Simultaneous targeting of two ligand-binding sites on VEGFR2 using biparatopic Affibody molecules results in dramatically improved affinity

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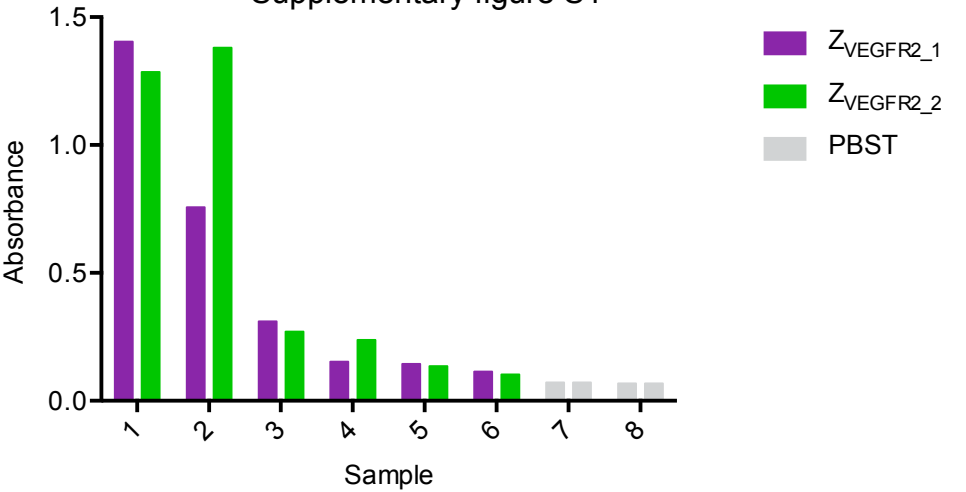
Supplementary Fig. S1-S12

Supplementary Table S1-S2

Supplementary Methods

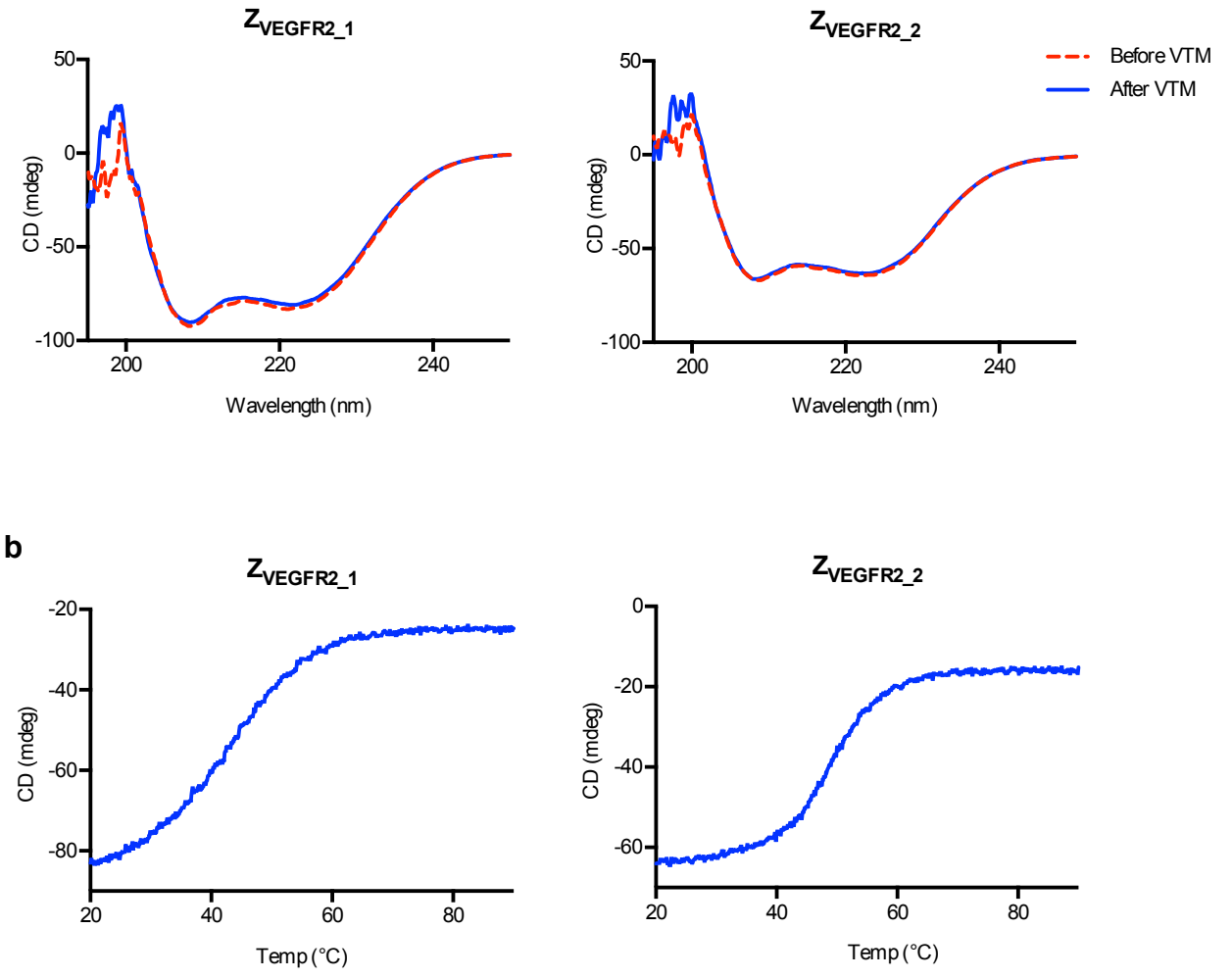
Supplementary figure S1



Sample	Treatment
1	1.8 nM mVEGFR2/Fc
2	1.8 nM hVEGFR2/Fc
3	1.8 nM mVEGFR2/Fc + 27 nM hVEGF-A
4	1.8 nM hVEGFR2/Fc + 27 nM hVEGF-A
5	20 nM hVEGFR1/Fc
6	20 nM hVEGFR3/Fc
7	PBST + 1.8 nM mVEGFR2/Fc
8	PBST + 1.8 nM hVEGFR2/Fc

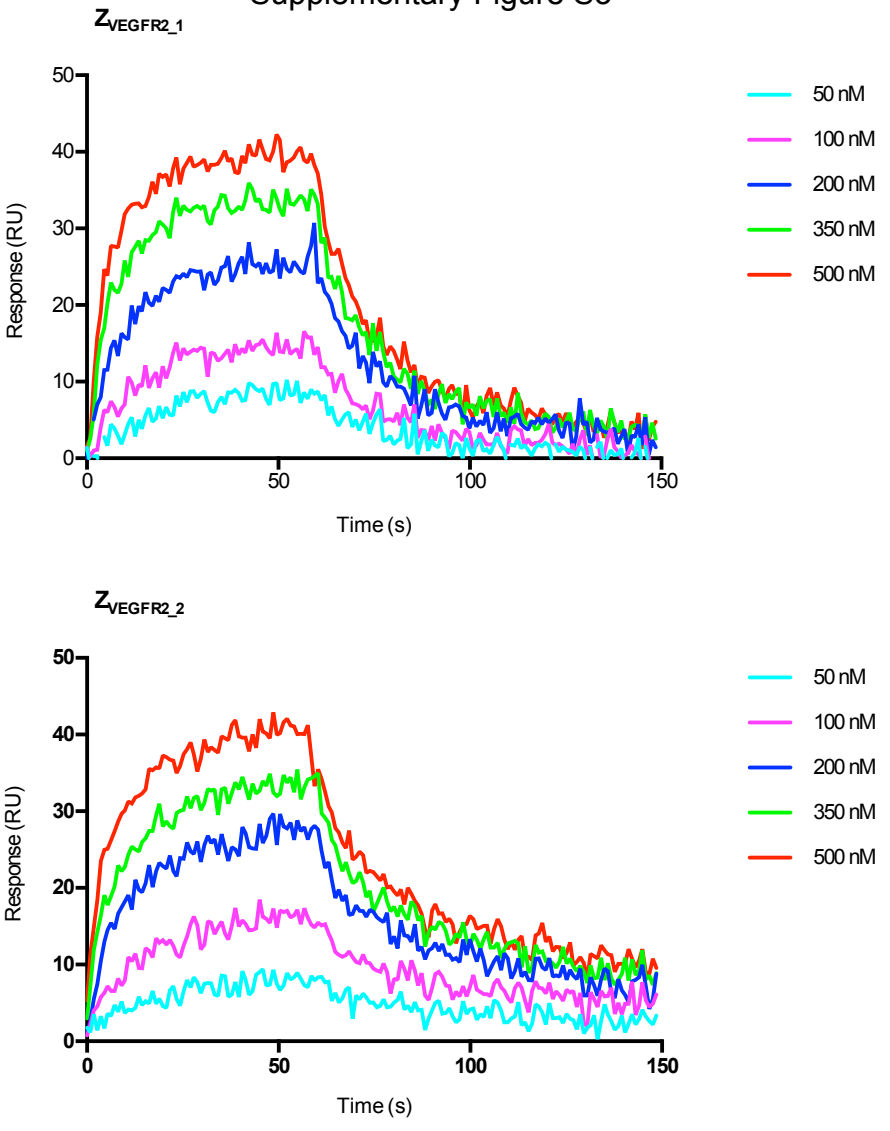
ELISA-based specificity and epitope analysis. Human and murine VEGFR2 both bind to Z_{VEGFR2_1} and Z_{VEGFR2_2}, but human VEGFR1 or VEGFR3 do not. Pre-incubation with human VEGF-A resulted in a decreased binding signal for both Affibody molecules.

Supplementary Figure S2



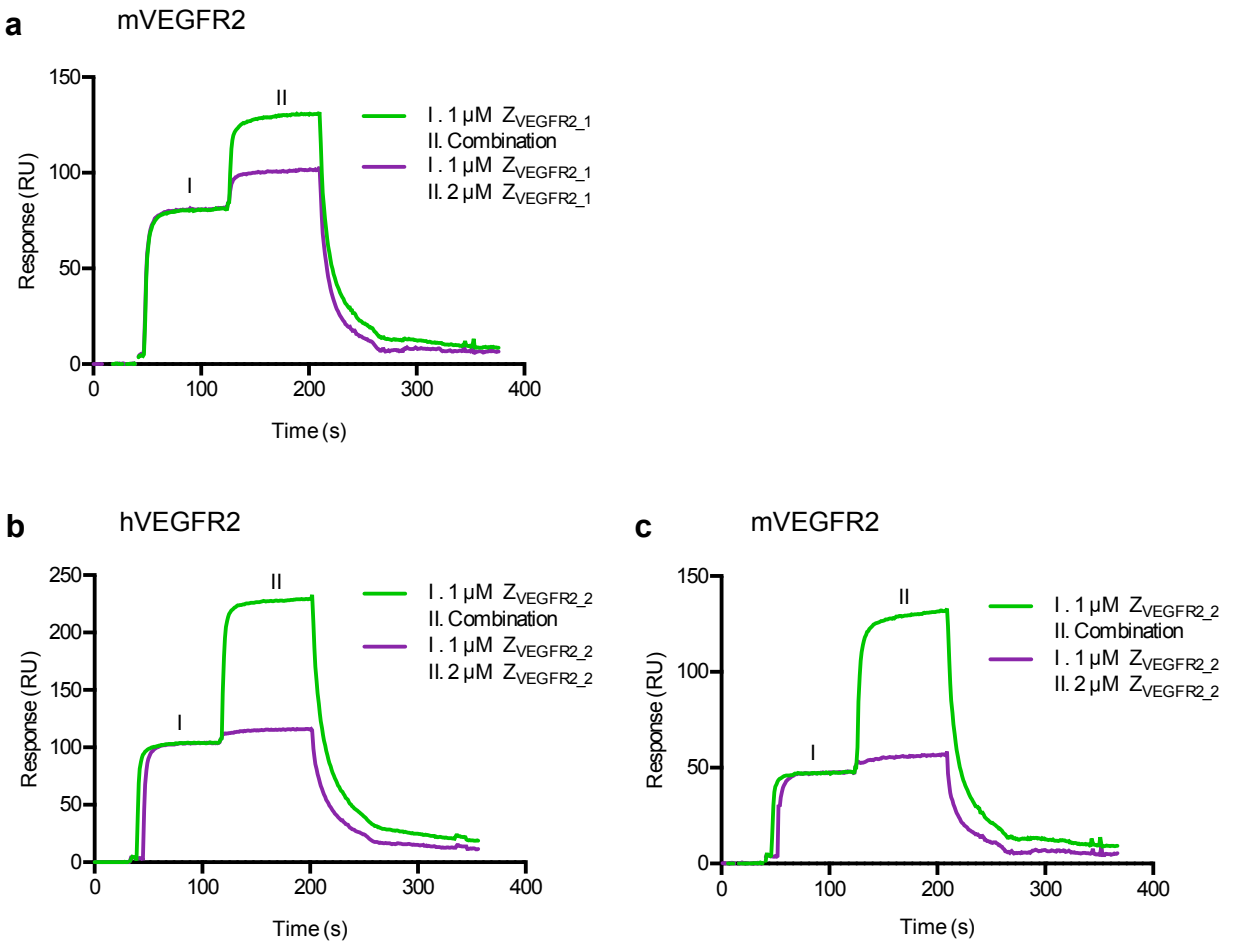
Circular dichroism (CD) analysis of Z_{VEGFR2_1} and Z_{VEGFR2_2}. a CD spectra of Z_{VEGFR2_1} and Z_{VEGFR2_2} at wavelengths ranging from 250 to 195 nm at 20 °C before and after the variable temperature measurement (VTM). The spectra recorded before and after VTM overlap well for both variants. **b** Variable temperature measurement (VTM) spectra obtained at 221 nm while heating Z_{VEGFR2_1} and Z_{VEGFR2_2} from 20 to 91 °C.

Supplementary Figure S3



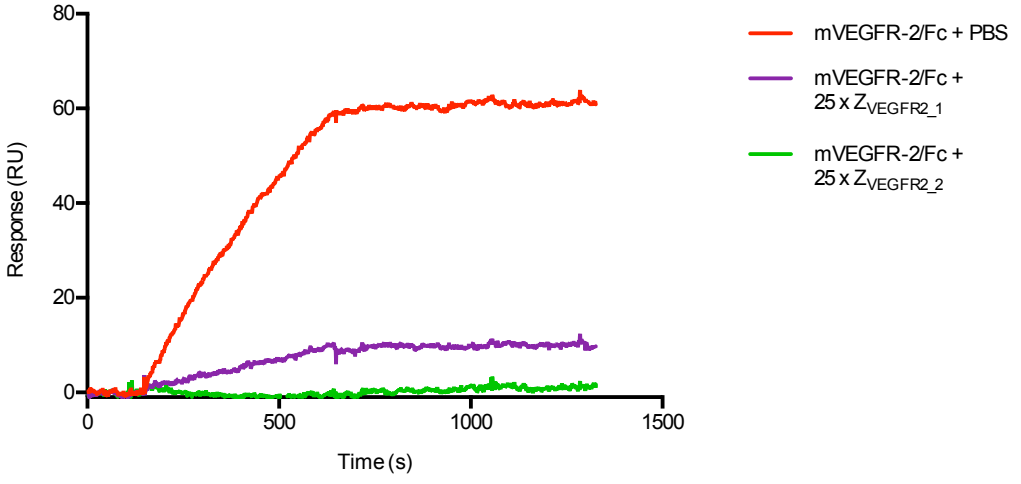
SPR analysis of Z_{VEGFR2_1} and Z_{VEGFR2_2} binding to murine VEGFR2. Representative sensorgrams from SPR analysis. Affibody molecules were injected at concentrations ranging from 50 nM to 500 nM. Data is double referenced by subtraction of simultaneous responses from reference surface and a buffer injection. The experiment was performed in duplicates.

Supplementary Figure S4



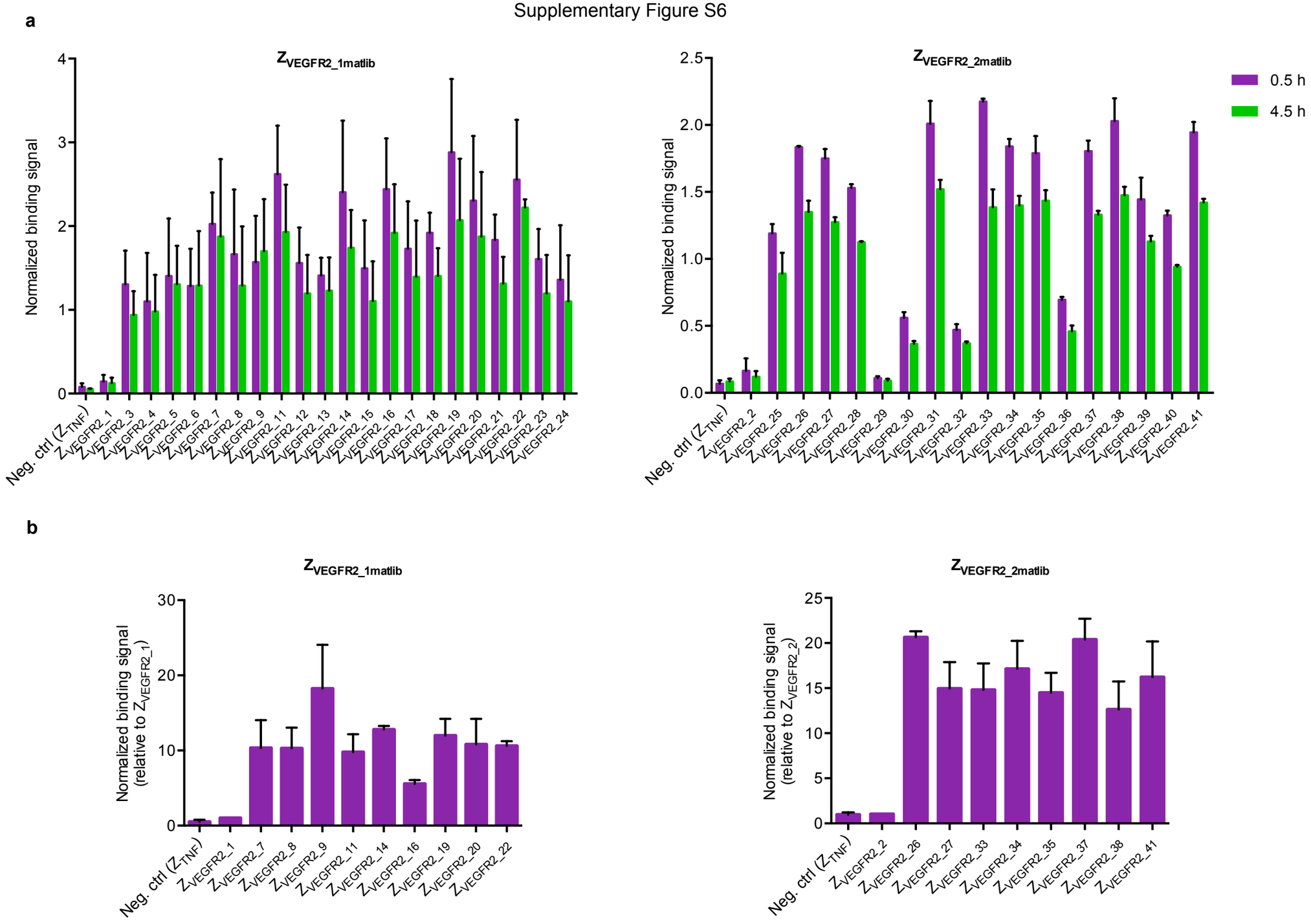
SPR-based competition assay. a Sensorgrams were obtained from a double injection, where 1 µM of Z_{VEGFR2_1} was injected (I) was immediately followed by a second injection (II) of either a combination of 1 µM of Z_{VEGFR2_1} and 1 µM of Z_{VEGFR2_2}, or 2 µM of Z_{VEGFR2_1}, over immobilized murine VEGFR2. **b** Sensorgrams were obtained from a double injection, where 1 µM of Z_{VEGFR2_2} was injected (I) was immediately followed by a second injection (II) of either a combination of 1 µM of Z_{VEGFR2_2} and 1 µM of Z_{VEGFR2_1}, or 2 µM of Z_{VEGFR2_2}, over immobilized human VEGFR2. **c** Sensorgrams were obtained from a double injection, where 1 µM of Z_{VEGFR2_2} was injected (I) was immediately followed by a second injection (II) of either a combination of 1 µM of Z_{VEGFR2_2} and 1 µM of Z_{VEGFR2_1}, or 2 µM of Z_{VEGFR2_2}, over immobilized murine VEGFR2. Data is referenced by subtraction of simultaneous responses from reference surface. The experiment was performed in duplicates.

Supplementary Figure S5



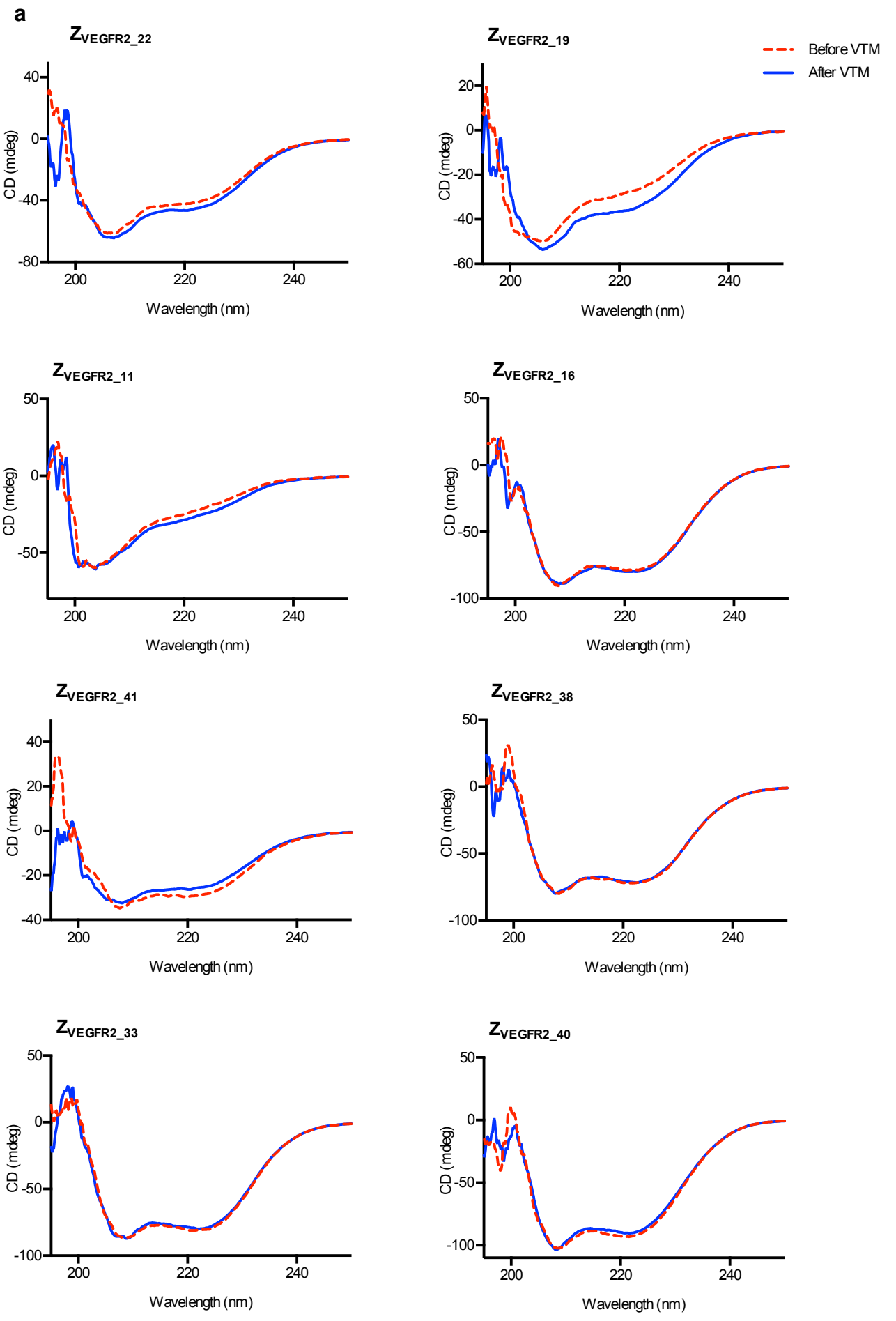
SPR-based analysis of murine VEGF blocking. Representative sensorgrams from SPR analysis. 40 nM of murine VEGFR2, which had been pre-incubated for 40 min with a 25 × molar excess of ZVEGFR2_1, ZVEGFR2_2 or PBS (control), was injected over a surface of immobilized murine VEGF-A. Data is referenced by subtraction of simultaneous responses from reference surface. The experiment was performed in duplicates.

Supplementary Figure S6



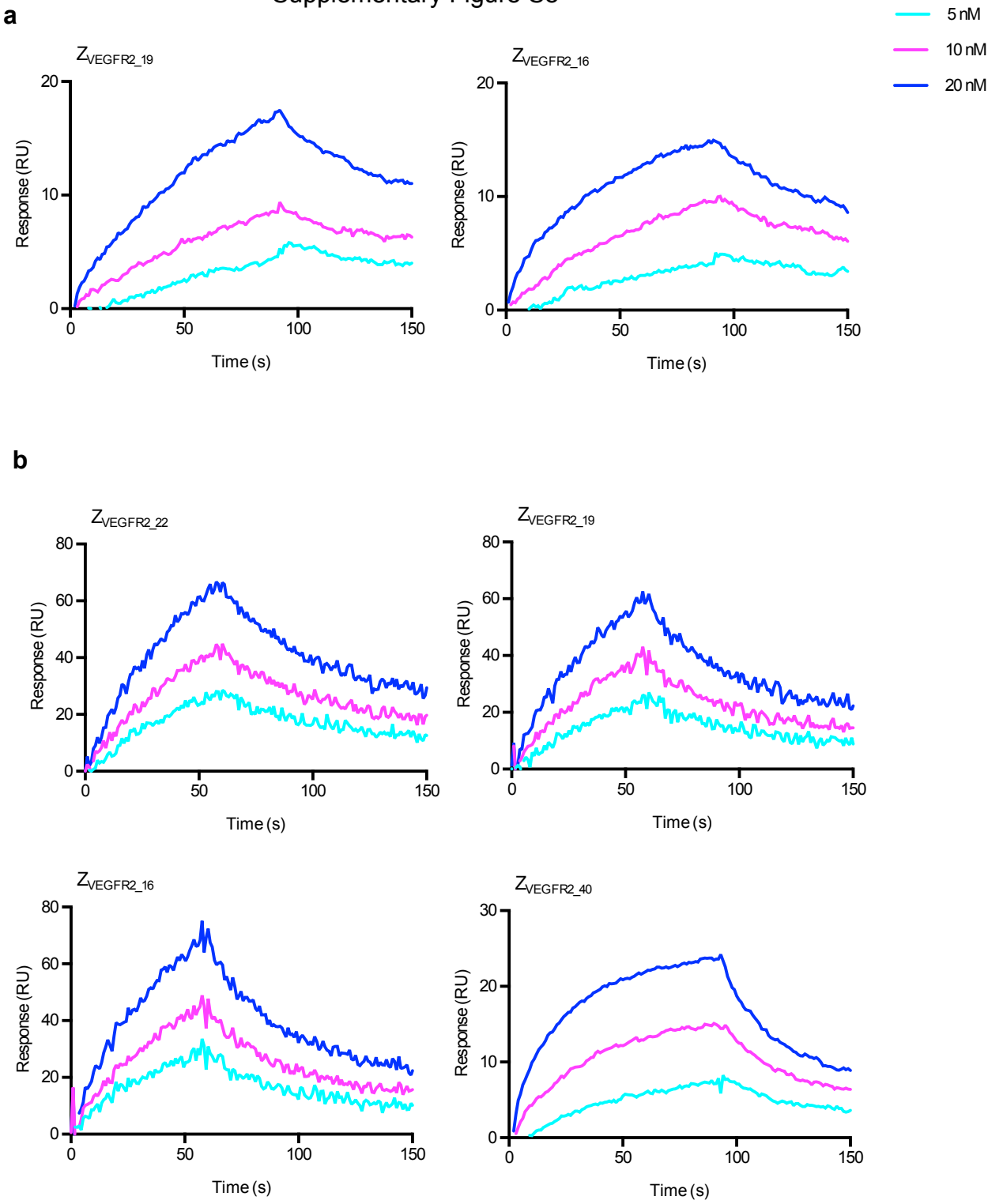
On-cell based ranking experiment for affinity-maturated Affibody molecules obtained from ZVEGFR2_1matlib and ZVEGFR2_2matlib, respectively. The normalized binding signal from the flow cytometric analysis of the binding cell-displayed Affibody clones to human VEGFR-2 (**a**) murine VEGFR2 (**b**) after an off-rate incubation step of 0.5 h (purple bar) or 4.5 h (green bar) is shown. The Affibody molecules are represented on the X axis and a ratio of FL-1 fluorescence intensity, corresponding to VEGFR-2 binding, and FL-6 fluorescence intensity, corresponding to surface expression level is represented on the Y axis. Error bars show the standard deviation of two independent experiments.

Supplementary Figure S7



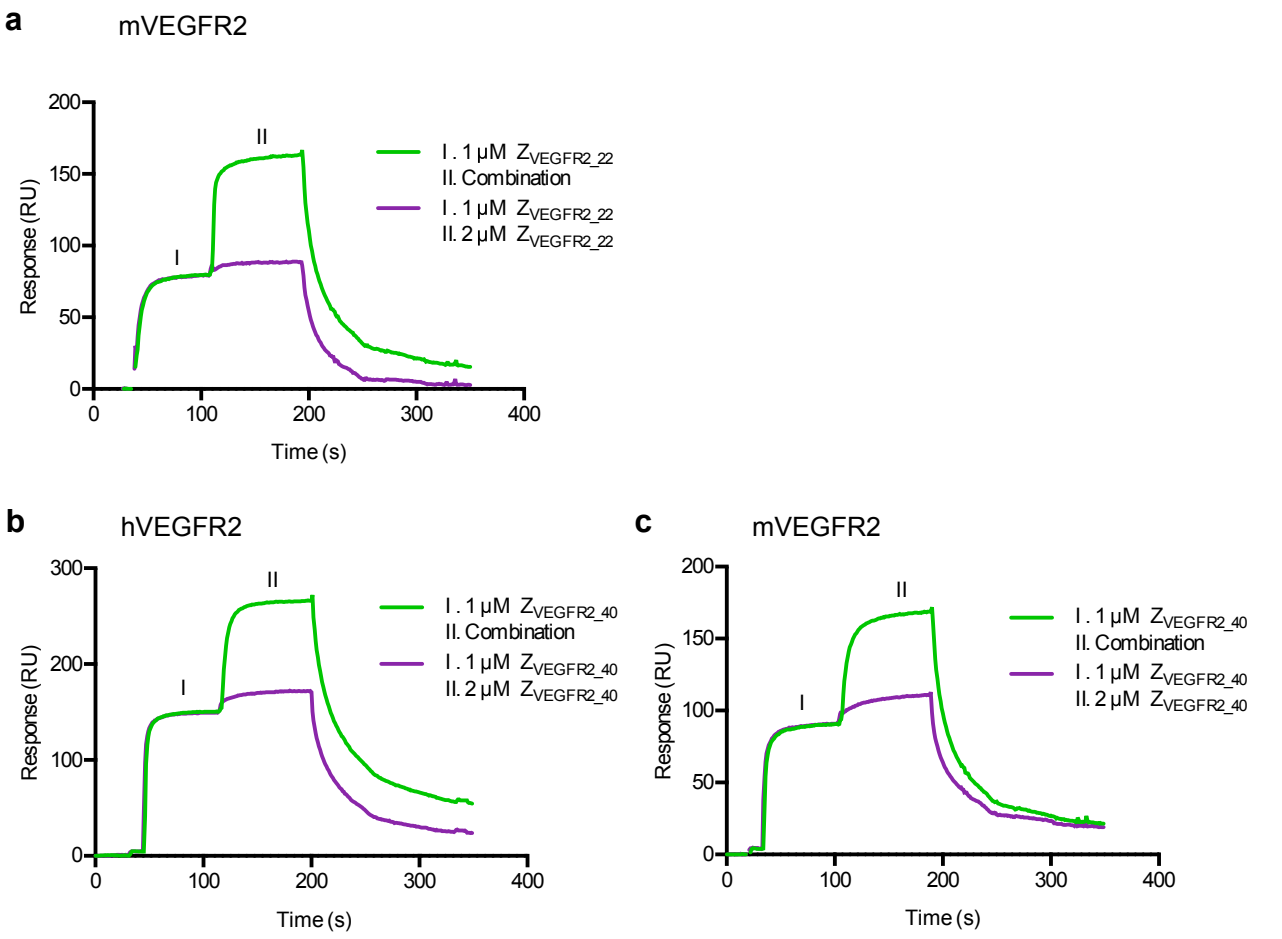
Circular dichroism (CD) analysis of Z_{VEGFR2_1} and Z_{VEGFR2_2}. a CD spectra of Z_{VEGFR2_1} and Z_{VEGFR2_2} at wavelengths ranging from 250 to 195 nm at 20 °C before and after the variable temperature measurement (VTM). The spectra recorded before and after VTM overlap well for both variants. **b** Variable temperature measurement (VTM) spectra obtained at 221 nm while heating Z_{VEGFR2_1} and Z_{VEGFR2_2} from 20 to 90 °C.

Supplementary Figure S8



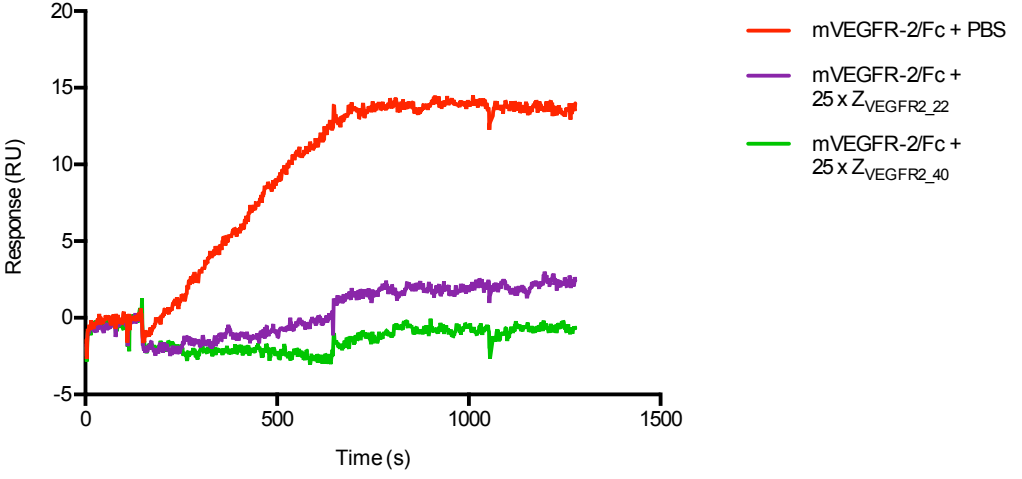
SPR analysis of the binding of affinity-maturated Affibody molecules to human and murine VEGFR2. Representative sensorgrams from SPR analysis. Affibody molecules were injected at concentrations of 5 nM, 10 nM and 20 nM over human VEGFR2 (**a**) or murine VEGFR2 (**b**). (Sensorgrams showing Z_{VEGFR2_22} and Z_{VEGFR2_40} binding to human VEGFR2 are shown in the main article). Data is referenced by subtraction of simultaneous responses from reference surface. The experiment was performed in duplicates.

Supplementary Figure S9



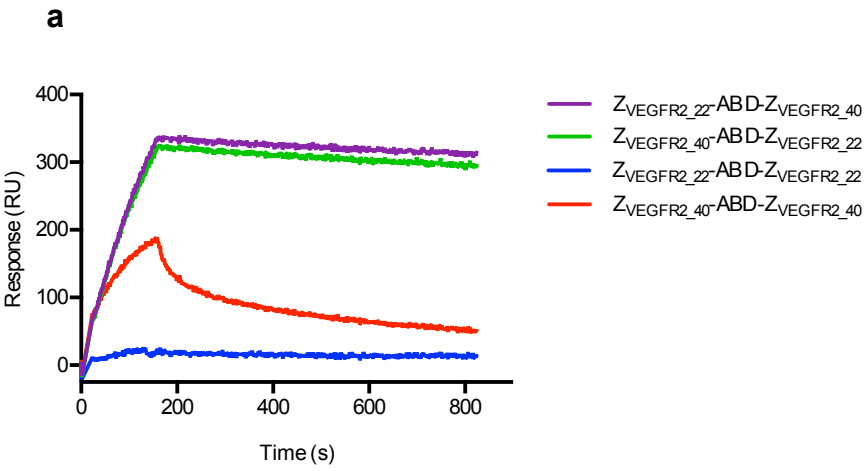
SPR-based competition assay. Representative sensorgrams from SPR analysis. **a** Sensorgrams were obtained from a double injection, where 1 µM of Z_{VEGFR2_22} was injected (I) was immediately followed by a second injection (II) of either a combination of 1 µM of Z_{VEGFR2_22} and 1 µM of Z_{VEGFR2_40}, or 2 µM of Z_{VEGFR2_22}, over immobilized murine VEGFR2. **b** Sensorgrams were obtained from a double injection, where 1 µM of Z_{VEGFR2_40} was injected (I) was immediately followed by a second injection (II) of either a combination of 1 µM of Z_{VEGFR2_40} and 1 µM of Z_{VEGFR2_22}, or 2 µM of Z_{VEGFR2_40}, over immobilized human VEGFR2. **c** Sensorgrams were obtained from a double injection, where 1 µM of Z_{VEGFR2_40} was injected (I) was immediately followed by a second injection (II) of either a combination of 1 µM of Z_{VEGFR2_40} and 1 µM of Z_{VEGFR2_22}, or 2 µM of Z_{VEGFR2_40}, over immobilized murine VEGFR2. Data is referenced by subtraction of simultaneous responses from reference surface. The experiment was performed in duplicates.

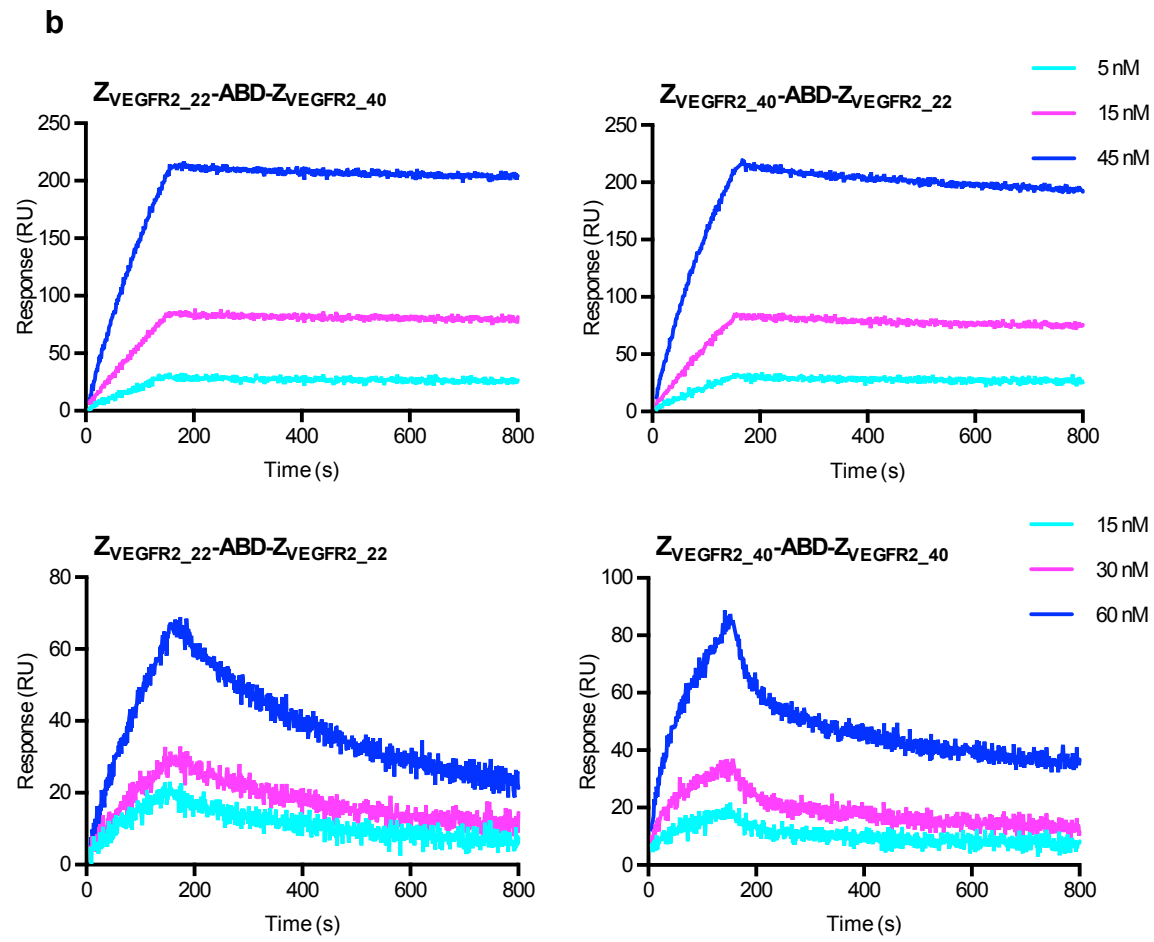
Supplementary Figure S10

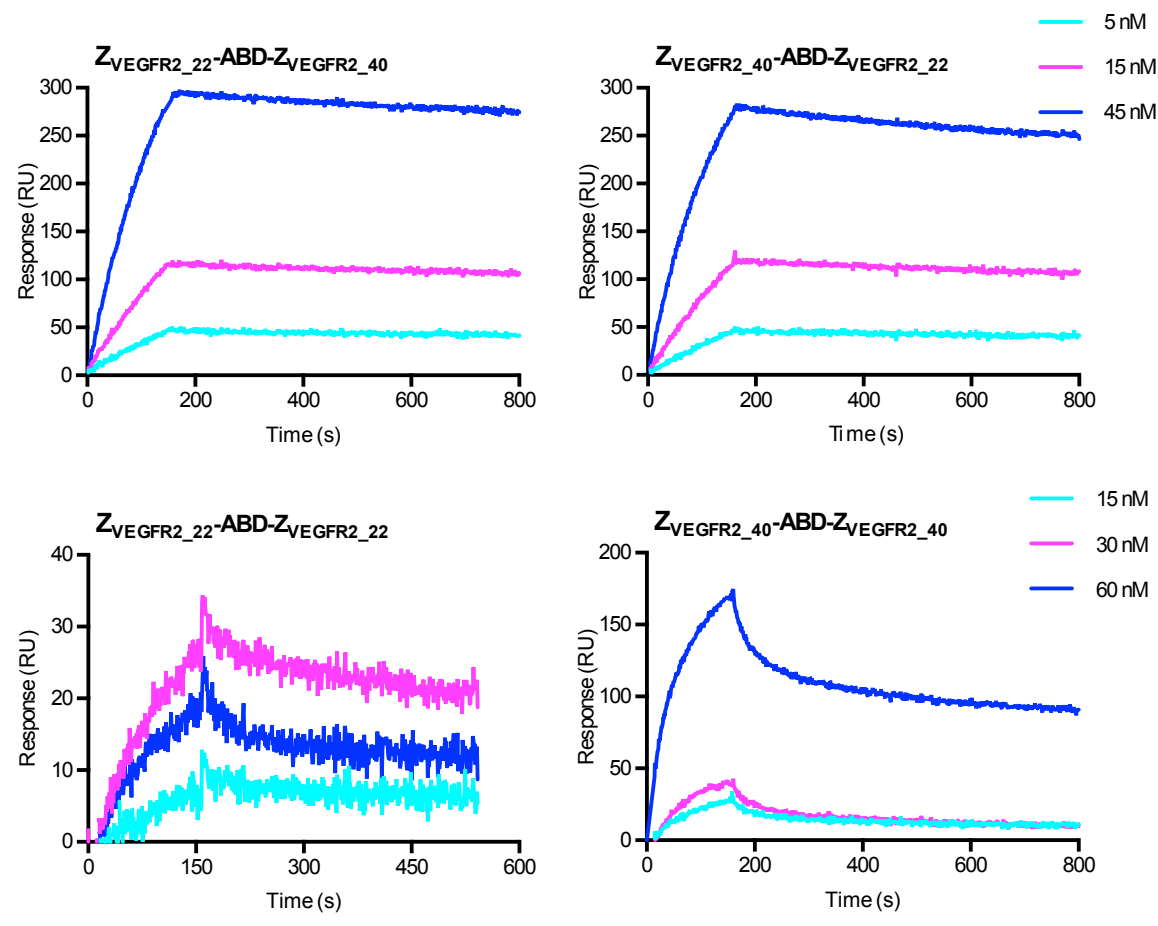


SPR-based analysis of VEGF blocking. Representative sensorgrams from SPR analysis. 40 nM of murine VEGFR2, which had been pre-incubated for 40 min with a 25 × molar excess of ZVEGFR2_22, ZVEGFR2_40 or PBS (control), was injected over a surface of immobilized murine VEGF-A. Data is referenced by subtraction of simultaneous responses from reference surface. The experiment was performed in duplicates.

Supplementary Figure S11

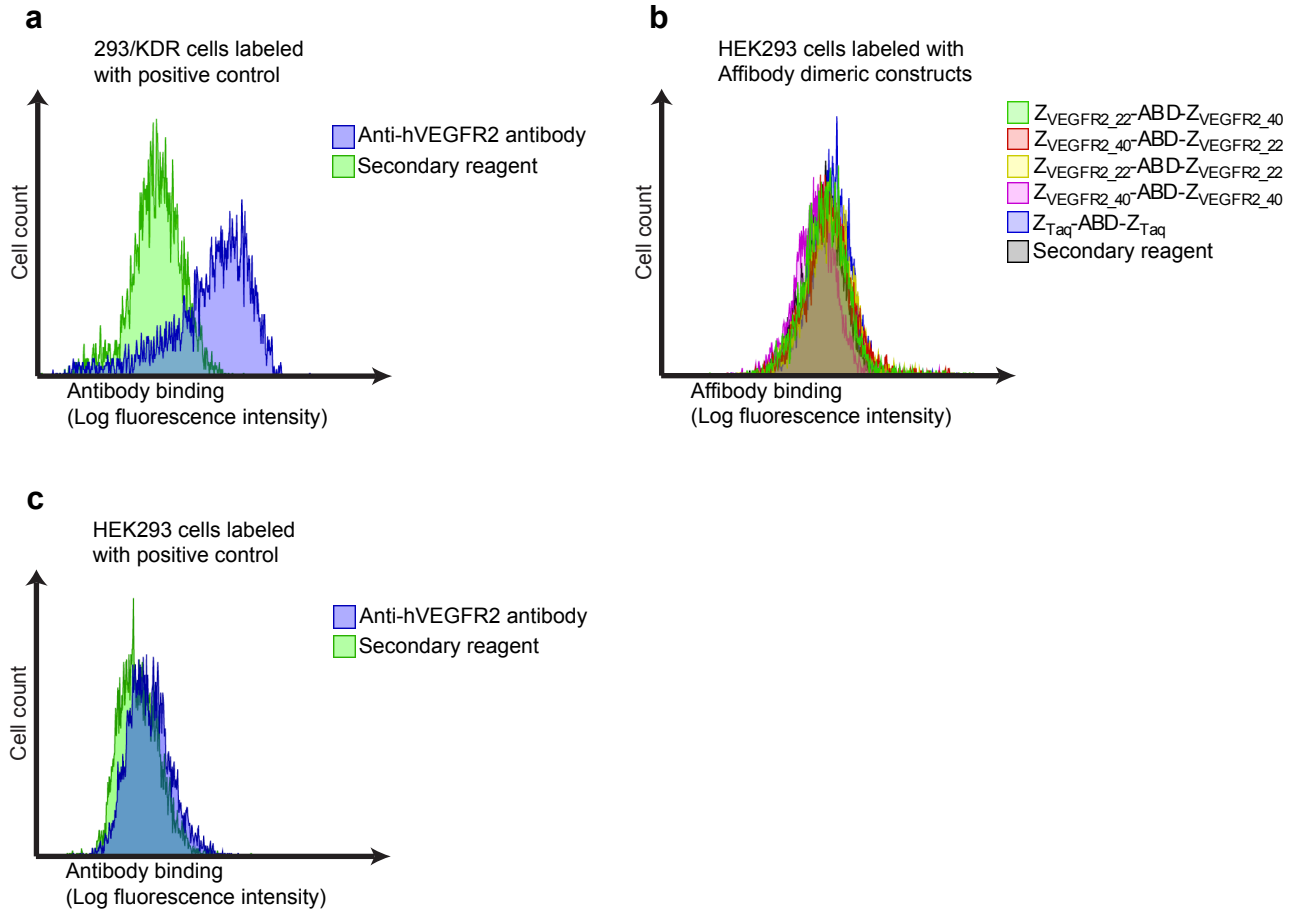






SPR analysis of dimeric VEGFR-2 binding Z variants. a SPR sensorgram showing the injection of 40 nM monomeric mVEGFR2 over each of the four dimeric Affibody molecules. **b and c** 200 nM of Z_{VEGFR2_22}-ABD-Z_{VEGFR2_40}, Z_{VEGFR2_40}-ABD-Z_{VEGFR2_22}, Z_{VEGFR2_22}-ABD-Z_{VEGFR2_22} or Z_{VEGFR2_40}-ABD-Z_{VEGFR2_40} was injected over a surface of immobilized HSA followed by a second injection of 5 nM, 10 nM and 45 nM (for the heterodimers) of human VEGFR2 (**b**) or murine VEGFR-2 (**C**), or 15 nM, 30 nM and 60 nM (for the homodimers) of human VEGFR2 (**B**) or murine VEGFR-2 (**C**). Data is referenced by subtraction of simultaneous responses from reference surface. The experiment was performed in duplicates.

Supplementary Figure S12



Flow-cytometric analysis of target specificity of dimeric Affibody constructs as well as verification of VEGFR2-expression using a VEGFR2-specific mAb. Binding of the Affibody constructs is monitored by the binding of fluorescently labeled HSA to the ABD tag, and binding of the positive control anti-hVEGFR2 antibody is monitored by the binding of a fluorescently labeled secondary antibody. **a** VEGFR2-expressing 293/KDR cells labeled with positive control anti-hVEGFR2 antibody. A shift in fluorescence intensity was observed, confirming that VEGFR2 was expressed on the 293/KDR cells. **b** Non-VEGFR2-expressing HEK293 cells labeled with dimeric Affibody constructs. No binding was detected for any of the constructs. **c** Non-VEGFR2-expressing HEK293 cells labeled with positive control anti-hVEGFR2 antibody. No binding was observed, confirming that VEGFR2 was not expressed on the HEK293 cells. The experiment was performed in duplicates.

Supplementary Table S1. Overview of the phage display selection using human VEGFR2/Fc as target.

Cycle	Selection track	Phage stock from library or selection track	Pre-selection	Target conc. (nM)	Number of ~1 min washes	Number of ~10 min washes	Constant high amount of phage to selection
1	1	Library	Yes	180	2	-	-
2	1-1	1	Yes	90	4	-	-
2	1-2	1	Yes	60	4	-	-
2	1-3	1	Yes	90	6	-	Yes
2	1-4	1	Yes	60	6	-	Yes
3	1-1-1	1-1	Yes	45	5	-	-
3	1-2-1	1-2	Yes	30	7	-	-
3	1-3-1	1-3	Yes	45	8	2	Yes
3	1-4-1	1-4	Yes	30	10	2	Yes
4	1-1-1-1	1-1-1	-	30	8	-	-
4	1-1-1-2	1-1-1	-	15	8	-	-
4	1-2-1-1	1-2-1	-	15	10	-	-
4	1-3-1-1	1-3-1	-	30	10	3	Yes
4	1-3-1-2	1-3-1	-	15	10	3	Yes
4	1-4-1-1	1-4-1	-	15	14	3	Yes

Overview of the phage display selection using human VEGFR2/Fc as target.

Supplementary Table S2. Sequences of VEGFR2-binding Affibody molecules.

Clone	Sequence
Z_{VEGFR2_1}	VDAKYAKE**NLK**A**SN**EI**AN**LPNLT**DK**Q**YI**AFI**Y**KL**L**DDPSQSSELLAEAKKLNDAQAPK
Z_{VEGFR2_2}	VDAKYAKE**FQS**A**DR**EI**RA**LPNLT**HG**Q**W**YAFI**V**KL**Y**DDPSQSSELLAEAKKLNDAQAPK

Randomized positions are indicated in bold.

Supplementary Methods

Selection of Affibody molecules using phage display

A combinatorial library of Affibody molecule variants displayed on bacteriophage was subjected to four rounds of selection using human VEGFR2/Fc (hVEGFR2/Fc, R&D Systems, Minneapolis, MN, USA), essentially as described by Grönwall *et al*. [1]. The library variants were fused to a gene encoding a *Taq* polymerase binding Affibody molecule Z_{Taq} ($Z_{Taq\ S1\text{-}1}$, [2]). The selection buffer consisted of phosphate-buffered saline (10 mM phosphate, 137 mM NaCl, 2.68 mM KCl, pH 7.4, PBS) supplemented with 0.1% Tween 20 (PBST 0.1) and 3% BSA (Sigma-Aldrich Company Ltd, Dorset, UK). PBST 0.1 was used as washing buffer. Pre-selections were performed in cycles 1-3 by incubation of phage stock with biotin-conjugated human IgG, Fc (Jackson Immuno Research Laboratories, West Grove, PA, USA) immobilized on streptavidin coated paramagnetic beads (SA-beads, Invitrogen, Carlsbad, CA) and with biotin-anti IgG Affibody molecule (Affibody AB, Solna, Sweden) immobilized on SA-beads (anti IgG-Z conjugated SA-beads). The selection was performed in solution in four cycles, one track in the first cycle and subsequently divided into six tracks in the last cycle. An overview of the selection strategy, describing an increased stringency in subsequent cycles obtained using a lowered target concentration and an increased number of washes, is shown in Online Resource 1.

Phage particles bound to Fc-fused target were captured on anti IgG-Z conjugated SA-beads before washing and phage particles were finally eluted at pH 2.2 (50 mM glycine-HCl). In half of the tracks in cycle 2-4, (see Online Resource 1), a constant high amount of phage particles were used for each round and in the rest of the tracks the number of phage particles used in the selection were about 2000 times the number of eluted phage particles in the previous cycle.

Soluble protein production and ELISA screening for VEGFR2 binding

The Affibody molecule variants were produced by inoculating single colonies of *Escherichia coli* containing phagemids from the selections into culture medium

supplemented with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG, Appollo Scientific Ltd). Bacteria containing the Affibody molecules expressed as fusion proteins with Z_{Taq} [2] were subjected to repeated freeze-thawing and pelleted by centrifugation to extract the proteins in the periplasmic fractions. Binding to hVEGFR2/Fc and murine VEGFR2/Fc (mVEGFR2/Fc) was investigated in a standard sandwich ELISA. In brief, half-area 96-well plates were coated with a polyclonal goat anti-Z IgG (Affibody AB) at a concentration of 4 μg/ml in PBSC (PBS supplemented with 0.5% casein). After washing of plates in water, the soluble Affibody-Z_{Taq} fusion variants in periplasmic samples were added to the wells. As a blank control, PBS supplemented with 0.05% Tween 20 (PBST 0.05) was added instead of the periplasmic sample. Plates were washed 4 times in PBST 0.05 followed by addition of hVEGFR2/Fc or mVEGFR2/Fc diluted in PBSC. The targets concentrations used for screening were 3.6 nM or 7.2 nM hVEGFR2/Fc and 0.9 nM or 9 nM mVEGFR2/Fc. After washing as described above, the plates were developed by addition of a horse-radish peroxidase-conjugated goat anti-human IgG (anti-human IgG-HRP, Southern Biotechnology Associates, Birmingham, AL) diluted 1:10,000 in PBSC followed by addition of TMB (Thermo Scientific, Rockford, USA). Absorbance was measured at 450 nm using a microplate reader (Victor^3, Perkin-Elmer Instruments and Life Sciences, Rodgau-Juegesheim, Germany). The DNA sequences of clones shown to be positive in ELISA were determined via PCR amplification of insert sequences and using an ABI PRISM® 3130xl Genetic Analyzer instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

ELISA-based specificity and epitope analysis

The same ELISA setup as described for the screening was used to investigate the specificity of two selected Affibody molecule variants as well as if these Affibody molecules could possibly interfere with the binding of VEGF-A to hVEGFR2. Selectivity to human and murine VEGFR2/Fc as well as human VEGFR1/Fc (hVEGFR1/Fc, R&D Systems) and human VEGFR3/Fc (hVEGFR3/Fc, R&D systems) were assayed at target concentrations of 1.8 nM hVEGFR2/Fc, 1.8 nM mVEGFR2/Fc, 20 nM hVEGFR1/Fc or hVEGFR3/Fc. To investigate if binding of

VEGFR2 to the Affibody molecules could be blocked by hVEGF, hVEGFR2/Fc or mVEGFR2/Fc at a concentration of 1.8 nM was pre-mixed with a approximately a 15-fold molar excess (27 nM) of human VEGF (R&D Systems) before addition to the ELISA plates. Both ELISA assays were developed as above and the plates were measured at 450 nm using a microplate reader (Victor^3, Perkin-Elmer).

Flow-cytometric analysis of alanine scan mutants

A single colony of each alanine (or valine) mutant was inoculated to 3 ml Tryptic Soy Broth medium supplemented with yeast extract (TSB+YE; 30 g/l; Merck, Darmstadt, Germany) supplemented with 10 μg/ml chloramphenicol (Sigma-Aldrich) and incubated at 37°C with shaking at 150 rpm for 18 h. 10^6 cells were pelleted by centrifugation (15000 *g*, 6 min, 4°C) and washed with PBS supplemented with 0.1% Pluronic F108 N1 Surfactant (PBSP; pH 7.4; BASF Corporation, Mount Olive, NJ, USA), re-suspended in 75 μl 100 nM human or murine VEGFR2/Fc (R&D Systems), and incubated at room temperature with gentle mixing for 50 min. After washing with ice-cold PBSP, cells were re-suspended in 100 μl of Alexa Fluor 488 Goat Anti-Human IgG (H+L) Antibody (Invitrogen) (1:1000 dilution) and 150 nM HSA (Human serum albumin; Sigma-Aldrich) conjugated to Alexa Fluor 647 (Invitrogen), and incubated on ice in the dark for 45 min. Cells were washed and re-suspended in ice-cold PBSP for flow-cytometric analysis. The experiment was performed in duplicates on different days using freshly prepared reagents.

SPR-based competition assays using Z_{VEGFR2_22} and Z_{VEGFR2_40}

Competition assays were performed using a BIAcore^{TM} 3000 instrument (GE Healthcare). In all experiments, PBST 0.1 was used as a running buffer and 10 mM HCl for regeneration. All experiments were performed in duplicates using freshly prepared reagents. Human or murine VEGFR2/Fc (R&D Systems) was immobilized on a CM-5 sensor chip (GE Healthcare). For detection of simultaneous binding, a double injection was performed, where 1 μM Z_{VEGFR2_22} was first injected, directly followed by an injection of either a combination of 1 μM of Z_{VEGFR2_40} and Z_{VEGFR2_22} or 2 μM Z_{VEGFR2_22}. In a separate experiment, the Affibody molecules were injected in the opposite order, i.e. 1 μM Z_{VEGFR2_40} was injected first, followed by a second

injection of either a combination of 1 μM Z_{VEGFR2_22} and 1 μM Z_{VEGFR2_40} or 2 μM Z_{VEGFR2_40}.

For analysis of competition with VEGF-A, VEGF-A (R&D Systems) was immobilized on a sensor chip surface. 40 nM of human or murine VEGFR2/Fc (R&D Systems), which had been pre-incubated with a 25-fold molar excess of Z_{VEGFR2_22} or Z_{VEGFR2_40} (or PBS, as a control) for 40 min, was injected over the surface.

1 Gronwall, C. *et al.* Selection and characterization of Affibody ligands binding to Alzheimer amyloid beta peptides. *Journal of Biotechnology* **128**, 162-183, doi:10.1016/j.jbiotec.2006.09.013 (2007).

2 Gunneriusson, E., Nord, K., Uhlen, M. & Nygren, P. Affinity maturation of a Taq DNA polymerase specific affibody by helix shuffling. *Protein Eng* **12**, 873-878 (1999).