbCH1-CL extended by VH and VL domains from the HER3 antibody RG7116 and trastuzumab using DVD-Ig technology (Fig. 3G). These tetrascpecific antibodies not only inhibit signaling mediated by the receptors in vitro and in vivo, but also disrupted HER-MET crosstalk.
Compared with 2-in-one antibodies and a series of bispecific antibodies in multiple tumor models, FL518 and CRTB6 were more broadly efficacious and able to inhibit the growth of anti-HER-resistant cancer cells.\(^70\)

Fischer and colleagues described an alternative and attractive strategy to generate natural bispecific antibodies based on a common heavy chain (CHC) without the requirement of heterodimeric Fc domains (e.g., KiH) using kappa and lambda light chains (LCs) and differential purification via kappa and lambda specific resins.\(^12\) As the specificity of antibodies frequently is determined by the heavy chains (HCs), we adapted this approach using a crossover of a common light chain (CLC) combined with the use of Ckappa and Clambda domains. In the respective format, the common light chain variable domain is now part of the identical heavy chains in the heterodimeric/asymmetric Kappa-Lambda-CrossMAb, whereas the 2 different VH domains fused to Ckappa and Clambda chains, respectively, remain in the respective light chains (Fig. 3H).\(^73\)

Last, but not least, the CrossMAb approach can be applied to generate bi-, tri- or multivalent Fc-free Tandem Fab-CrossFab antibodies (Fig. 3I)\(^74\) similar in design and application to what has been described using site directed mutagenesis by Wu and colleagues for using a redesigned Fab interface.\(^24\) Such molecules can also contain linkers between the respective light chains. Similarly, Fc-free bispecific Fab fusion proteins using various alternative fusion partners can be imagined using the CrossMab Fab approach (Fig. 3J).

**Generation of CrossMAbs**

An overview of the design principle applied for the generation of bispecific CrossMAb antibodies with CH1-CL crossover is shown in Fig. 4. Design of the crossing point is based on the fact that the VH, V\(k\) and V\(\lambda\) domains, as well as their constant counterparts, are structurally extremely similar. Thus, we have extracted the 4 domains VH, VL (kappa), CH1, CL (kappa) from the crystal structure of trastuzumab (PDB:1N8Z)\(^75\) and VL (lambda) and CL (lambda) from the crystal structure of a lambda Fab (PDB: 7FAB\(^76\)). Superpositions of the 3 variable domains and the 3 constant

![Figure 4](image-url). Design of CrossMAbs with CH1-CL crossover: Top: Typical structures of VH, Vk and V\(\lambda\) domains and their superposition in the sense that the C\(\alpha\) atoms of the \(\beta\)-sheets adjacent to the elbow region (displayed as spheres in the structures, gray in the sequences) match in space. Middle: the same applied to the CH1, C\(k\) and C\(\lambda\) domains. Bottom: wildtype and designed CrossMAb sequences; \(\beta\)-sheets adjacent to the elbow regions are colored gray. The newly designed sequence is shown in red.
domains were performed separately by application of a root mean square fit that included only the Cα-atoms of the β-sheets adjacent to the elbow region (displayed as spheres in the structures of Fig. 4, gray in the sequences) match in space. The superposition of separate domains avoids a bias for specific elbow angles. The new elbow can then be composed from the original sequences, aiming for optimal sequence homology, structural overlap, and contact between variable and constant domains, while also avoiding the generation of potential T cell epitopes.

From many possibilities, the elbow sequence “SSAS” (red sequence in Fig. 4) was selected in the initial CrossMAb for both the VH-Cκ as well as the Vκ and Vλ—CH1 transitions successfully. This means that on both sides of the CrossMab this stretch is part of a wildtype sequence (SSAS-TKGPS for Vκ-CH1 and GTLVTV-SSAS for VH-Cα); new solvent-exposed sequences do not occur as consequences of the crossover. Since the constant domains are not involved in antigen binding, there is no need to design separate VH-Cκ and VH-Cλ sequences; thus, typically VH-Cκ is used.

As mentioned above, several elbow sequences and variations in length are imaginable, and therefore we conducted a matrix approach, testing variations in length between VH-Cα and VL-CH1 by addition or deletion of amino acids and utilizing different amino acids in individual positions. In general, there are many elbow variations possible and functional. To identify the most suitable elbow sequences, we assessed the thermal stability of crossed Fabs, measured as aggregation onset temperature by dynamic light scattering, in dependency of an increasing temperature. To our surprise, there was no better elbow sequence included than the initially proposed one (unpublished data).

Table 1 and Fig. 4 give an overview of typical elbow sequences for Cα and Cλ containing light chains. Thermal stability and productivity can slightly differ between these elbow variants.

**Table 1. Typical elbow sequences, typically Cα is used.**

| Kappa crossover | Lambda crossover |
|-----------------|------------------|
| Vκ-CH1          | KVEIKSSASTKGKP   |
| WH-Cα           | VTVSSKPSAAPSV    |
| Vλ-CH1          | KVSKSSASTKGKP    |
| WH-CH1          | VTVSSRTPAAPSV    |

**Conclusion**

Based on the ongoing clinical validation of several CrossMAB-based bispecific antibodies and the successful application by independent academic researchers, the CrossMab approach and platform has been validated as a powerful strategy to generate diverse bi- and multispecific antibodies. Additional CrossMAB-based bispecific antibodies are expected to advance into clinical trials in the future, and ultimately they may contribute to the improvement of patients’ outcomes.

**Disclosure of potential conflicts of interest**

All authors are employees of Roche. CK, WS and JR are co-inventors on various CrossMAB technology related patent applications and issued patents.

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