**REPORT**

**Insertion of scFv into the hinge domain of full-length IgG1 monoclonal antibody results in tetravalent bispecific molecule with robust properties**

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**ABSTRACT**

By simultaneous binding two disease mediators, bispecific antibodies offer the opportunity to broaden the utility of antibody-based therapies. Herein, we describe the design and characterization of Bs4Ab, an innovative and generic bispecific tetravalent antibody platform. The Bs4Ab format comprises a full-length IgG1 monoclonal antibody with a scFv inserted into the hinge domain. The Bs4Ab design demonstrates robust manufacturability as evidenced by MEDI3902, which is currently in clinical development. To further demonstrate the applicability of the Bs4Ab technology, we describe the molecular engineering, biochemical, biophysical, and in vivo characterization of a bispecific tetravalent Bs4Ab that, by simultaneously binding vascular endothelial growth factor and angiopoietin-2, inhibits their function. We also demonstrate that the Bs4Ab platform allows Fc-engineering similar to that achieved with IgG1 antibodies, such as mutations to extend half-life or modulate effector functions.

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**Introduction**

During the past two decades antibody engineering methods have been used to design and develop multispecific antibodies, including numerous bispecific (BsAb). BSABs described thus far can be divided into five classes based on their molecular format: 1) BsAb with IgG1-like structure, which are monovalent for each antigen; 2) BsAb prepared by appending binding domains to the IgG, which are bivalent for each antigen; 3) BsAb prepared using antibody fragments, which are monovalent for each antigen and often lack the Fc region; 4) bispecific fusion proteins, which are formed of an antibody fragment genetically linked to a protein to add additional functionality or specificity; and 5) bispecific conjugates, which are prepared by chemical conjugation of antibody fragments. While some such bispecific molecules have demonstrated manufacturing robustness, in vivo drug-like properties, suitable pharmacokinetics (PK) and efficacy have been achieved, platforms that yield multifunctional bispecific antibodies with different spatial configurations and flexibility suitable for a variety of target and disease applications are still needed. Furthermore, bispecific antibody engineering is often not “plug and play” for all antibody combinations, and multiple formats may need to be tested to achieve suitable expression, optimal physical stability characteristics and clinical application.

We previously reported three bispecific antibody formats, Bs1Ab, Bs2Ab and Bs3Ab. Herein, we describe another format, termed Bs4Ab, which yields a novel bispecific tetravalent antibody. The Bs4Ab consists of a full-length IgG1 antibody with a scFv inserted into the hinge region. Among the plethora of BsAb formats, only Bs4Ab is engineered for bispecificity via the insertion of an additional binding unit into the IgG hinge. Bs4Ab can be efficiently produced in mammalian cells, purified using traditional methods and has demonstrated IgG1-like biochemical and biophysical properties, as evidenced by MEDI3902, which is a Bs4Ab targeting the *Pseudomonas aeruginosa* serotype-independent type III secretion system virulence factor PcrV and persistence factor Psl exopolysaccharide. MEDI3902 is currently in a Phase 2 clinical study (Clinical-Trials.gov Identifier: NCT02696902) in mechanically ventilated patients for the prevention of nosocomial pneumonia caused by *Pseudomonas aeruginosa*.

As an additional example of the applicability of the Bs4Ab design, we describe the molecular engineering, as well as the biochemical, biophysical and in vivo characterization of a Bs4Ab that neutralizes vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang2). VEGF and Ang2 are well-characterized factors that play key roles during angiogenesis. Dual blockade of VEGF and Ang2 has already been demonstrated to result in potent tumor growth inhibition in xenograft
mouse models by limiting angiogenesis and increasing apoptosis.\textsuperscript{14-17} In addition, the effects of neutralization of VEGF and Ang2 with a bispecific monovalent CrossMab antibody (RG7221, vanucizumab)\textsuperscript{15} in combination with chemotherapy are currently being investigated in a Phase 2 clinical trial (ClinicalTrials.gov NCT02141295) of patients with metastatic colorectal cancer. Therefore, the work described herein does not focus on confirming that dual targeting of VEGF and Ang2 can potentiate or differentiate the activity over the individual parental antibodies or their combination, but instead describes the molecular strategies that have been used to design the Bs4Ab bispecific antibody platform and to provide additional evidences of its applicability. Furthermore, we demonstrate that the Bs4Ab design allows Fc-mutations engineered to modulate Fc effector functions and half-life similarly to IgG1.

**Results**

**Molecular design of Bs4Ab**

A schematic representation of Bs4Ab is shown in Fig. 1a. Bs4Ab consists of a scFv inserted into the hinge of human IgG1 (Fig. 1b). Bs4Ab retains intact interchain disulfide bridges between the heavy-light chains and at the hinge as in an IgG1. Two linker sequences (Fig. 1a, Bs4Ab-L1 and Bs4Ab-L2) and three linker sequences (Fig. 1a, Bs4Ab-L3, Bs4Ab-L4 and Bs4Ab-L5) were tested to link the N-terminus and C-terminus of the scFv to the IgG1 hinge, respectively. The amino acid sequence of the IgG1 hinge with identification of residues C\textsubscript{220} and C\textsubscript{226} is shown in Fig. 1b. Bs4Ab-L1, Bs4Ab-L3 and Bs4Ab-L5 linkers have the mutation D\textsubscript{221}G (EU numbering\textsuperscript{18}) designed to prevent cleavage at the hinge. As shown in Fig. S1a, a Bs4Ab that retains D\textsubscript{221} at the hinge before and after the scFv results in cleavage in phosphate-buffered saline (PBS) at pH 7.2 as revealed by reduced SDS-PAGE. The reduced SDS-PAGE analysis showed the presence of four peptide chains, which, by analyzing their apparent molecular weight, can be assigned to the full-length heavy chain (i.e., VH-CH1-scFv-Fc), the VH-CH1-scFv, the Fc, and the light chain (Fig. S1b). This analysis suggests that cleavage occurs at a single point in the heavy chain, likely at the linker between the C-terminus of the scFv and the N-terminus of the Fc (Fig. S1c). While carrying out this analysis, we observed that the presence of ethylenediaminetetraacetic acid (EDTA) decreased the proteolytic cleavage while the presence of copper sulfate increased the cleavage (data not shown), which suggests the involvement of metal ions in the proteolysis. It has been reported that metals such as copper significantly increase the fragmentation rate of IgG1 molecules.\textsuperscript{19,20} Moreover, it has been proposed that the predominant IgG1 copper cleavage site resides in the hinge region sequence S\textsubscript{219}CDKTHTC\textsubscript{226} (EU numbering\textsuperscript{18}), with cleavage occurring at the N-terminus of D\textsubscript{221}, with H\textsubscript{224} being the metal.

**Figure 1.** Schematic representation of Bs4Ab. (a) Bs4Ab is composed of a full-length IgG1 with a scFv inserted into the hinge domain. Bs4Ab retains intact Fab and Fc, as a human IgG1. The linker sequences used to insert the scFv into the hinge domain and their designation are schematically shown. The scFv can be in the orientation VH-Linker-VL or vice versa, as is schematically shown in the inset. The scFv contains the mutations VH\textsubscript{44C} and VL\textsubscript{100C} introduced to form an interdomain disulfide bond to stabilize the scFv. The symbols ∥ and ⊥ in the sequence and inset represent the linking points for the scFv. (b) Sequence and numbering (according to EU) of the human IgG1 hinge. Cysteine at position 220 that forms the interchain disulfide bond with the cysteine 214 at the light chain is highlighted in magenta; cysteine 226 that forms the first of the two disulfide bridges in the hinge is highlighted in yellow; the cysteine replacement with valine at position 220 is highlighted in cyan; the G mutation at position 221 is underlined.
binding residue.\(^1\) It is possible that inserting the scFv at the IgG1 hinge may increase metal-mediated cleavage at the hinge due to increased flexibility and better solvent accessibility.

To prevent cleavage, the Bs4Ab linkers L1, L3, and L5 (Fig. 1a) were engineered to have the mutation D\(_{221}\)G. Bs4Ab-L1 retains residues K\(_{222}\) and T\(_{223}\) and contains a (G\(_4\)S\(_2\)) linker, while Bs4Ab-L2 has a (G\(_4\)S\(_2\)) linker after position 220; these two linkers precede the first N-terminal residue of the scFv. Bs4Ab-L3, Bs4Ab-L4 and Bs4Ab-L5 are used to link the last C-terminal residue of the scFv at the hinge (Fig. 1a). Bs4Ab-L3 has a (G\(_4\)S\(_2\)) linker after the last C-terminal residue of the scFv and contains C\(_{220}\)V mutation to eliminate potential difficulties associated with a free sulfhydryl, such as disulfide scrambling with the other cysteines at the hinge (Fig. 1a). We substituted the cysteine with valine, instead of the commonly used serine, to prevent any potential post-translation modification when serine is used as replacement to cysteine, such O-glycosylation. Bs4Ab-L4 has a (G\(_4\)S\(_2\)) linker between the last C-terminal residue of the scFv and cysteine 226 at the hinge. Bs4Ab-L5 has a (G\(_4\)S\(_2\)) linker after the last C-terminal residue of the scFv and does not have cysteine 220 at the hinge (Fig. 1a). The choice of which connecting linker sequences to use must be determined experimentally based on the optimal biochemical, biophysical and functional properties of the Bs4Ab. The scFv in Bs4Ab can be in the VL-Linker-VH or VH-Linker-VL orientation, and the scFv linker can be 3 or 4 G\(_4\)S repeats. Preferred scFv orientation is VH-Linker-VL, with a linker of 4 G\(_4\)S repeats and with V\(_{14}\) and V\(_{L100}\) cysteine mutations to introduce an interdomain disulfide bond to stabilize the scFv.\(^2\)

**Examples of antibodies derived from the Bs4Ab technology**

The Bs4Ab platform was previously used to construct MEDI3902,\(^13\) which targets *Pseudomonas aeruginosa* PcRV and PsL and is currently in a Phase 2 study (ClinicalTrials.gov NCT02696902), and a Bs4Ab targeting EGFR and IGF1R (USPTO Psl and is currently in a Phase 2 study (ClinicalTrials.gov NCT02696902), and a Bs4Ab targeting EGFR and IGF1R (USPTO Psl and is currently in a Phase 2 study (ClinicalTrials.gov NCT02141295), and a Bs4Ab targeting EGFR and IGF1R (USPTO Psl and is currently in a Phase 2 study (ClinicalTrials.gov NCT02141295)).

**Design and characterization of Bs4Ab-VA that concurrently neutralizes VEGF and Ang2**

VEGF neutralization by bevacizumab (Avastin\(^\circ\)) is clinically approved as a treatment of human cancer.\(^23,24\) Ang2 has been described to provide compensatory tumor growth during anti-VEGF therapy.\(^25,26\) Therefore, concurrent blockade of both VEGF and Ang2, as demonstrated by vanucizumab,\(^13\) which is currently in a Phase 2 study (ClinicalTrials.gov NCT02141295) may result in improved anti-angiogenic efficacy not achievable with a single agent. We sought to determine whether the Bs4Ab design can be used to concurrently block VEGF and Ang2. The resulting molecule, Bs4Ab-VA, was prepared using an anti-Ang2 binding site from the antibody LCO6,\(^27\) and an anti-VEGF binding site from bevacizumab.\(^28\) In particular, the scFv domain of Bs4Ab-VA is from LCO6 and the Fab domain is from bevacizumab (Fig. 2a and Fig. S2). The scFv is in the orientation VH-(G\(_4\)S\(_4\))-VL and has V\(_{14}\) and V\(_{L100}\) residues mutated to cysteine, which form an interdomain disulfide in the scFv designed to improve stability of the scFv.\(^22\) The transient expression levels of Bs4Ab-VA in human embryonic kidney (HEK)293 cells was 150 mg/L, which is similar to that of the parental anti-VEGF and anti-Ang2 antibodies. The monomeric content after protein A purification was 97% and 90% for the two parental antibodies and Bs4Ab-VA, respectively. Aggregates in Bs4Ab-VA were removed using ceramic hydroxyapatite chromatography (CHT). Purified Bs4Ab-VA and parental antibodies were analyzed using analytical size-exclusion chromatography-high performance liquid chromatography (SEC-HPLC) (Fig. 2b-d), reducing and non-reducing SDS-PAGE (Fig. 2e, f). SEC-HPLC analysis showed that Bs4Ab-VA has a retention time, and hence a molecular weight, distinct from the two parental antibodies (Fig. 2b-d). Reduced SDS-PAGE analysis (Fig. 2e) showed that the Bs4Ab-VA heavy chain has a molecular weight of about 80 kDa and the light chain retains a molecular weight similar to that of the light chain of the anti-VEGF antibody (Fig. 2e). The non-reduced SDS-PAGE showed that the Bs4Ab-VA has a molecular weight higher than the two parental antibodies (Fig. 2f).

Reduced reverse phase chromatography showed that the light chain of Bs4Ab-VA has the same retention time as the anti-VEGF antibody (Fig. 3a, b), but the heavy chain of the Bs4Ab-VA has a different retention time compared with the heavy chains of the anti-VEGF and the anti-Ang2 antibodies (Fig. 3a-c).

Differential scanning calorimetry (DSC) analysis showed that Bs4Ab-VA undergoes endothermic unfolding in 25 mM histidine-HCl pH 6.0 with three transition peaks (Fig. 4a). These observed endothermic transitions are typical for IgG1 and multispecific antibodies.\(^1,2\) The three endothermic transitions can be further deconvoluted into distinct endothermic unfolding transitions with a T\(_m\) of 69°C, 77°C and 82°C. By comparing the deconvolution unfolding transitions for the anti-VEGF (Fig. 4b) and the anti-Ang2 antibodies (Fig. 4c), the 69°C and 82°C unfolding transitions of Bs4Ab can be assigned to the antibody C\(_{\gamma 2}\) and C\(_{\gamma 3}\) domains, respectively. This indicates that Bs4Ab-VA has an Fc that unfolds similar to IgG1. The 77°C unfolding transition for Bs4Ab-VA may correspond to the unfolding of the Fab and scFv domains.

Reduced and intact mass spectrometry analysis confirmed the molecular weight of the light chain, heavy chain and intact Bs4Ab-VA (Fig. 5a, b), as well as the glycosylation profile, which is similar to that of a human IgG1. As comparison, the reduced and non-reduced mass spectrometry data for the two parental antibodies is also shown in Fig. 5c-f.

The isoelectric focusing properties of Bs4Ab-VA were shown to be similar to that of the two parental antibodies, as assessed by capillary isoelectric focusing (Fig. 5). An accelerated stability study of Bs4Ab-VA, anti-VEGF and anti-Ang2 was performed to determine physical stability under stressed conditions. The bsFv antibody and the two parental antibodies were concentrated to 25 mg/mL, subjected to three cycles of freeze-and-thaw and then incubated at 4°C, 25°C and 40°C for one month. Samples were removed after one, two and four weeks and purity loss (i.e., sum of aggregates and fragments) was determined using analytical SEC. As shown in Table 1, purity loss of Bs4Ab-
VA at the end of the studies is similar to the two parental antibodies.

Using an ELISA assay, we demonstrated that Bs4Ab-VA binds to VEGF (Fig. 6a) and Ang2 (Fig. 6b) similarly compared with the parental anti-VEGF and anti-Ang2 antibodies. BIAcore was used to demonstrate concurrent binding of Bs4Ab-VA to both VEGF and Ang2 (Fig. 7). Concurrent binding was demonstrated to be independent of protein immobilization or the sequence of ligands injected. When Bs4Ab-VA was immobilized on the BIAcore sensor chip, concurrent binding was observed irrespective of order of injected ligands (Fig. 7a: VEGF first, followed by Ang2; Fig. 7b: Ang2 first, followed by VEGF). Similarly, concurrent binding was observed when either Ang2 or VEGF were immobilized on the BIAcore sensor chip, followed by injection of Bs4Ab-VA with the cognate ligand (Fig. 7c, d). Concurrent binding to the two antigens is specific to Bs4Ab-VA since no binding was detected between the two antigens,
Ang2 and VEGF, using similar BIAcore experiments (Fig. S4).

To compare the inhibition of ligand-induced phosphorylation of Bs4Ab-VA versus the two parental antibodies, we used Ad293 and HEK293 cells that have been engineered to stably express the human Ang2 and VEGF receptors, pTie2 and pVEGFR2, respectively. Fig. 8a shows that Bs4Ab-VA effectively blocks Ang2-induced phosphorylation of Tie2 receptor with an IC₅₀ of 1.37 nM, which is similar to the IC₅₀ of the parental anti-Ang2 antibody (IC₅₀ of 1.24 nM). Suppression of

Figure 3. Reduced reverse phase chromatography of Bs4Ab-VA and the two parental antibodies. (a) Reduced reverse phase chromatography of Bs4Ab-VA, (b) anti-VEGF, and (c) anti-Ang2. The identity of each peak is schematically labeled. The retention time in minutes for the light and heavy chains is schematically shown.
Tie2 phosphorylation was confirmed by western blot analysis using an anti-Tie2 phosphotyrosine-Y992 specific antibody (Fig. 8b). Fig. 8c showed that Bs4Ab-VA also effectively blocks the VEGF-induced phosphorylation of VEGFR2 with an IC_{50} of 4.52 nM, which is similar to the IC_{50} of the anti-VEGF and anti-Ang2 antibodies, respectively. The terminal half-life and clearance of the bispecific antibody were 111 hours and 1.2 mL/hr/kg, respectively (Supplementary Table 1). The PK studies show that Bs4Ab-VA possesses IgG1-like PK properties.

Figure 4. Differential scanning calorimetry of Bs4Ab-VA and the two parental antibodies. Differential scanning calorimetry of (a) Bs4Ab-VA, (b) anti-VEGF and (c) anti-Ang2 antibodies. Bs4Ab-VA unfolds similarly to the two parental antibodies, indicating an IgG1-like structural stability.

Next, we confirmed that Bs4Ab-VA has affinity for human FcRn similar to that of an IgG1 antibody, as determined using BIAcore (Table 2). The equilibrium K_{D} at pH 6.0 of Bs4Ab-VA for human FcRn is 680 nM, which is similar to the K_{D} determined for the anti-VEGF (840 nM) and anti-Ang2 (800 nM) parental antibodies. As expected, no binding was observed at pH 7.4 (Table 2). In addition, Bs4Ab-VA retains equilibrium affinity similar to the parental anti-VEGF and anti-Ang2 antibodies for binding to FcyRI, FcyRIIA, and both allotype variants of FcyRIIIA using ProteOn (Table 3).

The maintenance of similar affinities to FcRn and FcγRs by Bs4Ab molecules is important to maintain IgG1-like serum half-life and Fc-effector functions in vivo. To this end, we determined the PK of Bs4Ab-VA and the parental anti-VEGF and anti-Ang2 antibodies using non-tumor bearing nude mice (Fig. 9). The single dose PK studies showed that dose-normalized serum peak concentrations (C_{max}/Dose) were comparable for the Bs4Ab-VA and the two parental antibodies (Supplementary Table 1). However, dose-normalized serum exposure of the Bs4Ab-VA was higher compared with the anti-VEGF and the anti-Ang2 antibodies, respectively. The PK studies show that Bs4Ab-VA possesses IgG1-like PK properties.

We next determined the in vivo anti-tumor activity of Bs4Ab-VA using a colon cancer (Colo205) mouse xenograft model, which expresses human Ang2 and human VEGF (Fig. 10). Colo205 tumor-bearing mice were treated when tumor reached a mean size of 200 mm³ with intraperitoneal injections two times a week for the duration of the study of 14 mg/kg of Bs4Ab-VA, 10 mg/kg of each of the anti-VEGF and anti-Ang2 antibodies, and 10 mg/kg of their combination (Fig. 10). Bs4Ab-VA yielded potent antitumor activity and its anti-tumor activity was statistically significant to the anti-VEGF monotherapy (P < 0.031, Fig. S5). There was, however, no statistically significant differentiation to anti-Ang2 monotherapy (P < 0.136, Fig. S5) or to the combination therapy of anti-VEGF and anti-Ang2 (P < 0.413, Fig. S5). Similar findings were recent published. Bs4Ab platform allows modifications of the Fc similar to IgG1 to extend half-life and to modulate FcγRs functions

Under some therapeutic conditions, it may be desired to either reduce or increase antibody Fc-mediated effector functions. For example, for antibodies that target cell-surface molecules, especially those on immune cells, removing Fc effector function is preferred. Contrarily, for antibodies intended for oncology or infectious disease applications, enhancing Fc effector functions may improve the therapeutic activity. Modification of IgG effector functions can be achieved by mutations in the Fc region. Two specific sets of Fc mutations, L234F-L235E-P331S (referred to hereafter as TM), and S239D-A330L-I332E (referred to hereafter as 3M), result in abrogating and in enhancing Fc effector functions, respectively. Increasing serum circulation of therapeutic antibodies is another way to improve their efficacy, allowing higher circulating levels and less frequent administration. Half-life extension of IgG1 can be achieved by enhancing the binding of the IgG1 to the neonatal FcRn, which is expressed on the surface of endothelial cells, and binds to the IgG1 in a pH-dependent manner. Several mutations in the Fc region of IgG1 were reported to increase serum circulation of IgG1. Among many reported mutations, the triple mutations M252Y-S254T-T256E (referred
to hereafter as YTE), resulted in increased binding to both cynomolgus monkey and human FcRn at pH 6, while preserving no binding at pH 7.4. To test whether the Bs4Ab platform allows Fc modifications similarly to IgG1, we prepared a Bs4Ab-VA with the YTE, TM and 3M mutations and performed a comparative analysis using the anti-VEGF antibody modified with the same Fc mutations. Proteins were transiently expressed using HEK293 cells and expression levels after 10 d of culture were 130 mg/mL, 125 mg/mL and 85 mg/mL for Bs4Ab-VA-YTE, Bs4Ab-VA-TM and Bs4Ab-3M, respectively. Expression levels were similar to that of the anti-VEGF antibody with the same Fc mutations, and were similar to the expression levels of the Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies without the Fc mutations. The monomeric content after protein A purification was 90%, 85% and 90% for Bs4Ab-VA-YTE, Bs4Ab-VA-TM and Bs4Ab-3M, respectively, as determined by SEC-HPLC. Similar aggregate levels were observed for the anti-VEGF Fc-mutant antibodies (90%, 90% and 95% for YTE, TM and 3M, respectively).

Table 1. Accelerated stability studies of Bs4Ab-VA, anti-VEGF and anti-Ang2 parental antibodies at 25 mg/mL and 4°C, 25°C and 40°C for one month.

| Analyte          | Concentration (mg/mL) | Temperature (°C) | Purity loss after 1 week (%) | Purity loss after 2 weeks (%) | Purity loss after 4 weeks (%) |
|------------------|-----------------------|------------------|------------------------------|------------------------------|------------------------------|
| Bs4Ab-VA         | 25                    | 4                | 0                            | 0.2                          | 0.4                          |
|                  |                       | 25               | 0.5                          | 1.0                          | 1.4                          |
|                  |                       | 40               | 1.5                          | 6.0                          | 10                           |
| anti-VEGF        | 25                    | 4                | 0                            | 0.1                          | 0.5                          |
|                  |                       | 25               | 0.1                          | 0.2                          | 0.7                          |
|                  |                       | 40               | 0.3                          | 0.4                          | 4                            |
| anti-Ang2        | 25                    | 4                | 0.2                          | 0.4                          | 0.8                          |
|                  |                       | 25               | 0.4                          | 2.0                          | 4.0                          |
|                  |                       | 40               | 10                           | 14                           | 22                           |

Purity loss represents the percentage of the sum of aggregates and fragments as determined by analytical SEC-HPLC. All analytes were subjected to 3 cycles of freeze-and-thaw before the accelerated stability studies. All analytes were formulated in 25 mM Histidine-HCl, 0.8% Sucrose, 0.02% Polysorbate-80, pH 6.0.

Figure 5. Isoelectric focusing analysis of Bs4Ab-VA and the two parental antibodies. pl markers (a), Bs4Ab-VA (b), anti-VEGF (c), and anti-Ang2 (d) have similar pl, as determined by capillary isoelectric focusing.
Figure 6. Binding of Bs4Ab-VA, anti-VEGF and anti-Ang2 to VEGF and Ang2. Bs4Ab-VA binds to (a) VEGF-165 and (b) Ang2 similar to the anti-VEGF and anti-Ang2 parental antibodies, as demonstrated by ELISA.

Figure 7. Concurrent binding of Bs4Ab-VA to VEGF and Ang2. Concurrent binding of Bs4Ab-VA to Ang2 and VEGF-165 is demonstrated using BIAcore. Concurrent binding is irrespective of which molecule is immobilized on the BIAcore sensor chip and of the order of ligands injection events.
respectively). The aggregates were removed using CHT chromatography and proteins were analyzed using the same analytical methods used to characterize the Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies without Fc mutations.

To determine whether the Fc mutations have any structural impact on the Bs4Ab structure, we compared the melting temperatures of Bs4Ab-VA-YTE, Bs4Ab-VA-3M and Bs4Ab-VA-TM vs. the anti-VEGF antibody modified with the same Fc mutations, using differential scanning calorimetry. Fig. 11 showed the combined structural and cartoon representation of the Bs4Ab with the Fc mutations mapped on the Fc (Fig. 11, left) and the correspondent DSC transition temperatures (Fig. 11, right). For comparison, the DSC data for the anti-VEGF antibody with the YTE, 3M and TM Fc mutations are shown in Fig. S6. As shown in Fig. 11, YTE, 3M and TM Fc mutations in Bs4Ab-VA resulted in similar transition temperatures compared with those observed for the parental anti-VEGF antibody (Fig. S6).

Next, we determined binding using ProteOn to human FcRn, FcγRI, FcγRIIA and FcγRIIIA (both allotype variants) for Bs4Ab-VA and the anti-VEGF Fc mutants. Compared to Bs4Ab-VA with native Fc (Table 2), Bs4Ab-VA YTE has increased binding to human FcRn, similarly to the anti-VEGF YTE antibody (Table 4). As expected, Bs4Ab-VA 3M and the anti-VEGF 3M have increased binding to FcγRIIIA (Table 4) compared with the native Bs4Ab-VA and anti-VEGF antibody (Table 3). Lastly, Bs4Ab-VA TM and the anti-VEGF TM have decreased binding to FcγRI and to both allotype variants of FcγRIII (Table 4) compared with the native Bs4Ab-VA and parental anti-VEGF antibody (Table 3).

**Discussion**

We have developed a novel, generic and efficient platform that yields tetravalent bispecific IgG1-like molecules. There have been several bispecific antibody formats described thus far,

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**Table 2.** Equilibrium KD (nM) determined by BIAcore for the binding to human FcRn for Bs4Ab-VA and for the anti-VEGF and anti-Ang2 antibodies.

| Human FcRn | anti-VEGF | anti-Ang2 | Bs4Ab-VA |
|------------|-----------|-----------|----------|
| pH 6       | 840       | 800       | 680      |
| pH 7.4/6   | Negative  | Negative  | Negative |

*Negative* denotes that FcRn binding at pH 7.4 relative to pH 6 was negligible (i.e., < 1%).

**Table 3.** Equilibrium binding to human FcγRs, Kd (nM), determined by ProteOn for Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies.

| Human FcγRs | anti-VEGF | anti-Ang2 | Bs4Ab-VA |
|-------------|-----------|-----------|----------|
| FcγRI       | 5.32      | 4.81      | 5.48     |
| FcγRIIA     | 900       | 543       | 910      |
| FcγRIIIA-1SBV | 321    | 261       | 308      |
| FcγRIIIA-1SBF | 1890   | 901       | 1990     |
mostly built by linking binding domains to the N- and or C-terminus of the heavy and or light chains of the IgG1. However, the modular architecture of the IgG1 offers tremendous opportunities to have additional binding modules inserted into the antibody structure. One insertion point is the IgG1 hinge, which also offers an intermediate paratopic distance in the bispecific that might be advantageous in some therapeutic applications. We envisioned that by using proper peptide linkages, we could insert a binding domain into the human IgG1 hinge while preserving structure and function of the IgG1, but adding an additional binding ability to the IgG1. To this end, we designed a tetravalent bispecific antibody where a scFv was inserted into the hinge domain of human IgG1.

The length and composition of the linkers used to connect the scFv to the hinge are critical to functional activity, structural stability and expression of Bs4Ab. For example, we discovered that proteolysis occurs at the C-terminus of the scFv in the lower hinge region of Bs4Ab, but proteolysis can be prevented by a single point mutation (D221G). In addition, we believe that using long linkers to insert the scFv domain should be avoided, as they may promote aggregation and favor proteolysis or can also induce crossover swap of the scFv variable domains. Another key design feature is at the lower insertion linker (i.e., the one that connects the C-terminus of the scFv to the lower hinge region), where the cysteine at position 220 was mutated to valine to prevent disulfide scrambling with any of the two adjacent cysteines 226 and 229 at the hinge, which form the inter-heavy chain disulfide bonds at the IgG1 hinge.

Bs4Ab technology is versatile in that it can yield molecules targeting soluble disease mediators, such as Bs4Ab-VA described here; membrane-bound targets on the surface of pathogens, as demonstrated by the clinical candidate MEDI3902 targeting the virulence factor PcrV and the persistence factor Psl exopolysaccharide of Pseudomonas aeruginosa; or receptors, as shown by a Bs4Ab that targets the EGFR and IGF1R described in the patent USPTO 20140302038. In the case of MEDI3902, it was determined that, for this combination of targets, the intermediate paratopic distance offered by the Bs4Ab format was optimal, and the molecule demonstrated significantly superior activity and efficacy compared with other bispecific antibody formats and antibody combination. The Bs4Ab platform maintains intact Fc, which is important for maintaining IgG1 Fc-like functions, such as half-life and effector functions. Moreover, the Bs4Ab platform allows Fc modifications to modulate half-life and effector functions similar to IgG1.

The progression of MEDI3902 to clinical studies demonstrates the developability, i.e., having drug-like properties and manufacturing feasibility, of molecules derived from the Bs4Ab technology. The IgG1-like properties and robust manufacturability of the Bs4Ab platform warrant its use as a molecular scaffold with the capacity to link other binding domains to the N- and or C-terminus of the Bs4Ab light and or heavy chain, thus engineering multispecific antibodies with higher than dual-target specificity. Illustrations of molecules that are theoretically possible to produce using the platform are shown in Fig. S7a-f. Insertion of more than one scFv, combining a scFv with other binding modules and insertion of alternatives to

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**Figure 9.** Pharmacokinetics of Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies in non-tumor bearing mice. Serum PK of Bs4Ab-VA (red line), anti-VEGF (blue line) and anti-Ang2 (green line) after intravenous administration in non-tumor bearing mice.

**Figure 10.** Tumor growth inhibition of Bs4Ab-VA, anti-VEGF and anti-Ang2, and their combination using a colon cancer (Colo205) mouse xenograft model. When the tumor reached a volume of approximately 200 mm³, mice (n = 6) were administered intraperitoneally twice per week for four weeks with 14 mg/kg of Bs4Ab-VA, 10 mg/kg of anti-VEGF, anti-Ang2, their combination, and a non-binding isotype antibody. Untreated mice were used as control. 10 mg/kg of each of the two parental antibodies were used for the combination study.
scFv binding modules are also possible, which additionally demonstrates the versatility of the Bs4Ab platform.

Materials and methods

Construction, expression and purification of Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies and isotype control antibody

VH and VL sequences of anti-Ang2 LCO627 and anti-VEGF28 were retrieved from USA patent application 2010/0111967. Amino acid sequences for the light and heavy chains of the Bs4Ab-VA used herein are shown in Fig. S2. DNA and amino acid sequences for other Bs4Abs, which were used to demonstrate applicability of the Bs4Ab technology platform, are shown in USA patent applications 2014/0302038, 2015/0284450, 2015/0023966. Oligonucleotides were synthetized and the Bs4Ab-VA construct, including linkers, anti-VEGF and anti-Ang2 antibodies, were assembled by overlapping PCR. Negative-control antibody, R347 is a proprietary molecule of MedImmune. The expression, purification and quantification of Bs4Ab-VA and parental antibodies were done following protocols as described previously.1,2 Briefly, recombinant proteins were transiently expressed using HEK293 cells and were

Figure 11. Combined cartoon and structural representation of Bs4Ab-VA with the Fc mutations mapped onto the Fc structure and differential scanning calorimetry analysis. Bs4Ab-VA with the YTE (a-left), 3M (b-left), and 3M mutations (c-left). The corresponding DSC analyses are shown on the right. The figure of the structure of the Fc was prepared using Pymol (http://www.pymol.org/), and structural information was retrieved from the Protein Data Bank (http://www.rcsb.org/) using entry code 3AVE.
purified using protein A chromatography (GE Life Sciences). Aggregates were removed after protein A purification using CHT (BIORAD) according to the manufacturer’s instructions. Fc mutations in Bs4Ab-VA and anti-VEGF antibody were prepared using standard molecular biology techniques with oligonucleotides containing the desired mutations. Mutagenesis, expression and purification of the Fc mutants were performed using similar methods used for Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies.

Analytical characterization

SEC-HPLC was used to analyze the purity of Bs4Ab-VA, anti-VEGF, anti-Ang2 antibodies and Fc mutants. SEC-HPLC was performed as described previously.\textsuperscript{1,2} Two hundred \textmu g of sample was used in each SEC-HPLC analysis. SDS-PAGE analysis was performed using 2 \textmu g of purified proteins. Samples were reduced using 5 \textmu L of NuPAGE reducing agent (Thermo Fisher Scientific). Reduced and non-reduced samples were incubated at 70°C for 10 minutes, spun down at 16,000 g for 5 minutes using a benchtop centrifuge and loaded onto 4–12% Bolt gel (Thermo Fisher Scientific). Electrophoresis was performed in MOPS buffer at 150 V for one hour. Novex Sharp Pre-Stained (Thermo Fisher Scientific) were used as protein molecular weight standards. For the reduced reverse phase chromatography (rRP-HPLC) analysis, Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies were reduced at 37°C for 20 minutes using 42 mM dithiothreitol (DTT) in PBS pH 7.2. Ten \textmu g of reduced sample was loaded onto a PLRP-S, 1000 Å column, 2.1 mm, Agilent) eluted at 80°C at a flow rate of 1 mL/min with a gradient of 5% B to 100% B over 25 minutes (mobile phase A: 0.1% trifluoroacetic acid in water, and mobile phase B: 0.1% trifluoroacetic acid in acetonitrile (EMD Bioscience). DSC was performed essentially as described by Dimasi N. et al.\textsuperscript{1,2} Briefly, Bs4Ab-VA, anti-VEGF, anti-Ang2 and Fc mutants were formulated at 1 mg/mL, and extensively dialyzed in 25 mM histidine-HCl pH 6. DSC experiments were performed using a VP-DSC (Malvern). The raw data were normalized for concentration and scan rate (1°C/minute). Data analysis and deconvolution were performed using the DSC module in Origin 7 using a non-2-state model and the best fits were obtained using 15 iteration cycles. The denaturation temperatures (T\textsubscript{m}) corresponding to the maximum of the transition peaks, were determined for each construct. Reduced liquid chromatography mass spectrometry (rLCMS) was carried on an Agilent 1200 series HPLC coupled to an Agilent 6520 Accurate-Mass Q-TOF LC/MS with an electrospray ionization source. Two \mu g of reduced sample was loaded onto a Poroshell 300SB-C3 column (2.1×75 mm, Agilent) and eluted at a flow rate of 0.4 mL/min using a step gradient of 60% B after 6 minutes (mobile phase A: 0.1% formic acid in water and mobile phase B: 0.1% formic acid in acetonitrile). Agilent MassHunter was used for data acquisition and chromatogram processing. Peaks were assigned based on their expected molecular mass.

Accelerated stability studies

Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies were formulated in 25 mM histidine-HCl pH 6 and were concentrated to 25 mg/mL using centrifugal filters with a 10,000 MWCO (Millipore). The proteins were subjected to three cycles of freeze-thaw followed by one month incubation at 4°C, 25°C and 40°C with 75% relative humidity. Samples were taken before and after the three cycles of freeze-thaw, after one, two and three week incubation at the respective temperatures and were analyzed for aggregation and fragmentation using analytical SEC-HPLC. Purity loss is expressed as percentage and is the sum of aggregates and fragments over the incubation time determined by peak integration of the SEC-HPLC chromatograms at 280 nm.

Imaged capillary isoelectric focusing

Imaged capillary isoelectric focusing was performed using an iCE3 analyzer (ProteinSimple). Pharmalytes pH 3–10 and 8–10.5 were obtained from Sigma. The FC cartridge and the Chemical Testing Kit for the performance evaluation of the iCE3 analyzer, including anolyte (80 mM phosphoric acid in 0.1% methyl cellulose), catholyte (100 mM sodium hydroxide in 0.1% methyl cellulose), 0.5% methylcellulose, hemoglobin standard, ampholytes and pH markers in 0.35% methyl cellulose were purchased from ProteinSimple. Deionized water was generated using a Milli-Q water purification system (Millipore). Bs4Ab-VA, anti-VEGF and anti-Ang2 were prepared at 1 mg/mL in deionized water before use. Sample preparation was performed by mixing 50 \mu l of 1 mg/ml of Bs4Ab-VA, anti-VEGF and anti-Ang2, 2 \mu l of 5.85 pL marker, 2 \mu l of 9.46 pL marker, 140 \mu l of 0.5% methylcellulose, and 2 \mu l of 3–10 and 6 \mu l of 8–10.5 Pharmalytes, respectively. The mixture was vortexed for 45 seconds and centrifuged at 10,000 rpm for 3 minutes. Samples were loaded into the capillary using an autosampler (ProteinSimple). Sample separation was performed by a pre-focus at 1000 kV for 1 minute/s, followed by 3000 kV for 7 minutes. Detection was performed using a deuterium lamp detector at 280 nm. Data were analyzed and figures were prepared using the iCE3 analyzer software.

ELISA

Sixty ng/well of recombinant human Ang2 (R&D Systems) and recombinant human VEGF-165 (Peprotech, cat. No. 100-20) in PBS were coated overnight on ELISA plates. Plates were washed.

Table 4. Equilibrium binding to human FcRn and Fc\gamma R3, Kd (nM), determined by ProteOn for Fc mutants for Bs4Ab-VA and the anti-VEGF parental antibody.

| Fc receptor | anti-VEGF | Bs4Ab-VA |
|-------------|-----------|----------|
| FcRn        | 118       | 123      |
| 3M          |           |          |
| Fc\gamma RIII-A-158F | 227       | 188      |
| TM          |           |          |
| Fc receptors | anti-VEGF | Bs4Ab-VA |
| FcRRI       | 259       | 273      |
| Fc\gamma RIII-A-158V | 2600      | 2900     |
| Fc\gamma RIII-A-158F | 10100     | 13100    |
with PBS+Tween-20 (PBST) and blocked using 3% non-fat milk in PBST (blocking buffer) for one hour. Bs4Ab-VA and parental antibodies were prepared at 66.7 μM in blocking buffer and added to the plates using a 2-fold dilution to a final concentration of 0.12 nM. Plates were then incubated for one hour at room temperature (RT) and washed three times with PBST and incubated with 1:3000 dilution of goat anti-human-Fc horseradish peroxidase (HRP) conjugated antibody (Southern Biotech, cat. No. 2047-05) in PBST for one hour at RT followed by the addition of the chromogenic substrate TMB (Sigma Aldrich) for five minutes at room temperature. The colorimetric reaction was stopped by the addition of 0.2 N HCl. Specific binding was detected by reading absorbance at 450 nm. EC₅₀ were calculated using non-linear regression analysis (log dose-response) and binding curves were plotted using Prism5 (GraphPad).

**Concurrent binding of Bs4Ab-VA to VEGF-165 and Ang2 using BIAcore**

Concurrent binding experiments of Bs4Ab-VA were performed using BIAcore 3000 (GE Healthcare) as described previously. Briefly, a CM5 BIAcore sensor chip (GE Life Sciences) was used to immobilize Bs4Ab-VA, human Ang2 (R&D Systems) and human VEGF-165 (Peprotech, cat. No. 100-20). Bs4Ab-VA, Ang2 and VEGF were prepared in HBS-EP buffer (GE Life Sciences) consisting of 10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% PS20. A flow rate of 30 μL/min was used for all binding measurements. For determining simultaneous binding to VEGF and Ang2 when Bs4Ab-VA was immobilized on the sensor chip, 1 μM of VEGF-165 was injected over the Bs4Ab-VA surface. Once the VEGF-165 binding level was stabilized, 1 μM of Ang2 mixed with 1 μM of VEGF-165 was injected. VEGF-165 was included in the mixture with Ang2 to prevent the signal loss due to potential VEGF-165 dissociation during the Ang2 binding phase. As a control, a second injection of 1 μM VEGF did not result in an increasing binding signal. To demonstrate that concurrent binding is independent of injection sequence, 1 μM of Ang2 was first injected on the Bs4Ab-VA surface followed by a mixture of 1 μM of Ang2 and 1 μM of VEGF-165. Also in this case, a second injection of 1 μM of Ang2 did not increase the binding signal. A similar procedure was used, but Ang2 and or VEGF-165 were immobilized on the sensor chip. To the immobilized antigens, 400 nM of Bs4Ab-VA was injected, followed by 1 μM of VEGF-165, when Ang2 was immobilized; and by 1 μM of Ang2, when VEGF-165 was immobilized. A further injection of 400 nM of Bs4Ab-VA after the sensor chip was saturated with the first injection of Bs4Ab-VA resulted in non-measurable binding. To rule out interaction between Ang2 and VEGF-165, the following BIAcore binding method was used. Fifty nM of VEGF-165 and Ang2 (R&D systems, cat. No. 623-AN-025/CF) were immobilized on a CM5 sensor chip to 800 RU using standard amine coupling chemistry and binding was performed using a BiaCore 3000 (GE Healthcare). A reference flow cell minus protein was prepared using same method. To test binding 500 nM of Ang2, VEGF-165 and Bs4-AB-VA were prepared in 50 mM phosphate, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% Tween-20 (system buffer). Injection of ligands was performed at a flow rate of 50 μL/min for 300 seconds and dissociation phase of the binding was performed at 100 μL/min for 600 second. To the immobilized Ang2, VEGF-165 and Bs4Ab-VA were used as ligands; and for the immobilized VEGF-165, Ang2 and Bs4Ab-VA were used as ligands. A blank injection of system buffer was used between each ligand injection. The background binding obtained from the reference cell and the one from a system buffer injection were used to correct the binding data for any injection artifact using the "double-referencing" technique. Data sets were processed using the BIAevaluation software (GE Life Sciences) and the figures were prepared using prism (GraphPad).

**FcRn binding using BIAcore**

Binding of the Bs4Ab-VA, anti-VEGF and anti-Ang2 to human FcRn using BIAcore was performed as described by Dimasi N. et. al. Binding was performed using a BIAcore 3000 instrument; human FcRn was prepared at MedImmune. Briefly, The Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies were immobilized on separate flow cells using a CM5 sensor chip following manufacturer recommendations (GE Life Sciences). Surface densities were around 2000 RU. A reference cell without protein was prepared using the same sensor chip. Two-fold serial dilutions of human FcRn were prepared, ranging from 3000 to 16 nM in PBS pH 6 with 0.05% Tween-20 (FcRn buffer). The instrument was primed with the FcRn buffer, and then each FcRn dilution was injected over the surface of each Bs4Ab-VA, anti-VEGF and anti-Ang2 antibodies and the reference cell at a flow rate of 5 μL/min. Binding data were collected over a 50 minute period, followed by a 1-minute pulse of a solution of PBS pH 7.4, 150 mM NaCl, 0.005% Tween-20. Data were processed as described for the concurrent binding to antigens.

**FcγRs and FcRn binding using ProteOn**

The equilibrium binding affinities for human FcγRI, FcγRIIIA-158V, FcγRIII-158F, and human FcRn to Bs4Ab-VA, anti-VEGF and the Fc mutants (i.e., YTE, 3M and TM) were performed using ProteOn XPR36 (BioRad). Bs4Ab-VA, anti-VEGF and the Fc mutants were immobilized using amino coupling chemistry on a GLC ProteOn sensor chip. The sensor chip was treated with 30 μL of 0.5% SDS, 50 mM NaOH, 100 mM HCl, followed by 30 μL of PBS, 0.005% Tween-20, 3 mM EDTA pH 7.4 (ProteOn running buffer) at 100 μL/minute. Bs4Ab-VA, anti-VEGF and the Fc mutants were prepared at 10 μM in acetate buffer pH 4 and flowed over the activated sensor chip surface at 30 μL/minute. The surface density for the immobilized proteins ranged from 3240 to 3630 resonance unit (RU). A reference surface was similarly prepared but without immobilized protein. After immobilization, the chip surfaces were inactivated using ethanolamine-HCl (Bio-Rad). Before the binding experiments, the chip surfaces were treated one more time with 30 μL of 0.5% SDS, 50 mM NaOH, 100 mM HCl and 30 μL of ProteOn running buffer at 100 μL/minute. FcγRIIIA-158V was prepared at 16 μM in ProteOn running buffer and serially diluted 1:3 to
0.19 nM. FcγRIII-158F was prepared in the same buffer, but at a starting concentration of 32 μM and serially diluted 1:3 to 0.395 nM; the FcγRI was also prepared in ProteOn running buffer at a starting concentration of 4 nM and serially diluted 1:3 to a final concentration of 5.49 nM. Human FcRn was prepared at 3 μM in ProteOn running buffer, but with pH 6 and serially diluted 1:3 to a final concentration of 4.11 nM. FcγRI, FcγRIIIA-158V, FcγRIII-158F, and FcRn dilutions were injected on the sensor chip with immobilized Bs4Ab-VA and anti-VEGF at 25 μL/minute for 8 minutes. Between injections, 25 μL of 5 mM HCl was used to regenerate the ProteOn chip surface. For the FcRn binding experiments, the chip surface regeneration was performed in ProteOn running buffer with pH 7.4. Data were blank subtracted, reference surface corrected, and equilibrium binding constant were determined using the ProteOn software (Bio-Rad).

**pTie2 immunoassay**

Inhibition of Ang2-induced phosphorylation of Tie2 receptor was performed as described previously. Briefly, 22,000 stably transfected HEK293 cells expressing human Tie2 on the cell surface, HEK293-Tie2, in DMEM-F12 media, 10% fetal bovine serum (FBS), 2 μg/mL puromycin (Life Technologies) were plated onto 96-well Corning CellBind tissue culture plate (Corning) and incubated overnight at 37°C and 5% CO2. Antibodies in concentrations ranging from 120 nM to 1 nM were combined with 6 μg/mL of recombinant human Ang2 (R&D Systems, cat. No. 623-AN-025/CF) at a 1:1 volume ratio. The antibody:Ang2 mixture was then added to each well in duplicate and incubated under gentle rotation for 15 minutes. Plates were washed and cells were lysed using RIPA lysis buffer (Boston BioProducts) containing protease and phosphatase inhibitors (Life Technologies). Total protein was quantified using the bicinchoninic acid assay (Pierce) and phosphorylated Tie2 was quantified using a Meso Scale Diagnostics (MSD) assay. MSD plates were coated overnight with 2 μg/mL of anti-human Tie2 antibody (Abcam, cat. No. ab24859), blocked for 1 hour at RT with 0.05% Tween-20 (blocking buffer) for one hour at room temperature. The membranes were washed three times with 0.1% Tween-20 (wash buffer) and incubated for 1 hour at RT. Plates were washed with WB, and incubated for 1 hour at RT. Plates were washed with WB, and incubated with 0.5 μg/mL of sulfo-tag goat anti-rabbit secondary antibody (MSD cat. No. R32AB-5) and incubated for 1 hour at RT. Finally, the plates were washed in WB, and 150 μL of Read buffer T (MSD) was added to each well and analyzed using a MSD Sector Imager 6000 (MSD). IC₅₀ were calculated using non-linear regression analysis (log dose-response) and binding curves were plotted using Prism5 software (GraphPad).

**pVEGFR2 immunoassay**

A synthetic gene coding for human VEGFR2 was prepared (Gene Art) using Uniprot database accession number P35968–1. The DNA was digested with XbaI and NotI and cloned into the lentivirus vector pCDHI-Ef1-puro (System Biosciences), which was digested with the same restriction enzymes. The resulting vector pCDHI-huVEGFR2-puro was mixed 1:5 with pPACKH1 packing plasmid (System Biosciences) and transected into adherent HEK (Ad293; ATCC) cells to produce pseudoviral particles. 2 × 10⁶ pseudoviral particles were then used to transduce Ad293 cells to allow integration of the VEGFR2 DNA. Stably transfected cells, Ad293-HuVEGFR2, were selected using 1 μg/mL of puromycin (Life Technologies). 15,000 Ad293-HuVEGFR2 cells per well were plated using 96-well Corning Cell Bind tissue culture plate in DMEM, 10% FBS and incubated overnight at 37°C and 5% CO2. The following day, the culture medium was removed and replaced with MEM, 0.2% FBS, 0.1% BSA and the cells were incubated overnight at 37°C and 5% CO2. The culture medium was removed and antibodies in concentrations ranging from 1330 nM to 0.02 nM were added in duplicate for 30 minutes at 37°C, followed by the addition of 4 nM of human VEGF-165. The plates were incubated at 4°C for 30 minutes to allow binding, followed by incubation at 37°C for 7 minutes, the medium removed, and the cell lysed using RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Total protein was quantified as described for the Tie2 assay, and phosphorylated VEGFR2 was quantified using a pVEGFR2 whole cell lysate kit following the manufacturer’s instruction (MSD, cat. No. k151dj2-2). IC₅₀ were calculated using non-linear regression analysis (log dose-response) and binding curves were plotted using Prism5 software (GraphPad).

**Western blot**

Cell lysates were obtained from Ang2 and VEGF-induced receptors phosphorylation as described above, and untreated cell lysates were centrifuged at 16,000 g at 4°C for 10 minutes to remove cellular debris. Forty μL of the Ang2-treated lysates obtained from the 20 nM, 2.96 nM and 0.19 nM incubations with Bs4Ab-VA and anti-Ang2, and 20 nM cell lysates obtained with the incubation of the isotype control antibody, were diluted to a final volume of 50 μL with 1X PBS pH 7.2. Fourteen μL of 4X SDS sample buffer (Invitrogen) containing 2.5% β-mercaptoethanol (Sigma) was added to each sample and incubated at 70°C for 10 minutes, followed by centrifugation at 16,000 x g for 1 minute. Fifty μL of the reduced samples and 7 μL of MagicMark XP molecular weight western standard (Invitrogen) were loaded on to a 4–12% Bolt Bis-Tris Plus gel and electrophoresed at 150 V in Bolt MOPS SDS running buffer for 75 minutes (Invitrogen). The proteins were transferred to a PVDF membrane using the iBlot2 Dry Blotting System (Invitrogen). The membranes were incubated with 3% non-fat dry milk dissolved in 50% Superblock (Pierce) in PBS with 0.1% Tween-20 (blocking buffer) for one hour at room temperature. The membranes were washed three times with PBS, 0.1% Tween-20 (wash buffer) and incubated in blocking buffer for one hour with a 1:1000 dilution of an anti-β-Actin rabbit antibody (Cell Signaling, cat. No. 4967) and 0.5 μg/mL anti-pTie2 rabbit antibody (R&D Systems, cat. No. AF2720), which is specific for phosphorylated tyrosine at position 992 of the Tie2 receptor. After three washes, a 1:3000 dilution of a goat anti-rabbit-HRP antibody (KPL, cat. No. 074–1516) in blocking buffer was added for one hour at room temperature.
After three washes, 5 mL of SuperSignal West Dura Extended Duration substrate (Invitrogen) was added and the blot was visualized using ImageQuant LAS4000 system (GE). Western blot analyses of the VEGF-induced cell lysates were processed as for the Ang2-induced cell lysates, but using 10 μL of cell lysates obtained from the 665 nM, 0.649 nM and 0.01 nM incubation with Bs4Ab-VA and anti-VEGF, and 20 nM cell lysates obtained with incubation of the isotype control antibody. A 1:3000 dilution of pVEGFR2-Y1175 rabbit antibody (Cell Signaling, cat. No. 2478T), an antibody that is specific for phosphorylated tyrosine at position 1175 of the pVEGFR2 receptor was prepared with the anti-β-Actin antibody and the western blot membrane was treated and developed as described for the pTie2 western blot.

In vivo efficacy

All procedures using mice were approved by the MedImmune Institutional Animal Care and Use Committee according to guidelines as set forth by The Association for Assessment and Accreditation of Laboratory Animal Care International. 5 × 10⁶ Colo205 cells in 50% matrigel were inoculated subcutaneously on the right flank into 5- to 6-weeks old female athymic nude mice (Harlan Laboratories). When tumors reached approximately 200 mm³, mice were randomized into groups (6 mice per group) based on tumor volume using Studylog software (Studylog). Endotoxin-free isotype control antibody R347, anti-VEGF, anti-Ang2, and Bs4Ab-VA were administered intraperitoneally twice per week at 14 mg/kg for Bs4Ab-VA, 10 mg/kg for the anti-VEGF and anti-Ang2, and control antibodies, and at 10 mg/kg each for the combination of the anti-VEGF and anti-Ang2 antibodies. Untreated mice were included as controls. Tumor growth inhibition was calculated on the last day of study relative to the initial and final mean tumor volume of the control group. Two-way ANOVA was used to compare the reduction in tumor volume in mice treated with the combination therapies vs. those treated with either agent alone. The tumor growth inhibition was plotted using Prism5 software (GraphPad). Tumor volumes are expressed as mean ± SEM (standard errors of the means).

Mouse pharmacokinetics

PK experiments were performed using 6- to 8-week-old non-tumor bearing athymic female (nu/nu) nude mice (Harlan Laboratories). Mice (3 per group) were administered a single intravenous dose of anti-VEGF and anti-Ang2 antibodies at 10 μg/kg, and Bs4Ab-VA at 14 μg/kg. Blood samples were collected into K₂-EDTA tubes (Fisher Scientific) from each mouse via orbital bleeding at 1, 6, 24, 48, 96, 168 hours, and via terminal bleeding at 240 hours post-dose. Concentration of Bs4Ab-VA, anti-VEGF and anti-Ang2 were assessed by ELISA with a limit of quantification of 2.058 ng/mL. ELISA plates (Corning) were coated overnight at 4°C with 30 μL/well of goat anti-human IgG-Fc antibody (Thermo Scientific, cat. No. G-102-C) at a concentration of 5 μg/mL in PBS pH 7.4. Plates were washed with PBS pH 7.4, 0.05% (v/v) Tween-20 (TBST) and blocked with 100 μL/well of non-fat dry milk for one hour at room temperature under shaking. Standard curves were prepared using Bs4Ab-VA, anti-Ang2 and anti-VEGF prepared in PBS pH 7.4. Standard curves concentration ranged from 500 ng/mL to 0.68 ng/mL. 50 μL of standards and samples (1:10 serial dilution) were added and plates were incubated at room temperature for two hours under shaking. Plates were washed with TBST four times and incubated with goat anti-human lambda HRP-conjugated antibody (Southern Bio, cat. No. 2070–05) for the detection of the anti-Ang2 antibody, or goat anti-human kappa HRP-conjugated antibody (Southern Bio, cat. No. 2060–06) for the detection of the Bs4Ab-VA and the anti-VEGF antibody. The goat anti-human lambda and goat anti-human kappa HRP-conjugated antibodies were diluted 1:8000 in 1% dry milk prepared in PBS and 100 μL of each were added per well. Plates were incubated one hour at room temperature under shaking. After four washes with TBST, the plates were incubated with 100 μL/well of 3,3',5,5'-tetramethylbenzidine liquid substrate (Thermo Fisher Scientific) for one minute at room temperature and the HRP activity stopped using 100 μL/well phosphoric acid stop solution. Absorbance of each plate at 450 nm was read using a plate reader (Thermo Fisher Scientific).

Pharmacokinetics data analysis

Sparse non-compartmental PK analysis was performed using Phoenix 64 WinNonlin 6.3 (Pharsight). PK parameters were summarized statistically and presented as mean and one standard deviation. Data were plotted and the figure was prepared using Prism5 software (GraphPad).

Disclosure of potential conflicts of interest

All authors are employees of MedImmune and stockholders of AstraZeneca.

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Author contributions

ND designed Bs4Ab. BB and RF performed cloning, expression, biochemical and biophysical characterization. CF and HZ performed in vivo efficacy and PK. XQY performed PK data analysis. CCL and KC performed inhibition of ligand-mediated phosphorylation. NG and SW prepared Ad293 cells expressing VEGFR2. CKS, HW and CG provided scientific support. ND wrote the manuscript. All authors edited and reviewed the manuscript.

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