Introduction

Bispecific antibodies combine the activity of two parental monoclonal antibodies, such as dual targeting, but can also utilize novel mechanisms of action, including effector cell retargeting, shunting the blood-brain-barrier, or mimicking the activity of natural proteins.\(^1\text{-}\text{6}\) Two bispecific antibodies are currently approved for therapy, blinatumomab for the treatment of acute myeloid leukemia and emicizumab as a substitute of factor VIII for the treatment of hemophilia A, and more than 50 bispecific antibodies are currently in clinical development.\(^7\text{-}\text{8}\) A wide variety of different formats are available to generate bispecific antibodies, which can be classified according to the absence or presence of an Fc region (IgG-like molecules), their overall architecture (symmetric or asymmetric), or the number of antigen-binding sites (valency).\(^7\text{-}\text{9}\)

In the past, the correct assembly of heavy and light chains to generate an intact bispecific IgG molecule was a challenge. Genetic engineering forcing the heterodimerization of the two heavy chains (heavy chain problem) and of the heavy and light chain (light chain problem) solved these issues, and allowed the generation of asymmetric IgG or Ig-like molecules.\(^1\text{0}\text{-}\text{1}\text{9}\) Symmetric IgG-like molecules can be generated by fusion of different binding sites to an Fc region or an IgG, resulting in bispecific and tetravalent molecules,\(^7\) including IgG-single-chain variable fragment (scFv) fusion proteins,\(^2\text{0}\) single-chain diabody-Fc fusion proteins,\(^2\text{1}\) dual-variable-domain antibody (DVD-Ig),\(^2\text{2}\) and cross-over dual variable Ig-like proteins (CODV-Ig).\(^2\text{3}\) However, these tetravalent bispecific formats often suffer from poor stability, restricted antigen-binding, or the extensive use of linker sequences to connect the variable domains.

Here, we present a novel and versatile platform for the generation of tetravalent symmetric Ig-like antibody molecules, designated diabody-Ig (Db-Ig). The binding sites of these molecules are formed by a bivalent diabody (either mono- or bispecific), fusing a \(V_H\) domain with a short, five amino acid long linker to a \(V_L\) domain. The diabody unit is stabilized by fusion to either homodimerizing domains, such as the second heavy chain domains of IgE (EHD2) or IgM (MHD2),\(^2\text{4}\text{-}\text{2}\text{5}\) or heterodimerizing domains, such as the second heavy chain domains of IgE (EHD2) or IgM (MHD2),\(^2\text{4}\text{-}\text{2}\text{5}\) and cross-over dual variable Ig-like proteins (CODV-Ig).\(^2\text{3}\) These Db-Igs retained the antigen-binding properties of parental antibodies, and demonstrated the ability to bind simultaneously both antigens, resulting in strong anti-proliferative activity and induction of apoptosis. In addition, the Db-Ig molecules showed high stability in human plasma, and the use of the Fc of an IgG revealed IgG-like pharmacokinetic properties in mice.
Results

**The Db-Ig format**

The Db-Fab arm of a Db-Ig molecule is formed by fusing a diabody moiety in the $V_H$-$V_L$ configuration to the N-terminus of a dimerization domain (DD), which can form either a homo- or a heterodimer (Figure 1(a)). Here, the second constant domain of the heavy chain of IgM (MHD2)$^{24}$ or IgE (EHD2)$^{24,25}$ was engaged as homodimerization domains, whereas $C_{H1}/C_L$ and a mutated version of EHD2 (hetEHD2) were used as heterodimerization domains. With respect to the hetEHD2 domain, cysteine residues forming one of the two interdomain disulfide bonds formed between residues C247 and C337 were substituted by serine residues, leading to the formation of heterodimers. Heterodimerization of the hetEHD2 domain was confirmed in SDS-PAGE analysis under non-reducing conditions using His-tagged scFv-EHD2(C247S) and Flag-tagged EHD2(C337S), which were co-transfected in HEK293E cells and purified with anti-Flag M2-agarose (Fig. S1). Importantly, the formation of homodimers of scFv-EHD2 (C247S) of EHD2(C337S) was not detected in SDS-PAGE analysis. Fusion of one chain of the Db-Fab arm to the N-terminus of an Fcγ1 results in dimeric assembly and formation of tetrameric molecules (Figure 1).

Modeling of crystal structures of a diabody moiety ($V_H$-$V_L$ orientation)$^{26}$ with the $C_{H1}$ and $C_L$ domains of an IgG$^{27}$ revealed close proximity of the C-termini of the diabody moiety and the N-termini of the constant domains enabling a direct genetic fusion without the insertion of an additional linker sequence (Fig. S2A). In addition, an alignment of the different crystal structures of human $C_{H1}/C_L$, human EHD2, and mouse MHD2 (as no human structure was available) showed high structural similarity, including the position of the N-terminus of each domain (Fig. S2B). Thus, for the generation of tetravalent Db-Ig molecules, only one short linker (e.g., 5 aa) connecting both variable domains ($V_H$-$V_L$) in the diabody moiety is required within each chain of the molecule.

**Tetravalent bispecific Db-Igs targeting EGFR and HER3**

The variable domains of a humanized version of anti-EGFR antibody cetuximab (hu225)$^{28}$ and anti-HER3 antibody IgG 3–43$^{29}$ were used to generate tetravalent bispecific Db-Ig molecules. In total, four different tetravalent bispecific molecules were produced using different dimerization domains (DD): $C_{H1}/C_L$, EHD2, MHD2, and hetEHD2 (Figure 2(a)). Thus, each tetravalent molecule consists of two antigen-binding sites for EGFR and two binding sites for HER3 (2+2 format). All Db-Ig molecules were produced in HEK293-6E suspension cells, which were co-transfected with both plasmids encoding for the heavy chain ($V_H$-$V_L$-DD-$C_{H2}$-$C_{H3}$) and for the light chain ($V_H$-$V_L$-DD). Proteins were purified for further analysis via a one-step affinity chromatography using either $C_{H1}$-CaptureSelect (using $C_{H1}/C_L$ as DD) or FcXL-CaptureSelect (using EHD2, MHD2, or hetEHD2 as DD). Productivities of the $C_{H1}/C_L$-based Db-Ig molecules were 6.4 mg/l or 9.9 mg/l using either $C_{H1}$-CaptureSelect or FcXL-CaptureSelect, respectively. In comparison, using FcXL-CaptureSelect, higher productivities of 35.3 mg/l, 21.6 mg/l, and 21.3 mg/l were obtained for Db-Ig molecules utilizing EHD2, hetEHD2, and MHD2, respectively, as dimerization domain.

Size-exclusion chromatography of the different tetravalent antibodies under native conditions confirmed correct assembly of the tetravalent antibodies, indicating protein preparations with high purity for the different Db-Ig molecules, with...
only very low amounts of multimers (below 5% using C1/C1, hetEHD2, or MHD2 as DD; below 10% using EHD2 as DD) (Figure 2(c)). The apparent Stokes radii ($S_R$) were ~5.4 nm for Db3–43xhu225-C1/C1-Fc and ~5.8 nm for the Db3–43xhu225-Ig molecules using EHD2, MHD2, or hetEHD2 as dimerization domain. SDS-PAGE analysis confirmed purity of the molecules, revealing two bands of ~40 kDa and 70 kDa under reducing conditions, representing the light and heavy chain (calculated molecular weight: 37.3 and 60.8 kDa), respectively (Figure 2(b)). In the case of EHD2, MHD2, and hetEHD2, a more or less pronounced double band was detected for each chain, which is most likely due to the presence or absence of N-glycans (all three domains exhibit one potential N-glycosylation site). Under non-reducing conditions, a major band of approximately 220 kDa was observed for Db-C1/C1-Fc, confirming disulfide-linkage of all four chains. This band was also observed for the other molecules, but bands of lower molecular weights were also observed to a varying extent, indicating a partial disulfide-bond formation between the light and heavy chains, which was especially observed for the EHD2 and MHD2-derived Db-Igs. This was confirmed by Western Blot analysis of the different Db-Ig molecules using horseradish peroxidase (HRP)-conjugated anti-human Fc detection antibody (Fig. S3).

Binding of the tetravalent antibodies was analyzed by ELISA using either EGFR-His or HER3-His as immobilized antigen. All Db-Igs showed a concentration-dependent binding to the two antigens, similar to the parental monospecific antibodies anti-EGFR IgG hu225 and anti-HER3 IgG 3–43 included as controls. The EC$_{50}$ values were in the range of 92 to 118 pM for EGFR binding and 140 to 173 pM for HER3 binding (Figure 3(a,b), Table 1). This finding confirms the correct assembly of the individual antigen-binding sites in all four Db-Ig formats. Furthermore, simultaneous binding of both antigens was confirmed in a second ELISA binding assay using EGFR-Fc fusion protein as immobilized antigen and HER3-His as second soluble antigen. Similar EC$_{50}$ values in the range of 160 to 204 pM were obtained for the tetravalent bispecific molecules, whereas, as expected, no binding was detected for the parental antibodies (Figure 3(c), Table 1).

We further generated a Db-Ig with a V$_L$-V$_H$ orientation of the diabody moiety using C1/C1 as heterodimerization module. Production and purification of the molecule were performed identically as described above for the Db-Ig molecule in the V$_H$-V$_L$ configuration. Of note, the Db3–43xhu225-C1/C1-Fc molecule in the V$_L$-V$_H$ orientation showed similar binding to both antigens in ELISA binding experiments (EGFR: EC$_{50}$ ~218 pM; HER3: EC$_{50}$ ~240 pM) compared to the V$_H$-V$_L$ Db-Ig molecule, but the protein preparation was associated with a heterogeneous purification profile with an increased portion of multimers (above 20%) (Fig. S4).

Cell binding of the bispecific antibodies was analyzed by flow cytometry using the head and neck cancer cell line FaDu (Figure 4(a)). The parental antibodies IgG hu225 and IgG 3–43 were included as a control. Similar to the ELISA binding studies, concentration-dependent binding was detected for all analyzed antibodies. As FaDu cells express very high amount of EGFR (~140,000 receptors per cell) and low amounts of HER3 (~3,000 receptors per cell) on their surface, cell binding was obviously dominated by EGFR, which resulted
in binding of IgG hu225 and of all bispecific antibodies with similar EC\textsubscript{50} values in the range between 129 and 238 pM (Table 1).

Bioactivity of the Db-Igs was investigated in a proliferation assay using FaDu cells in starvation medium kept either unstimulated or stimulated with heregulin (HRG). Here, all bispecific antibodies, as well as the combination of both parental antibodies (IgG hu225 and IgG 3–43), showed a strong reduction of proliferation, with strongest effects observed for the bispecific antibodies under HRG-stimulation (Figure 4(b)). The bispecific Db-Ig antibody harboring C\textsubscript{H}1/C\textsubscript{L} as dimerization domain was further analyzed for the induction of apoptosis in FaDu cells. FaDu cells were incubated with monospecific antibodies, the combination thereof, or the bispecific Db-Ig antibody for 24 h in rich medium. Induction of apoptosis was analyzed by flow cytometry using annexin V and PI staining. Increased amounts of annexin V-stained (early apoptosis) and annexin V/PI-stained (late apoptosis) cells were measured for IgG hu225 and the combination of the monospecific antibodies compared to untreated and IgG 3–43-treated cells, indicating the dominant effect of EGFR-targeting on FaDu cells. Interestingly, the bispecific Db-Ig antibody revealed the highest amount of cells in the early and late apoptosis (Figure 4(c); Fig. S5).

In vitro stability of the tetravalent bispecific Db-Ig antibodies was tested by incubating proteins in human plasma at 37°C. All tetravalent antibodies showed retained the binding capacity to both antigens after 7 days of incubation (Figure 5(a)), indicating high stability of these molecules in human plasma. In addition, pharmacokinetic profiles of tetravalent bispecific antibodies were determined in immunocompetent CD-1 mice receiving one single dose intravenous (i.v.) injection. Except for Db3-43xhu225-hetEHD2-Fc, which revealed a slightly reduced pharmacokinetic profile, all other Db-Ig molecules showed similar terminal half-lives in the range of 71 and 105 h, as well as similar drug exposure (AUC), independent of the antigen used for detection (Figure 5(b); Table 2).

**Discussion**

With the Db-Ig platform, we established a new format for the generation of tetravalent bispecific IgG-like molecules. Variable domains of monoclonal antibodies can be fused directly into the platform, generating novel multivalent antibodies. We found that the diabody moiety in the V\textsubscript{H}-V\textsubscript{L} orientation is ideally suited for the fusion to the N-terminus of the different dimerization domains. Advantages include: 1) the C-termini of both Db-assembled V\textsubscript{L} domains are closely located to the N-termini of the different dimerization domains; 2) both antigen-binding sites are facing away from each other enabling unhindered access to both antigens; 3) Db exhibit (besides a V\textsubscript{H}-V\textsubscript{L} pairing within each Fv head) a strong V\textsubscript{H}-V\textsubscript{H} back-to-back interaction stabilizing a Fab-like, closed conformation of the antigen-binding sites. This particular structure of a diabody in the V\textsubscript{H}-V\textsubscript{L} orientation was further confirmed by other studies\textsuperscript{30,32} and nicely reviewed by Kwon et al.\textsuperscript{33} In general, we assume that Db-Ig molecules should exhibit Ig-like flexibility, for example, the elbow angle, within each Db-Fab arm or the hinge angle between both Db-Fab arms,\textsuperscript{34,35} due to the high structural similarity within the utilized dimerization domains (C\textsubscript{H}1, C\textsubscript{L}, MHD2, and EHD2). In our examples, neither the inter-peptide linker within the
antigen-binding site (diabody moiety) nor the composition of the antigen-binding sites within one Db-Fab arm of the antibody needed to be optimized for the generation of novel bispecific antibodies. In contrast, a requirement for linker optimization has been described for other formats, e.g., DVD-Ig,\textsuperscript{22,36} CODV-Ig.\textsuperscript{23} Here, the four different linkers with a length of up to 30 aa, which connect the variable domains with each other or with the constant domains, as well as the arrangement of the variable domains, need to be adapted for optimal binding of the antibody. Of note, the orientation of the variable domains on the diabody moiety seems to be critical for correct assembly of the Db-Igs. Carmichael and coworkers published the crystal structure of a diabody in the V\textsubscript{L}-V\textsubscript{H} orientation.\textsuperscript{37} In comparison to the crystal structure of the V\textsubscript{H}-V\textsubscript{L} orientation (1LMK),\textsuperscript{26} the position and orientation of the different domains are rearranged. This divergent structure resulted most likely from the hydrophilic interactions of the loops originated from both, V\textsubscript{H} and V\textsubscript{L}, domains.\textsuperscript{33} As a result, the C termini of this V\textsubscript{L}-V\textsubscript{H} diabody are located ~ 60 Å from each other. With a distance of ~ 40 Å for the N-termini of a C\textsubscript{H1}/C\textsubscript{L} heterodimer originating form an IgGl (1HZH),\textsuperscript{27} the direct (linker-less) fusion with a diabody in the V\textsubscript{L}-V\textsubscript{H} orientation results in a discrepancy of ~ 20 Å, which could be responsible for the heterogeneous preparation compared to fusion with a diabody in the V\textsubscript{H}-V\textsubscript{L} configuration (~ 40 Å between both C-termini).

Different dimerization domains were used in the Db-Ig platform for the stabilization of the diabody moiety: C\textsubscript{H1}/C\textsubscript{L}, hetEHD2, EHD2, and MHD2. Homogeneous protein preparation, as well as similar binding to both antigens and FaDu cells or similar plasma stability, was associated with all bispecific antibodies independent of the utilized dimerization domains. Besides EHD2 and MHD2, other constant domains of an antibody, which tend to form homodimers like C\textsubscript{L}/C\textsubscript{L}, IgG-derived C\textsubscript{H3}/C\textsubscript{H3}, or IgM- or IgE-derived C\textsubscript{H4}/C\textsubscript{H4}, could potentially also be used as dimerization domains in the Db-Ig platform. In addition to these homodimers, we also designed and generated a EHD2-based heterodimerization domain, hetEHD2. In general, the two EHD2 domains are disulfide-linked between residue C241 (domain a) and C337 (domain b) and vice versa between C337 (domain a) and C241 (domain b). Besides the two disulfide bridges, the interface of the EHD2 dimer is dominated by polar residues and involves only 11 hydrogen bonds.\textsuperscript{38} In the absence of these dimer-forming disulfide bridges, the
EHD2 domains had no measurable affinity to each other, indicating the importance of the disulfide bridges for dimerization. Thus, eliminating one disulfide bond by substituting the involved cysteine residues with serine residues forces the formation of heterodimers. As these mutated cysteine residues are located at the interface of the EHD2 dimer, the conformational structure of the hetEHD2, including structurally dependent properties like plasma stability, should be mainly unaffected by the introduction of this mutational change. Recently, Cooke and co-workers developed a heterodimerization domain, EFab, on the basis of EHD2 by introducing knob-into-hole-like mutations.

Asymmetric bivalent and bispecific molecules were generated using this EFab arm in combination with a ‘classical’ second Fab arm and a heterodimeric Fc part (knob-into-hole). Besides the formation of multimers (and half antibodies), disorders regions were identified in the fusion of the variable domains with EFab. In line with these results, EFab showed increased proteolysis in the presence of proteinase K compared to the wild-type EHD2 or a ‘classical’ Fab.

We show that the different Db-Ig formats can be purified via a one-step purification procedure using either C_{\text{H}1}/CaptureSelect or FcXL-CaptureSelect with less than 5% to 10% of multimers formation. Interestingly, different dimerization domains affected the production of Db-Ig molecules, with EHD2 showing the highest productivity (~ 35 mg/l). This productivity is within the range of our in-house production of monospecific IgG antibodies (30 to 50 mg/l). In addition, as protein G exclusively binds to the constant domains of the heavy chain of IgGs, this affinity chromatography can also be used for purification of Db-Ig molecules. In contrast, standard purification of antibodies via protein A affinity chromatography purification resulted in heterogeneous preparation of antibodies (not shown), as V_{\text{H}}, which can be also bound by protein

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**Table 2.** Determination of initial (t_{α}) and terminal (t_{β}) half-lives as well as of drug exposure (area under the curve; AUC) of bispecific Db3-43xhu225-Ig molecules. Mean ± SD, n = 3.

| antigen Db3-43xhu225-C_{\text{H}1}/C_{\text{L}}-Fc | Db3-43xhu225-EHD2-Fc | Db3-43xhu225-hetEHD2-Fc | Db3-43xhu225-MHD2-Fc |
|--------------------------------------------------|----------------------|------------------------|---------------------|
| t_{α} [h] EGFR                                  | 3.0 ± 0.7            | 3.4 ± 1.9              | 1.4 ± 0.1           |
| t_{α} [h] HER3                                  | 3.3 ± 1.3            | 2.6 ± 0.5              | 3.9 ± 3.7           |
| t_{β} [h] EGFR                                  | 89 ± 3               | 103 ± 16               | 64 ± 14             |
| t_{β} [h] HER3                                  | 86 ± 3               | 95 ± 5                 | 74 ± 8              |
| AUC [%·h] EGFR                                  | 3,899 ± 533          | 4,467 ± 1,010          | 3,442 ± 380         |
| AUC [%·h] HER3                                  | 3,695 ± 621          | 3,768 ± 388            | 2,603 ± 364         |

**Figure 5.** Plasma stability and PK analysis of bispecific Db3-43xhu225-Ig molecules. (a) The Db-Ig molecules were diluted in 50% human plasma and incubated at 37°C for 1, 3, or 7 days. Protein content was detected by ELISA using both antigens, EGFR-His or HER3-His. (b) Pharmacokinetic profile of Db-Ig molecules were analyzed in female CD-1 mice (n = 3). Mice received a single i.v. injection of 25 µg of the protein. Serum protein concentrations were determined by ELISA using both antigens, EGFR-His or HER3-His.
is present in both chains of the antibody, resulting also in the purification of the light chain dimer.

ELISA binding studies confirmed unhindered accessibility of both antigen-binding sites of the Db-Ig platform to their antigens, resulting in retained binding properties of the parental antibodies, and in simultaneous binding of both antigens. For other multivalent antibody platforms, for example, the DVD-Ig platform, reduced binding of the inner Fv compared to the parental antibody was reported for a molecule targeting tumor necrosis factor (TNF), most likely due to steric hindrances.

Results from the plasma stability assay as well as IgG-like PK profiles underline correct assembly of the polypeptide chains for all four analyzed Db-Ig molecules, with high stability in vitro and in vivo. The half-life of the different anti-EGFR x anti-HER3 Db-Ig antibodies (2 to 4 days) is similar to that of the parental anti-HER3 antibody IgG 3-43 (2.5 days). Due to the cross-reactivity of IgG 3-43 to mouse HER3, target-mediated disposition could be an explanation for these pharmacokinetic properties. With respect to stability, the importance of the introduction of dimerization domains within the Db-Igs becomes apparent when compared with other diabody-based molecules, which lack an additional dimerization domain. For example, the tetravalent ‘Di-Diabody’ showed reduced antigen binding either after incubation in mouse serum at 37°C for three days or after circulation in mice for 7 days, most likely due to the dissociation of the two non-covalently linked polypeptide chains. In line with these results, reduced serum stability was also reported for non-covalently linked bivalent diabody molecules, as well as for tetravalent tandem diabodies (tandAbs).

In summary, we present a novel Db-Ig platform for the generation of tetravalent bispecific IgG-like antibodies, with a diabody as a binding moiety. Db-Ig molecules are stabilized either by homo- or heterodimerization domains, resulting in high stability. In total, only one short inter-peptide linker was introduced within the binding moiety, whereas the other domains, which are human antibody-derived, are ‘naturally’ fused to each other. Our results indicate that variable domains of parental antibodies can be directly inserted into the Db-Ig platform without time-consuming optimization of the composition of antigen binding sites or optimization of the inter-peptide linker, retaining parental binding properties. Our results need to be validated in future studies that include other variable domains. Of note, the Db-Ig platform also allows the generation of asymmetric molecules by introducing a heterodimeric assembled Fc. Thus, tetravalent asymmetric molecules, for example, tetraspecific (1 + 1 + 1 + 1), trispecific (2 + 1 + 1), or bispecific (2 + 2; each Db-Fab arm is monospecific), can be generated by using two different dimerization domains in each Db-Fab arm (Figure 6). Due to the modular composition of the Db-Ig platform, other moieties can be fused to one chain of the heterodimerized Fc part, including scFv or Fabs, resulting in trivalent molecules (1 + 2, 1 + 1 + 1), or cytokines such as single-chain derivatives of the TNF superfamily, resulting in bifunctional molecules. In addition, bivalent Db-Ig molecules (1 + 1) can be generated by fusing both chains of the Db-Fab arm directly to a heterodimeric Fc part. Thus, the Db-Ig format represents a versatile novel platform for the generation of molecules with varying valency and specificity for a plethora of applications.

Materials and methods

Material

HRP- and phycoerythrin (PE)-conjugated anti-human Fc antibodies were purchased from Sigma-Aldrich (A0170; P9170), whereas anti-His-HRP (HIS-6 His-Probe-HRP, sc-8036) was purchased from Santa Cruz Biotechnology. Antibodies for immuno-blotting were purchased at Cell Signaling (Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb #3777; Phospho-HER2/
Antibody production and purification

Heavy and light chains of different tetravalent bispecific antibodies were cloned into variants of pSecTagA vector, and were transiently transfected into HEK293-6E suspension cells using polyethylenimine (PEI; linear, 25 kDa, Sigma-Aldrich) for transfection. HEK293-6E cells were provided by National Research Council of Canada (Ottawa, Canada), and cultivated in F17 Freestyle expression medium (ThermoFisher Scientific), supplemented with 0.1% Kolliphor P-118 (Sigma), 4 mM GlutaMAX (ThermoFisher), and 25 µg/ml G418. By adding TN1 (20% tryptone N1 (Organotechnie S. A.S., France) in F17 medium) to feed the culture 24-h post transfection, protein production was initiated; cells were cultivated at 37°C and subsequently medium exchanged to rich medium (10% fetal bovine serum (FBS)) and cultivated for 5 days at 37°C. The trypsin-EDTA reaction was stopped using 50 µl 1 M H2SO4 and absorption was measured at a wavelength of 450 nM. In general, plates were washed three times with PBS + 0.005% Tween20 and twice with PBS between each incubation step and in advance of the detection.

Antibody characterization

Antibodies were analyzed by SDS-PAGE (4 µg) and stained with Coomassie-Brilliant Blue G-250. The purity and integrity of molecules (9–30 µg in 30 µl) were analyzed via size-exclusion chromatography using a Waters 2695 HPLC in combination with a TSKgel SuperSW mAb HR column (822854, Sigma Aldrich) at a flow rate of 0.5 ml/min using 0.1 M Na2HPO4/NaH2PO4, 0.1 M Na2SO4, pH 6.7 as mobile phase. Standard proteins: thyroglobulin (669 kDa, Rs 29 nm), apoferritin (443 kDa, Rs 61 nm), β-amylose (200 kDa, Rs 5.4 nm), bovine serum albumin (66 kDa, Rs 3.55 nm), carbonic anhydrase (29 kDa, Rs 2.35 nm). Stokes radii of antibodies were interpolated from standard proteins.

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well plates were coated with EGFR-Fc, EGFR-His (extracellular domain (ECD) of EGFR: aa 25–645 with C-terminal His-tag), or HER3-His (ECD of HER3: aa 20–643 with C-terminal His-tag) (200 ng/well in PBS) overnight at 4°C and residual binding site were blocked with 2% (w/v) skim milk powder in PBS (MPBS, 200 µl/well). The antibodies were diluted in MPBS and titrated 1 to 3 in duplicates starting from 100 nM and incubated for 1 h at room temperature. Bound antibodies were detected with HRP-conjugated antibodies specific for human Fc (for the bispecific antibodies) or His-tag (for bound HER3-His). Detection antibodies were incubated for one additional hour at room temperature. TMB (1 mg/ml; 0.006% (v/v) in 100 mM Na-acetate buffer, pH 6) was used as the substrate, reaction was terminated using 50 µl 1 M H2SO4 and absorption was measured at a wavelength of 450 nM. In general, plates were washed three times with PBST (PBS + 0.005% Tween20) and twice with PBS between each incubation step and in advance of the detection.

Flow cytometry analysis

FaDu cells (1x10^5 per well) were incubated with a serial dilution of different antibodies (starting from 3 nM) for 1 h at 4°C. After washing cells twice, bound antibodies were detected using a PE-labeled anti-human Fc antibody (9170, Sigma-Aldrich). Flow cytometry was performed using MACSQuant Analyzer 10 or MACSQuant VYB (both Miltenyi Biotec). Relative mean fluorescence intensities (MFI) were calculated as followed: rel. MFI = ((MFI_{sample} - MFI_{detected-MFI_{cells}})/MFI_{cells}).

Cell proliferation assays

FaDu cells (2x10^3 per well) were seeded in 96-well plate and cultivated at 37°C in rich medium (10% fetal bovine serum (FBS)) for 24 h, and subsequent medium exchange to starvation medium (0.2% FBS and 1x penicillin/streptomycin). After 24 h of starvation, cells were treated with different antibodies (single: 50 nM; combination: 50 nM each) for 60 min at 37°C. Cells were kept unstimulated or were stimulated with heregulin (HRG; 30 ng/ml; Sigma-Aldrich: St. Louis, MO, USA) and cultivated for 5 days at 37°C. Cell viability was measured using CellTiter-Glo 2.0 Assay (Promega). Luminescence of untreated and unstimulated cells was set as 100%.

Induction of apoptosis assay

FaDu cells (5x10^4 per well) were seeded in 12-well plates and cultivated at 37°C in rich-medium (10% FBS) for 24 h, and subsequently medium exchanged to rich medium (10% FBS and 1x penicillin/streptomycin) with added antibodies (single: 50 nM; combination: 50 nM each). After 24 h of incubation at 37°C, supernatant was collected, and the cells were washed with PBS and harvested using Trypsin-EDTA (1x). The trypsin-EDTA reaction was stopped using matched collected supernatant. After centrifugation (5 min, 500 x g), supernatant was discarded and cells were resuspended in 100 µl AnnexinV Binding Buffer (556454; Heidelberg, BD Biosciences). Next, 5 µl of PI (556463, Heidelberg, BD Biosciences) and 5 µl of annexinV-GFP were added, following 15 min of incubation at room temperature. Flow cytometry was performed using MACSQuant Analyzer 10 (both Miltenyi Biotec). Gates were set for untreated cells.
**Plasma stability**

Proteins were diluted in 50% human plasma obtained from healthy donors (Klinikum Stuttgart, Germany) with a concentration of 200 nM. Proteins were either directly stored at −20°C (day 0) or incubated at 37°C for 1, 3, or 7 days prior storing at −20°C. Levels on intact protein were analyzed via ELISA using either EGFR-His or HER3-His as antigen. Bound antibodies were detected using an HRP-conjugated anti-human Fc antibody as described above.

**Pharmacokinetics**

Animal care and all performed experiments were in accordance with Federal and European guidelines and were approved by university and state authorities. Twenty-five micrograms of proteins were injected into the tail vein of female CD-1 mice (Charles River, three animals per molecule) in a total volume of 100 µl. Blood samples were taken after 3 min, 30 min, 1 h, 2 h, 6 h, 24 h, 72 h, and 168 h after injection, and incubated on ice immediately to obtain serum samples after centrifugation (16,000 x g, 4°C, 20 min), which were stored at −20°C until analysis. Serum concentration of antibodies were determined via ELISA using either EGFR-His or HER3-His as antigen. Bound antibodies were detected using HRP-conjugated anti-human Fc antibody as described above.

**Statistics**

All data are represented as mean ± SD. Significances were calculated by GraphPad Prism 5.0.1 and results were compared by one-way ANOVA followed by Tukey’s multiple comparison test (post-test).

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| AUC          | area under the curve |
| CI           | constant domain of heavy chain |
| CL           | constant domain of light chain |
| CODV-Ig      | cross-over dual variable immunoglobulin |
| Db-Ig        | diabody immunoglobulin |
| DD           | dimerization domain |
| DVD-Ig       | dual-variable domain immunoglobulin |
| EHD2         | IgE heavy chain domain 2 |
| EGFR         | epidermal growth factor receptor |
| Fab          | fragment antigen-binding |
| Fc           | fragment crystalizable |
| HER3         | human epidermal growth factor receptor 3 |
| hetEHD2      | heterodimeric EHD2 |
| HRG          | intravenous |
| i.v.         | immunoglobulin |
| IgM          | IgM heavy chain domain 2 |
| MHD2         | protein data bank |
| PDB          | pharmacokinetic |
| PK           | single-chain fragment variable |
| scFv         | tumor necrosis factor |
| TNF          | variable domain of heavy chain |
| Vh           | variable domain of light chain |

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**Disclosure of potential conflicts of interest**

O.S., F.R., and R.E.K. are named inventors on patent applications covering multivalent binding proteins.

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