Supporting Information

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SI Materials and Methods

Model Building. Structural models are based on the X-ray structure of the bevacizumab antigen binding fragment (Fab) in complex with vascular endothelial growth factor (VEGF) (Protein Data Bank ID 1bj1). The missing residues at the C-terminal end of both chains, the missing segment 128–133 (SKSTSG) in the heavy chain constant domain 1 (CH1), and the first amino acids of the hinge region (DKTHT) have been inserted from a different antibody structure (1). Models were generated and optimized using Moloc (2).

Construction of Expression Vectors. All antibody genes were ordered as gene syntheses and cloned via unique restriction sites using standard cloning procedures (3) into separate expression vectors enabling secretory expression in HEK cells growing in suspension. Amino acids of antibody chains are numbered and referred to according to European Union numbering (4, 5).

Transient Antibody Expression in HEK293-F. Transfection into HEK293-F cells (Invitrogen) was performed according to the cell supplier’s instructions using Maxiprep (Qiagen) preparations of the antibody vectors, Opti-MEM I medium (Invitrogen), 293fectin (Invitrogen), and an initial cell density of 1–2 × 10^6 viable cells/mL in serum-free FreeStyle 293 expression medium (Invitrogen). Antibody containing cell culture supernatants were harvested after 7 d of cultivation in shake flasks or stirred fermenters by centrifugation at 14,000 × g for 30 min and filtered through a sterile filter (0.22 μm). The antibodies were purified directly from the supernatant, or the supernatant was stored at −80 °C until purification.

Determination of Protein Concentration. The protein concentration of purified antibodies and derivatives was determined by measuring the OD at 280 nm, using a molar extinction coefficient calculated according to Pace et al. (6).

Determination of Antibody Concentration in Supernatants. The concentration of antibodies and derivatives in cell-culture supernatants was measured by protein A HPLC chromatography. Cell-culture supernatants containing antibodies and derivatives which bind to protein A were applied to a HiTrap protein A column (GE Healthcare) in 50 mM potassium phosphate, 300 mM NaCl, pH 7.3, and were eluted from the matrix with 550 mM acetic acid on a Dionex HPLC-System. The eluted protein was quantified by UV absorbance. A purified IgG1 antibody served as a standard. This method detects complete antibodies and derivatives binding to protein A.

Protein Purification. Proteins were purified from filtered cell-culture supernatants referring to standard protocols. Antibodies were applied to a protein A Sepharose column (GE Healthcare) and washed with PBS. Elution of antibodies was achieved at pH 3.0 followed by immediate neutralization of the sample. Aggregated protein was separated from monomeric antibodies by size-exclusion chromatography (Superdex 200; GE Healthcare) in 20 mM histidine, 140 mM NaCl, pH 6.0. Monomeric antibody fractions were pooled, concentrated if required using a MILLIPORE Amicon Ultra (30 molecular weight cutoff) centrifugal concentrator, and stored at −80 °C. An aliquot of the samples was retained for subsequent protein analytical characterization (e.g., by SDS/PAGE, size-exclusion chromatography, mass spectrometry) and also by endotoxin determination.

SDS/PAGE. Samples were prepared according to the manufacturer’s protocol using NuPAGE reagents (Invitrogen). Denaturation was performed for 5 min at 70 °C. A total of 8 μg protein per lane was loaded on a 4–20% Tris-Glycine gel (Invitrogen) and separated at 125 V for approx. 90 min. Gels were stained with Simply Blue Safe stain.

Capillary Electrophoresis Sodium Dodecyl Sulfate (CE-SDS). For the quantification of incompletely assembled molecules, a chip-based capillary electrophoresis system (BioAnalyzer 2100; Agilent Technologies) and a Protein230 Series II chip were used. Samples were prepared according to the manufacturer’s protocol. Denaturation was achieved by 5 min. incubation at 95 °C. No reducing agent was added.

Analytical Size-Exclusion Chromatography. For analytical size-exclusion chromatography, samples were applied to two Tosoh TSKgel G3000SW columns connected in series in 300 mM NaCl, 50 mM potassium phosphate, pH 7.5, on an Agilent HPLC 1100 system. The eluted protein was quantified by UV absorbance at 280 nm.

Surface Plasmon Resonance Analysis. Antigen binding. Experiments were performed on a BiacoreT100 instrument (GE Healthcare Bio-Sciences AB) using PBS, 0.05% polysorbate 20 as running and dilution buffer. Anti-human IgG polyclonal antibodies (Jackson ImmunoResearch Europe Ltd.) were immobilized to the surface of a CM5 sensorchip using standard amine-coupling chemistry to a surface density of ∼200 response units (RU). After capturing the bispecific antibodies to the surface, either human angiopoietin-2 (hAng-2) or human VEGF (hVEGF) was injected at increasing concentrations at a flow rate of 30 μL/min. The contact time (association phase) was 3 min. The dissociation time was 10 min for hAng-2 and 30 min for hVEGF. After each binding cycle, regeneration of the sensorchip was performed using a single injection of 0.85% phosphoric acid for 5 min. The sensorchip was washed thoroughly with running buffer before the next injection. For the interaction analysis, the hVEGF was injected for 1 min. The contact time was 30 min at a flow rate of 30 μL/min. The dissociation time was 10 min at a flow rate of 5 μL/min. Binding to human fragment crystallizable (Fc) γ receptor IIIa. Fc γ receptor IIIa (FcyRIIIa-His (V158) was coupled to a CMS sensor chip using standard amine-coupling chemistry. CrossMab Anti-C1L-C1L or LC06 was injected into the flow cell, and the derived binding signals were compared. Additionally, the binding was inhibited with increasing concentrations of FcγRIIIa to estimate the apparent steady-state affinity (solution affinity).

Mass Spectrometry. The total mass of deglycosylated as well as deglycosylated and reduced CrossMabs was determined by electrospray ionization mass spectrometry (ESI-MS) [Bruker MaXis MS system equipped with a NanoMate (Advion) source]. Briefly, 100 μg of purified protein was deglycosylated with 50 μU N-Glycosidase F (ProZyme) in 100 mM potassium phosphate, pH 7, at 37 °C for 12–24 h at a protein concentration of up to 2 mg/mL. A sample was divided into two equal aliquots. One aliquot was incubated with 60 μL 1 M tris(2-carboxyethyl)phosphine (Pierce) and 50 μL 8 M guanidine-hydrochloride (Sigma) for 30 min at 37 °C. Both aliquots were subsequently desalted via...
HPLC on a Sephadex G25 column (GE Healthcare) in a buffer containing 40% acetonitrile and 2% formic acid. Finally the mass of the total CrossMab and the respective heavy and light chains was determined.

Stability Assessment. All the following experiments were performed with samples dialyzed into 20 mM citric acid/sodium citrate, 20 mM L-arginine, 190 mM sucrose, pH 5.5, and adjusted to a protein concentration of ~1 mg/mL. For stability during storage, samples were stored without agitation at 40°C for 3 wk. Control samples were stored at ~80°C. The formation of high-molecular-weight (HMW) and low-molecular-weight (LMW) species was monitored by size-exclusion chromatography using a TSK3000SWXL column (Tosoh) and isocratic elution with 200 mM sodium phosphate, 250 mM KCl, pH 7.0. Protein peaks eluting earlier than the main peak are referred to collectively as “HMWs”; peaks eluting after the main peak are referred to as “LMWs.” Differential scanning calorimetry (DSC) measurements were performed using a N-DSC II microcalorimeter (Calorimetry Science Corporation). The protein melting point (T_m) is defined as a peak in the temperature-dependent specific heat capacity graph.

Animals. Female SCID beige mice were purchased from Charles River. Continuous health monitoring was carried out. The mice were kept under specific pathogen-free conditions according to international guidelines (Gesellschaft für Versuchstierkunde; Federation for Laboratory Animal Science Associations, and Tierschutzgesetz, Animal Welfare Act). The experiments were conducted when the mice were 4–6 wk old. All experiments were reviewed and approved by the local government (Regierung von Oberbayern; registration no. 211.2531.2–22/2003).

Single-Dose Pharmacokinetics in Non–Tumor-Bearing Mice. Experiments were performed in female NMRI mice using a single i.v. bolus administration of 10 mg/kg. Concentrations of the human antibodies in mice serum were determined by ELISA using 1% mouse serum. Pharmacokinetic parameters were determined by noncompartmental analysis, using the pharmacokinetic evaluation program WinNonlin, provided by PharSight, www.pharsight.com.

Tumor Implantation and Treatment Schedule. Colo205 cells (ATCC) [10^6 cells suspended in 100 μL BD Matrigel Matrix (BD Biosciences)/PBS (1:1)] were injected s.c. in the right flanks of 8- to 10-wk-old SCID beige mice. Tumor growth was quantified weekly by caliper measurements. Tumor volume was calculated by measuring with a caliper the largest diameter (A) and its perpendicular (B) according to the formula, 0.5 × A × B^2. Because there is comparable exposure for i.v. and i.p. routes of administration (7, 8), mice were treated i.p. with 10 mg/kg bevacizumab, with 10 mg/kg anti-Ang-2 antibody LC06, or with the two combined at a dose of 10 mg/kg each, and with the Ang-2-VEGF Cross-Mab^{CH1-CL} at an equivalent dose of 20 mg/kg. Omalizumab, a humanized anti-IgE IgG1 antibody, was used as a negative control at 10 mg/kg (i.p.) Mice were treated once weekly for a period of 6 wk, and tumor volume was monitored.

Mouse Cornea Micropocket Angiogenesis Assay. Female BALB/c mice (8- to 10-wk-old) were purchased from Charles River. The protocol was modified according to the method described in ref. 9. Micropockets with a width of about 500 μm were prepared under a microscope at ~1 mm from the limbus to the top of the cornea using a surgical blade and sharp tweezers in the anesthetized mouse. The disk (Nylafo; Pall Corporation) with a diameter of 0.6 mm was implanted, and the surface of the implantation area was smoothed. Discs were incubated in corresponding growth factor or in vehicle for at least 30 min. After 7 d, the eyes were photographed, and the vascular response was measured. The assay was quantified by calculating the percentage of the area of new vessels per total area of the cornea.

Statistical Analysis. All raw data were processed using statistics software SAS-JMP version 7 (SAS Inc.).

Fig. S1. Schematic representation of the antibodies discussed in the text. The desired antibody (A) and undesired side products (B, C, and D) resulting from application of the knobs-into-holes (KiH) technology. (E) CrossMab\textsuperscript{Fab}. (F) Unspecific monovalent antibody and (G) unspecific Fab observed as main side products in the generation of E. (H) CrossMab\textsuperscript{VH-VL} and (I) its major side product which results from Bence-Jones interaction of the wrong light chain with the domain-exchanged heavy chain. (J) CrossMab\textsuperscript{CH1-CL} as used in the in vivo experiments.

Fig. S2. Connection of the Fab to the Fc part as it occurs in (A) the WT protein and the CrossMab\textsuperscript{VH-VL} and (B) the CrossMab\textsuperscript{CH1-CL} and the CrossMab\textsuperscript{Fab}. Dark blue indicates heavy-chain sequences. Light blue indicates light-chain sequences.
Fig. S3. (A) Superimposition of the CL domain of the bevacizumab Fab structure (light blue) on the CH1 domain (dark blue). (B and C) Sequences of the “elbow” regions between variable and constant Fab domains as occurring in the WT antibody (B) and the CrossMab\textsuperscript{VH-VL} as well as CrossMab\textsuperscript{CH1-CL} (C). Dark blue indicates heavy-chain sequences. Light blue indicates light-chain sequences.

Fig. S4. Comparison of the bevacizumab X-ray structure (Fab) with models of the three CrossMabs. WT: Crystal structure of the Fab fragment (blue) of the VEGF antibody bevacizumab in complex with VEGF (Protein Data Bank ID 1bj1); the VEGF dimer is shown in yellow/orange. A second Fab fragment binding to the opposite side on top of the ligand dimer has been omitted. The three figures on the right show molecular models of bevacizumab with crossover of the Fab domains, VH and VL domains, or CH1 and CL domains within the Fab region. Light blue indicates light-chain domains. Dark blue indicates heavy-chain domains. The short green stretch at the bottom of each of the structures denotes the hinge region which connects the Fab fragment to the Fc part of the antibody.
Fig. S5. Overlay ESI-MS of CrossMabs (brown: CH1-CL, green: VH-VL, red: Fab) after deglycosylation. The upper panel shows the full m/z spectrum with an envelope for all CrossMabs which is typical for spectra of antibodies. The lower panel shows a zoom into a region around the charge state +51. Shoulders and peaks at higher m/z values close to the molecular ion result from sodium and phosphate adducts (especially CrossMax(CH1-CL)), lysine heterogeneity, and unspecific glycation.
Fig. S6. Overlay ESI-MS of CrossMabs (brown: CH1-CL, green:VH-VL, red: Fab) after deglycosylation and reduction. The upper panel shows the full m/z spectrum for all CrossMabs with envelopes typical for an antibody containing four chains. The lower panel shows a zoom into the m/z range from 900–1,000. The three bispecific CrossMabs clearly show the presence of two different light chains and two different heavy chains.
Fig. S7. Concentration-dependent binding of human Ang-2 to the parental antibody LC06 and the three bispecific CrossMabs as determined by surface plasmon resonance. The x axis shows time, and the y axis shows response units.
Fig. S8. Concentration-dependent binding of human VEGF-A to the parental antibody bevacizumab and the three bispecific CrossMabs as determined by surface plasmon resonance. The x axis shows time, and the y axis shows response units.
Fig. S9. Comparison of the FcγRIIIa-binding properties of the Ang-2–VEGF CrossMab\textsuperscript{CH1-CL} and the monospecific IgG1 antibody LC06 as measured by surface plasmon resonance. Both antibodies were expressed in HEK293 cells and showed comparable glycosylation patterns. (A) Binding of LC06 (green) and CrossMab\textsuperscript{CH1-CL} (red) to immobilized FcγRIIIa-His (V158). (B) Inhibition of receptor binding by free FcγRIIIa-His. Dissociation constants (KD) were 109 nM for LC06 and 115 nM for the Ang-2–VEGF CrossMab\textsuperscript{CH1-CL}. Colors are as in A.

Fig. S10. Comparison of the side-product profile of the quadroma, KiH, and CrossMab technologies.