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Variants of the Antibody Herceptin That Interact with HER2 and VEGF at the Antigen Binding Site

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The interface between antibody and antigen is often depicted as a lock and key, suggesting that an antibody surface can accommodate only one antigen. Here, we describe an antibody with an antigen binding site that binds two distinct proteins with high affinity. We isolated a variant of Herceptin, a therapeutic monoclonal antibody that binds the human epidermal growth factor receptor 2 (HER2), on the basis of its ability to simultaneously interact with vascular endothelial growth factor (VEGF). Crystallographic and mutagenesis studies revealed that distinct amino acids of this antibody, called bH1, engage HER2 and VEGF energetically, but there is extensive overlap between the antibody surface areas contacting the two antigens. An affinity-improved version of bH1 inhibits both HER2- and VEGF-mediated cell proliferation in vitro and tumor progression in mouse models. Such “two-in-one” antibodies challenge the monoclonal antibody paradigm of one binding site, one antigen. They could also provide new opportunities for antibody-based therapy.

The binding of antibodies to specific single antigens has prompted their use for numerous targeted therapies (1). However, the notion of an antibody that recognizes more than one antigen is intriguing. It has been suggested that multi-specificity may evolve and play a role in the highly efficient antibody repertoire for immune protection with one antibody performing more than one task (2, 3). However, the few examples of such multi-specificity are lim-

ited to antibodies that bind small haptens (4), and a strategy to generate a single antigen binding fragment (Fab) capable of recognizing two unrelated proteins has not yet been reported.

We set out to explore whether dual specific antibodies can be derived from a monospecific antibody with the following approach: A repertoire of Herceptin (Genentech, South San Francisco, CA) antibody variants with mutations in the light chain (LC) complementarity determining regions

(CDRs) were generated, and Fabs that can bind a new protein antigen while maintaining human epidermal growth factor receptor 2 (HER2) binding were identified. The approach is based on the understanding that modifications of the LC sequence can modulate the binding specificity of antibodies (5, 6). In addition, many antibodies, with Herceptin as a prime example (7, 8), bind the antigens by using mainly the heavy chain (HC) CDRs, suggesting that mutations in the LC CDRs might allow preservation of the original antigen binding specificity.

The framework regions of Herceptin variable domains (V_H and V_L, the variable domain of HC and LC, respectively) belong to subtypes that are prevalent in the human antibody repertoire (V_H3, V_Lkappa1). Thus, mutations within the antigen binding site of Herceptin that confer a second specificity may indicate a potential for a dual specific antibody to evolve from the natural repertoire. As Herceptin is a validated therapeutic for breast cancers that overexpress HER2 (9), recruitment of a second binding specificity to Herceptin may add to it a distinct pharmacological activity.

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Table 1. The representative antibodies from the LC library of Herceptin. Mutations from Herceptin are shown in *italics*. Dashes indicate positions where no residue is present. Antigen binding affinity (K_d) was determined by surface plasmon resonance using Fab.

NB indicates that no binding is detected. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

	CDR-L1										CDR-L2				CDR-L3						Specificity		Affinity (nM)	
	28	29	30	30a	30b	30c	30d	31	32	33	50	51	52	53	91	92	93	93a	93b	94	Antigen1	Antigen2	K _d 1	K _d 2
Herceptin	D	V	N	—	—	—	—	T	A	V	S	A	S	F	H	Y	T	—	—	T	HER2	NB	0.1	NB
	N	V	W	—	—	—	—	D	W	V	P	A	S	S	G	W	Y	I	—	A	VEGF	NB	15	NB
bH1	D	I	P	R	S	I	S	G	Y	V	W	G	S	Y	H	Y	T	—	—	T	HER2	VEGF	26	300
bH3	D	I	G	L	—	—	—	G	S	V	W	A	S	Y	H	Y	T	—	—	T	HER2	VEGF	8	19,000
bH4	D	I	R	S	—	—	—	G	S	V	W	G	S	Y	H	Y	T	—	—	T	HER2	VEGF	11	3,500
4-1	D	I	W	N	R	—	—	R	A	L	E	G	S	S	G	G	S	Y	S	S	DR5	NB	120*	NB
4-5	N	V	G	—	—	—	—	R	P	V	G	G	S	S	Y	G	S	F	G	T	DR5	NB	150*	NB
bD1	N	V	S	—	—	—	—	K	H	V	W	G	S	Y	S	Y	S	—	—	G	HER2	DR5	200*	14,000*
bD2	N	I	R	N	G	—	—	G	G	L	S	A	S	F	H	Y	T	—	—	T	HER2	DR5	100*	67,000*

*IgG was used to determine the K_d.

We constructed the repertoire of Herceptin LC variants by randomizing a subset of solvent exposed LC CDR positions to mimic the natural diversity in amino acid composition and length (tables S1 and S2) (10). Selection of the Herceptin variant library against vascular endothelial growth factor (VEGF), death receptor 5 (DR5), and the complement binding fragment of immunoglobulin G (IgG) (Fc) generated over one hundred specific clones containing 3 to 17 amino acid substitutions and/or insertions compared with

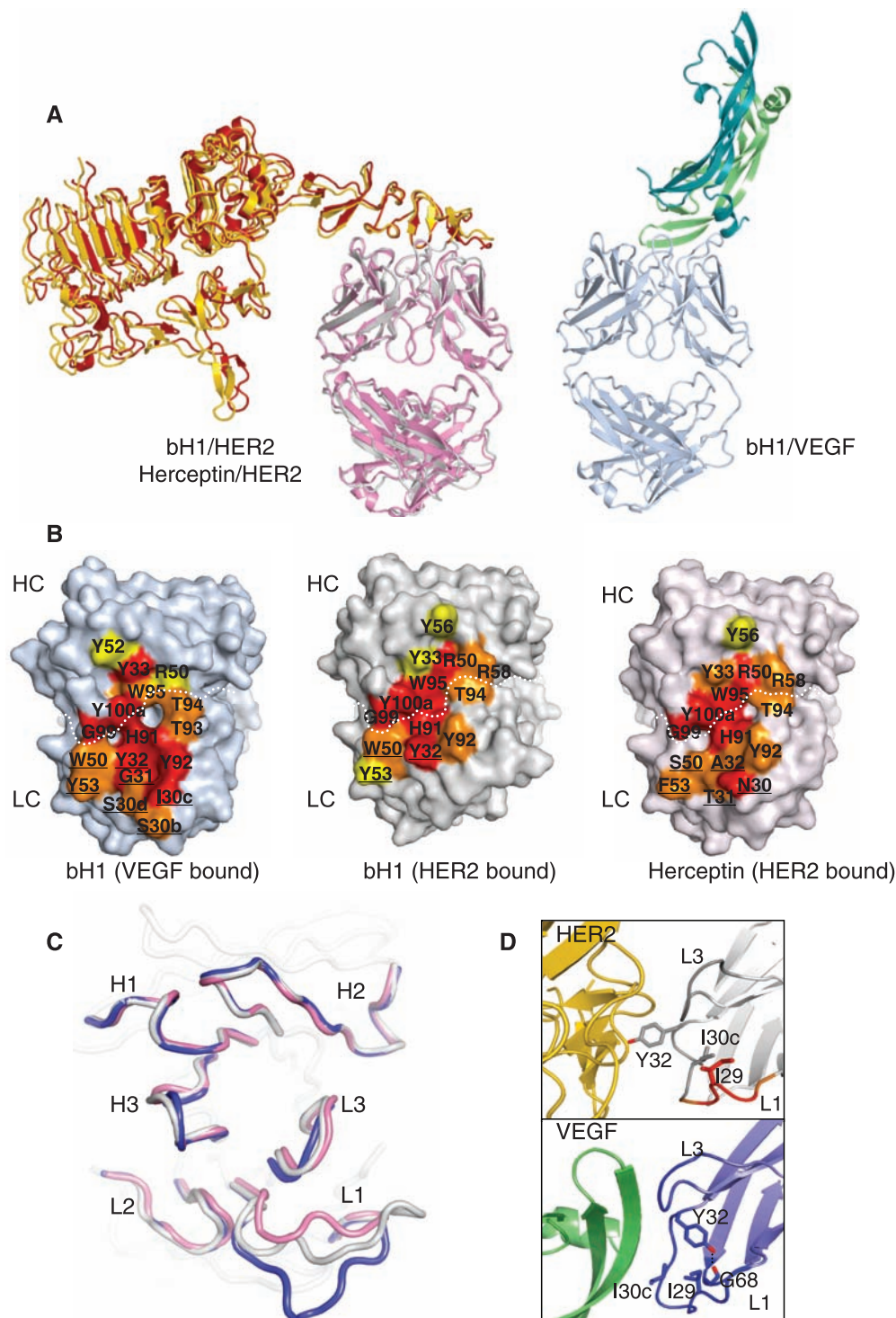
Herceptin (tables S3 and S4). Some of the clones lost binding affinity for HER2 and switched binding entirely to the new antigen, whereas others maintained the ability to bind HER2 and thus were dual specific.

Representative VEGF and DR5-binding clones were expressed as Fab and IgG proteins to assess their specificity and affinity (Table 1 and fig. S1). VEGF and DR5 are therapeutic targets of known structure. Equilibrium binding affinities (K_d) of the LC library-derived mono-

specific antibodies ranged from 15 to 150 nM (Table 1). The dual specific antibodies bound the new antigens (i.e., VEGF or DR5) with high nanomolar to micromolar affinity while preserving HER2 binding in the low nanomolar affinity range. The antibody bH1 exhibited the highest dual affinity for the two completely different protein antigens VEGF ($K_d = 300$ nM) and HER2 ($K_d = 26$ nM).

To understand the molecular basis of the dual specificity, we determined the crystal struc-

Fig. 1. The crystal structures of bH1 Fab bound to HER2 or VEGF. **(A)** The bH1 Fab (gray)/HER2 (gold) superimposed on to the Herceptin (pink)/HER2 (red) complex (left), and bH1 Fab (light blue)/VEGF (green, teal) complex (right). **(B)** Fab surface residues are colored according to the extent buried in the complex (red, >75%; orange, >50 to 75%; yellow, >25 to 50%). The underlined amino acids differ between bH1 and Herceptin. The white dotted line separates the LC and HC. **(C)** Superposition of the CDR loops of VEGF and HER2-bound bH1 (blue, gray) and HER2-bound Herceptin (pink) in the same orientation as in (B). **(D)** CDR-L1 regions of the two bH1 complexes shown in the same orientation. The residues with temperature factors higher than average are shown in red and orange. VEGF would clash with Tyr32 of bH1 in its HER2-bound conformation.



tures of the bH1 Fab bound to the extracellular domain of HER2 (residues 1 to 624) and to the VEGF receptor-binding domain (residue 8 to 109) at 2.9 and 2.6 Å resolution, respectively (Fig. 1A and table S5). The structure of the bH1/HER2 binding domains (bH1 Fv and domain IV of HER2) superimposes onto the Herceptin/HER2 complex with a root mean square deviation of 0.8 Å, showing that the Herceptin binding epitope on HER2 is retained (Fig. 1A) (7). The HER2 binding sites on bH1 and Herceptin differ only in the CDR-L1 and -L2 regions where the bH1 sequence differs from Herceptin (Fig. 1B).

In the structure of the bH1/VEGF complex, the Fab recognizes an epitope that overlaps with the binding sites of the VEGF receptors and other VEGF binding antibodies (11–13). Consistent with this observation, bH1 blocks the interaction between VEGF and its receptors (fig. S2).

Comparison of the bH1/VEGF and the bH1/HER2 complexes shows an extensive overlap of the VEGF and HER2 binding sites on bH1 (Fig. 1B and table S6); 11 out of the 13 bH1 residues that are in close contact with HER2 also make contact with VEGF. In the HER2 com-

plex, the LC and HC CDRs contribute approximately equal antigen contact area (53% and 47%, respectively), whereas in the VEGF complex, the LC CDRs constitute nearly 70% of the buried surface (fig. S3).

With the exception of CDR-L1, the CDR conformations of the bH1 Fab bound to VEGF are markedly similar to those observed in bH1 or Herceptin bound to HER2 (Fig. 1C). The capability of CDR-L1 to rearrange appears to be necessary for the dual specificity of bH1 (Fig. 1D). In the HER2 complex, CDR-L1 is minimally involved in antigen interaction, and part of the loop appears to be flexible, as evidenced by poorly defined electron density (Fig. 1D). In contrast, in the VEGF complex, this loop is well structured and forms extensive contacts with VEGF. Similar conformational flexibility of CDR-L1 has been reported to play a role in antigen recognition of natural antibodies (14, 15).

Next, we performed shotgun scanning mutagenesis studies to assess the energetic contributions of individual CDR residues to VEGF and HER2 binding (S11 and S12 in the supporting online material) (10). We determined the change

in free energy ($\Delta\Delta G$) as a result of mutation to alanine (alanine scan) or a homologous residue (homolog scan) (table S7), revealing the CDR residues that make up the functional paratopes of the Fab in the two complexes (Fig. 2A).

In contrast to the extensive overlap between buried surfaces in the interfaces of bH1 bound to either VEGF or HER2, the two functional paratopes show only limited overlap (Fig. 2B and fig. S4). Energetically, the VEGF binding interaction is mediated primarily by the LC CDRs, whereas HER2 binding is dominated by HC CDRs. Compared to Herceptin, bH1 maintains the same core hotspot residues for HER2 binding (Arg50, Trp95, and Tyr100a of HC) (fig. S4).

The structural and functional studies show that the interactions between bH1 and the two entirely unrelated large proteins are distinct (Fig. 2 and fig. S5) and are characterized by conformational adaptation of the antigen binding site (table S8) and differential engagement of V_L and V_H (Fig. 2). The molecular versatility observed in the dual specific bH1 is reminiscent of other antibodies binding multiple small haptens or peptides (4, 16) or of an antibody binding its

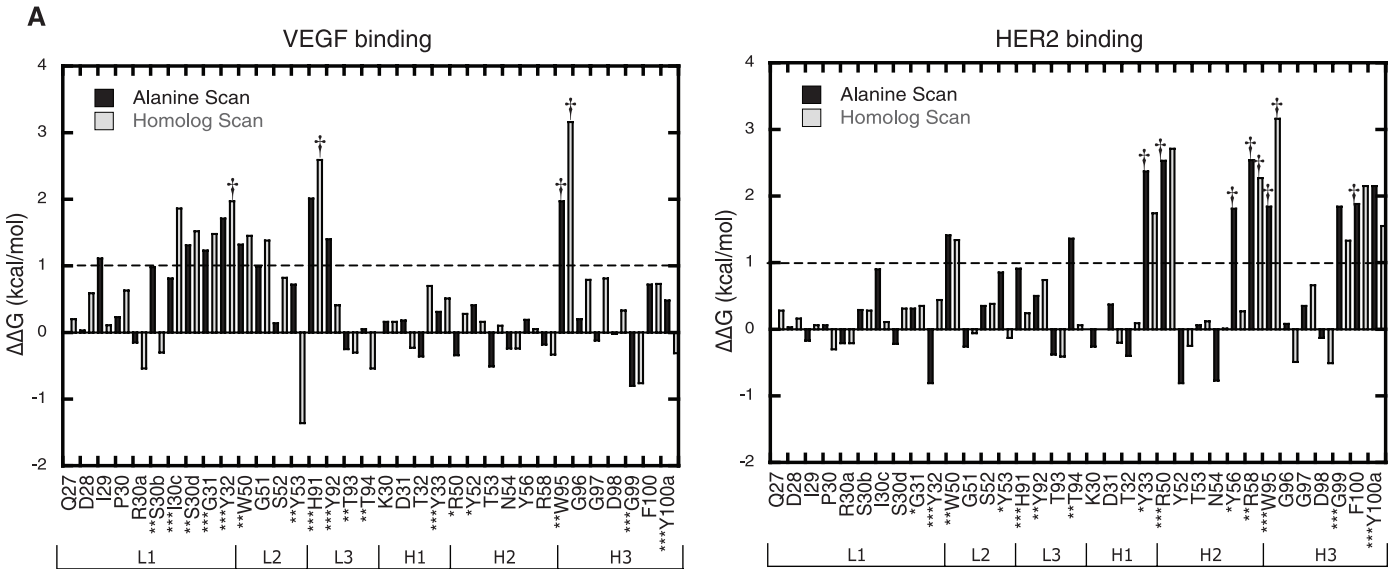
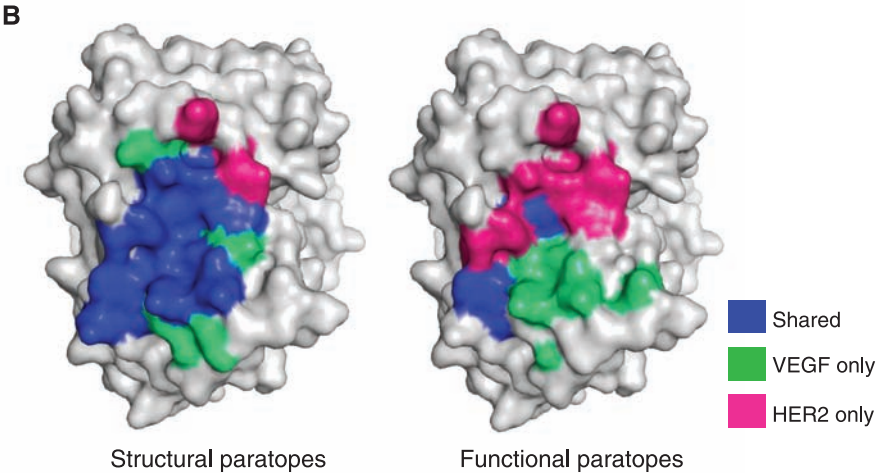


Fig. 2. The distinct interactions of bH1 with HER2 and VEGF. **(A)** The $\Delta\Delta G$ values (y axis, kilocalories per mole) of each mutation to alanine (black) or a homologous amino acid (white) for VEGF or HER2 binding based on shotgun scanning mutagenesis (10). The extent of the bH1 residues buried upon VEGF or HER2 complex formation is indicated (single asterisk, >25 to 50% buried; double asterisk, >50 to 75%; triple asterisk, >75%). The dagger symbol represents a lower limit (table S7). **(B)** The residues that make structural contacts (>25% buried) or energetic interaction ($\Delta\Delta G > 10\%$ total binding energy) with HER2 (pink), VEGF (green), or both (shared, blue) are mapped on the surface of HER2-bound bH1.



cognate antigen and an anti-idiotypic antibody (17, 18). Further, the differential engagement of residues within a common binding site also echoes the plasticity observed in other protein-protein interactions, such as the recognition of distinct antigens (major histocompatibility complex-bound peptides) by T cell receptors (19) or growth factor binding to different receptors (20).

We further examined whether the dual binding specificity can translate into dual activity in vivo. To facilitate the investigation of the in vivo activity of bH1, we generated versions of bH1 with high affinity and specificity for HER2 and VEGF (figs. S1, S2, S6, S7, and S8 and table S9). bH1, the affinity-improved bH1-81 ($K_d = 58/6$ nM VEGF/HER2), and bH1-44 ($K_d = 3/0.2$ nM VEGF/HER2) inhibit VEGF-induced proliferation of human umbilical vein endothelial cells (HUVECs) and the growth of the HER2 overexpressing breast cancer cell line, BT474 (Fig. 3A).

The potencies of the bH1 variants correlate with their relative affinities. The bH1-44 variant inhibits the growth of HUVEC and BT474 cells with potencies similar to bevacizumab or Herceptin, respectively.

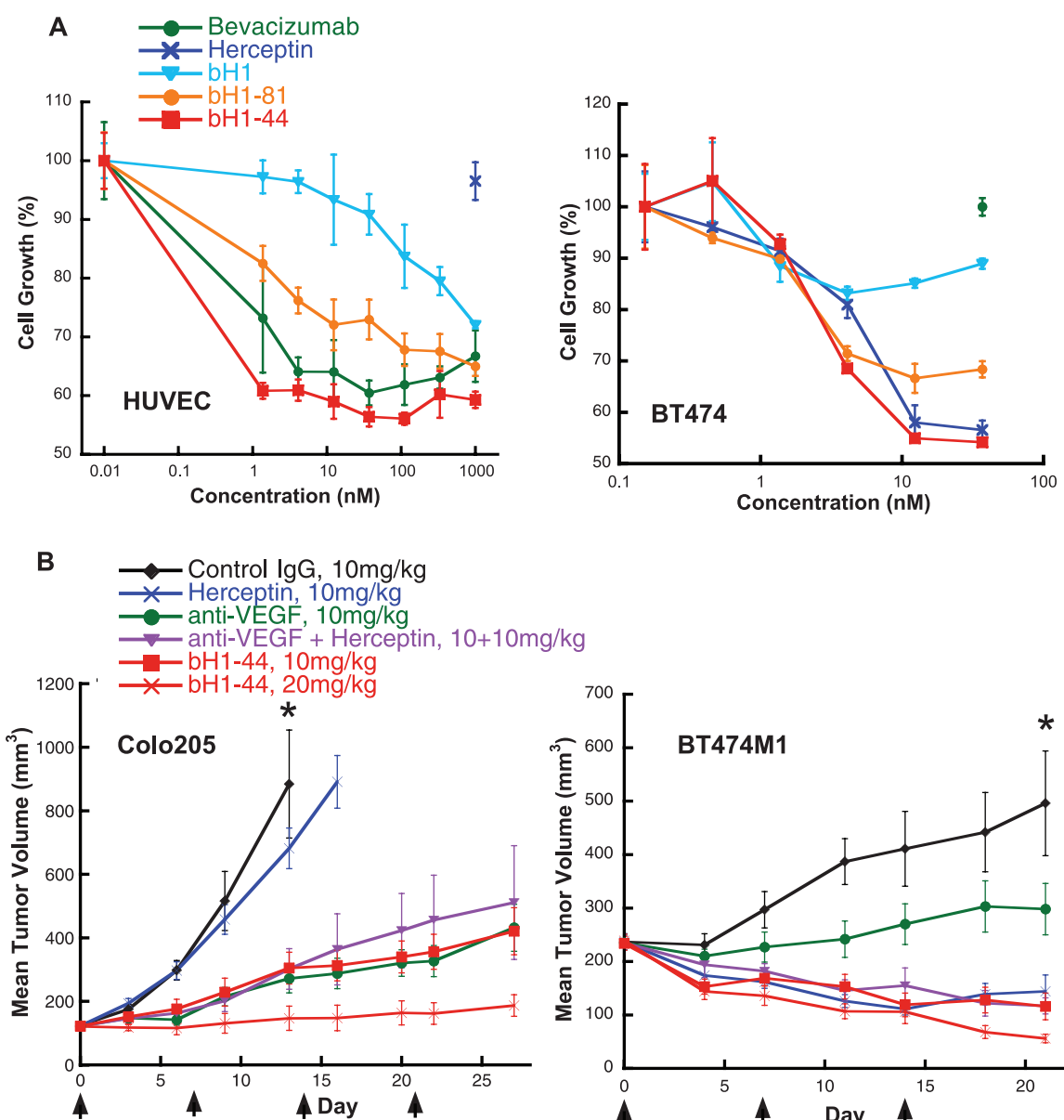
To examine the two unique activities of bH1-44 in vivo, we studied mouse xenograft tumor models known to be responsive to treatment by anti-VEGF antibodies (Colo205, a human colorectal cancer cell line) or Herceptin (BT474M1, human breast cancer cell line). We compared the bH1-44 treated groups with those treated with anti-VEGF (B20-4.1) (21), Herceptin, or the combination (Herceptin + anti-VEGF) (table S10) (10). The results of the Colo205 model demonstrate that the VEGF binding component of bH1-44 is inhibiting tumor growth ($P < 0.0001$, $n = 10$ mice, compared with the control IgG treated group), as Herceptin alone has no effect ($P = 0.12$, $n = 10$). The BT474M1 model indicates that the

HER2 binding component of bH1-44 is active ($P < 0.0001$, $n = 7$), because the effect observed with the anti-VEGF control antibody is not significant ($P = 0.6$, $n = 7$) (Fig. 3B).

Hence, bH1-44 appears to have the pharmacological activity of a VEGF blocking antibody and a Herceptin-like antibody, although we do not yet know whether bH1-44 is as effective as Herceptin or anti-VEGF administered separately or in combination. Whether there is a therapeutic benefit to co-targeting the tumor cell proliferation mediated by HER2 and tumor angiogenesis mediated by VEGF is a question currently being evaluated in clinical trials combining Herceptin and bevacizumab in breast cancer patients (22–24). If the drug combination is found to be beneficial, then dual specific antibodies like bH1-44 may merit further exploration as potential therapeutics.

Various bi-targeting antibody formats that assemble two distinct antibody fragments into one

Fig. 3. Inhibition of VEGF and HER2 function in vitro and in vivo. **(A)** bH1 and the affinity improved variants bH1-81 and bH1-44 as IgGs inhibit VEGF-stimulated proliferation of HUVECs and human breast cancer cell BT474 in a dose-dependent manner. Herceptin and bevacizumab (anti-VEGF) were used as controls. Error bars represent the average deviation of triplicates ($n = 3$). **(B)** Tumor growth inhibitions of bH1-44 in Colo205 and BT474M1 xenografts in immunocompromised mice. The graph shows tumor volume as a function of time. The arrows indicate the time points for antibody (human IgG) administration. Error bars indicate SEM (10). An asterisk indicates the time at which the effects on tumor growth inhibition were compared. For the statistical analysis, we used one-way analysis and Student's *t* tests. For BT474M1, 5 out of the 8 mice (5/8) dosed with bH1-44 at 10 mg/kg had partial responses (PR, 50 to 99% regression from initial volume), similar to Herceptin (PR = 6/8) or the anti-VEGF/Herceptin combination (PR = 5/8). Anti-VEGF treatment alone did not yield any partial response in this model.



molecule have previously been described (25, 26). In contrast to these bispecific formats, the dual specific "two-in-one" antibody we describe has the molecular structure of a regular IgG (or Fab). It has all the favorable attributes of an IgG for therapeutic development, such as predictable pharmacokinetic properties, well established manufacturing protocols, choice of Fc-mediated effector functions, and bi- or mono-valencies (25).

In summary, we have demonstrated that an antigen binding site is capable of interacting with two unrelated protein antigens with high affinity. The dual specific antibodies reported here are derived from a monospecific antibody through mutations in the periphery of the antigen binding site in the LC CDRs. This strategy is a general one and can be applied to create dual specific antibodies against two distinct antigens. The mutational analysis of bH1 and bH1-44 (Fig. 2 and fig. S8) suggested that the dual specificity could be switched to monospecific binding to either antigen (10). Indeed, bH1-44 lost binding to VEGF but retained HER2 binding when mutating two LC residues. Similarly, two alanine mutations in the HC drastically reduced the affinity for HER2 while preserving tight binding for VEGF (fig. S9). This finding highlights how a limited number of mutations in the antigen binding site can alter specificity or add a distinct specificity. During development of the natural antibody repertoire, the antigen binding sites often undergo diversification by exchanging the V_L that pairs with a V_H (6). Somatic mutations also occur frequently, in particular among the res-

idues in the periphery of the antigen binding site (27–30). Our studies reveal a mechanism by which one antibody can diverge into many antibodies with distinct specificity profiles. This mechanism may contribute to the large capacity of the natural antibody repertoire for diverse antigen recognition.

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31. We thank the Protein Engineering structure group, L. Haber, and D. McMahon for technical consultation and C. Eigenbrot for helpful advice. We also thank members of the DNA synthesis, DNA sequencing, in vivo cell culture, and protein chemistry groups at Genentech. We are grateful for the support of R. Neutze and Chalmers University of Technology, Sweden. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under contract no. DE-AC02-05CH11231. The atomic coordinates of the bH1/HER2 and bH1/VEGF were deposited in the Protein Data Bank with accession numbers 3BDY and 3BE1, respectively. G.F. and J.B. are inventors of the patent application "Multispecific Antibodies" (Pub. App. No. 20080069820), which relates to this work. All authors are or were under the employment of Genentech and declare competing financial interests.

Supporting Online Material

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Ankyrin-G Promotes Cyclic Nucleotide–Gated Channel Transport to Rod Photoreceptor Sensory Cilia

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Cyclic nucleotide–gated (CNG) channels localize exclusively to the plasma membrane of photosensitive outer segments of rod photoreceptors where they generate the electrical response to light. Here, we report the finding that targeting of CNG channels to the rod outer segment required their interaction with ankyrin-G. Ankyrin-G localized exclusively to rod outer segments, coimmunoprecipitated with the CNG channel, and bound to the C-terminal domain of the channel $\beta 1$ subunit. Ankyrin-G depletion in neonatal mouse retinas markedly reduced CNG channel expression. Transgenic expression of CNG channel β -subunit mutants in *Xenopus* rods showed that ankyrin-G binding was necessary and sufficient for targeting of the $\beta 1$ subunit to outer segments. Thus, ankyrin-G is required for transport of CNG channels to the plasma membrane of rod outer segments.

Cyclic nucleotide–gated (CNG) channels initiate the electrical responses to light in photoreceptors and to chemical stimuli in olfactory neurons (1). CNG channels are segregated to sensory cilia, where visual and olfactory signal transduction takes place. This precise intracellular localization is dependent on the channel's β subunit (CNG- $\beta 1$, CNGB1) in both

classes of neurons (2–4). However, the molecular mechanism(s) of CNG channel targeting to the plasma membrane of sensory cilia, where this channel normally functions, are unclear.

Ankyrin-G is a versatile membrane adaptor involved in the formation and maintenance of diverse specialized membrane domains (5–9). Ankyrin-G is localized exclusively to rod outer

segments (ROSSs), where it was found along with CNG channels that have been localized to the ROS plasma membrane (10, 11) (Fig. 1, A and B). In contrast, the plasma membrane of the inner segment was lined with ankyrin-B, which is required for the coordinated expression of the Na^+ - and K^+ -dependent adenosine triphosphatase (ATPase), Na^+ and Ca^{2+} exchanger, and $\beta 2$ -spectrin (12) (Fig. 1A). Localization of ankyrin-G to the plasma membrane was evident in isolated mouse ROSSs, but was better demonstrated in frog ROSSs, which are three to four times as large in diameter (Fig. 1B). Ankyrin-G also localized in the olfactory sensory cilia and the principal piece of sperm flagella, together with CNG- $\beta 1$ (4, 13) (fig. S1). We treated isolated bovine ROSSs with a cleavable cross-linker, solubilized them in 0.1% SDS, and used ankyrin- or CNG- $\beta 1$ -specific antibodies for immunoprecipitation. We observed the reciprocal coimmunoprecipitation of CNG- $\beta 1$ and ankyrin-G (Fig. 1C). The interaction with ankyrin-G was specific, because CNG- $\beta 1$ was not precipitated by nonimmune or ankyrin-B-specific antibodies,

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Supporting Online Material for

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Variants of the Antibody Herceptin That Interact with HER2 and VEGF at the Antigen Binding Site

MATERIALS AND METHODS

Light chain library construction and selection

The phagemid pV1384 was modified from pS2018 for bivalent display of Herceptin scFv on M13 phage (1). The light chain (LC) framework (FR) mutation R66G was incorporated because Gly is more common than Arg at this position in kappa LCs. A stop codon was incorporated in CDR-L1. Combinatorial phage-displayed antibody libraries were constructed by mutagenesis using mixtures of oligonucleotides to encode the desired frequency of amino acids and CDR loop lengths (Table S1, S2). Library phage was produced in *E. Coli* and purified (1) to yield $\sim 10^{10}$ unique members. Screening showed that 25% of displayed antibodies fragments retained HER2 binding. 150 displayed clones were sequenced to examine the actual diversity. 30% of the displayed clones retained Herceptin CDR-L2 and/or CDR-L3 sequence due to incomplete mutagenesis and were excluded from the analysis. The actual diversity did not deviate significantly ($p > 0.05$, odds-ratio test) from the designed, except at three positions (29, 51

and 53) where small deviations were observed.

Selection for new binding specificity was performed by four rounds of selection on immobilized targets (VEGF₈₋₁₀₉, DR5 and human Fc) (1) followed by a final round of selection on HER2 ECD. Clones were assayed using ELISA for specific binding to the target, HER2 and a non-target protein (BSA) and sequenced. The relative binding affinity was determined by competitive phage ELISA (1).

Antibody characterization

The gene segments containing V_L of selected clones was cloned into Herceptin Fab or human IgG1 vectors(2). Fabs and IgGs were expressed in *E. Coli* and 293 cells respectively, and purified as described (2). Surface plasmon resonance measurements with BIAcore3000 were used to determine the binding affinities of Fabs and IgGs as described (2). The antigens (hVEGF₈₋₁₀₉, HER2 ECD and DR5) were immobilized at low density on a BIAcore CM5 chip. Serial dilutions of Fab and/or IgG of the antibodies from the primary library were flowed over the immobilized antigens. 1:1 Langmuir binding models, or bivalent analyte-binding models, were used to calculate the k_{on} , k_{off} and K_D for Fabs and IgGs, respectively. Steady state binding analysis was applied for weak binding clones. The K_D s of bH1 affinity-improved variants as Fabs were determined as above. For IgG, the K_D was determined by capturing IgG on a BIAcore CM5 chip by immobilized anti-Fc antibody, followed by incubation with dilutions of hVEGF₈₋₁₀₉, murine VEGF₁₋₁₀₈ and HER2-ECD (Table S9). 1:1 Langmuir binding models were used to determine the K_D for HER2, while bivalent analyte models were used for VEGF, which is a homodimer.

Crystal structures

bH1 Fab, human VEGF₈₋₁₀₉, and HER2₁₋₆₂₄ were expressed and purified as described (3, 4). The complexes were purified by size-exclusion chromatography in 25 mM Tris-HCl, pH 7.5, 0.3 M NaCl for the Fab/VEGF and with 25 mM Tris-HCl, pH 8 and 0.15 M NaCl for the Fab/HER2 complex. Crystals of bH1 Fab/VEGF were grown by 1:1 mixing of 10 mg/ml protein complex with 0.15 M D, L Malic Acid pH 7.0, 20% PEG₃₃₅₀, and bH1 Fab/HER2 crystals by mixing 11 mg/ml protein solution with 25% w/v PEG₂₀₀₀ and 0.1M MES pH 6.5, followed by hanging-drop vapor-diffusion experiments at 19 °C. Before data collection the crystals were flash frozen in liquid nitrogen using 15% Glycerol (bH1/VEGF) or 20 % Ethylene Glycol (bH1/HER2) in artificial mother liquor as cryo-protectants. Data was collected at the beam line 5.0.1 of the Advanced Light Source (Berkeley, CA).

The diffraction data were processed with Denzo and Scalepack(5). The structures were solved by molecular replacement using Phaser (6, 7). The bH1 Fab/VEGF structure was solved using coordinates of VEGF from 2FJG and Herceptin Fab (1N8Z). Fragments of HER2 and Herceptin Fab (1N8Z) were used to solve the bH1/HER2 structure. Refinement and model building were performed using Refmac (8) and Coot(9). Stereo chemical parameters were analyzed using MolProbity (10). Fig. 1, 2, S4 and S5 were generated using PYMOL (DeLano Scientific, San Carlos, CA).

Shotgun scanning mutagenesis

A phagemid was created to display bH1 Fab or bH1-44 Fab on phage with the light chain C-terminally fused to an epitope (gD) tag (2). Separate heavy chain and light chain

alanine and homolog scanning libraries were constructed (11). The degeneracy ranged from 10^5 to 10^8 and the actual library size was 10^{10} . Selections were performed on immobilized VEGF₈₋₁₀₉, HER2-ECD, protein L or anti-gD mIgG followed by ELISA screening for target binding, and sequence analysis of ~100 unique sequences from each selection. The wild type/mutation (wt/mut) ratio was calculated (11). The wt/mut ratios of VEGF and HER2 binding clones were corrected for protein folding and expression effects by division with anti-gD binding wt/mut ratios to yield the $F_{wt/mut}$. $\Delta\Delta G$ numbers of each mutation were calculated using the formula $\Delta\Delta G = RT \ln(K_{a,wt}/K_{a,mu}) = RT \ln(F_{wt/mut})$ as described (12) (See Table S7 for details).

Affinity maturation

The light chain homolog shotgun scan library was subjected to selections for improved VEGF binding (2). 96 clones were screened using competitive phage ELISA and sequenced (2). The clone bH1-81 was isolated (Table S9). A second affinity maturation library was constructed based on the phagemid encoding bH1-81. Oligonucleotides were designed to encode ~50% wild type and 50% of all other 19 amino acids at each targeted position (Table S9) (13). The library was selected for VEGF and HER2 binding in solution and screened as described above. The highest affinity variant bH1-44 was identified. The affinity (K_D) of bH1-81 and bH1-44 as Fab and IgG was determined using BIAcore (SPR) (Table S9).

Cell proliferation assays

VEGF-stimulated human umbilical vein endothelial cells (HUVECs) were treated with

increasing concentrations of human IgG and the proliferation inhibition after 2 days of incubation measured as described (14). HER2 expressing BT474 breast cancer cells were treated with human IgG and incubated at 37 °C for 5 days. 10% Alamar Blue (Biosource International, Camarillo, CA) was added. The level of cell proliferation was determined by measuring the fluorescent signal after 6 hours.

Tumor growth inhibition

All animal studies were in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the Genentech Institutional Animal Care and Use Committee. The BT474M1 and Colo205 (ATCC, Manassas, VA) cells were cultured in RPMI media/10% fetal bovine serum. BT474M1 is a sub-line of BT474 (ATCC, Manassas, CA) selected for optimal *in vivo* growth in nude mice. 5×10^6 BT474M1 cells suspended in Hank's Buffered Salt Solution (HBSS) and matrigel (1:1) mixture were injected into the mammary fat pad of Harlan beige nude XID mice (Indianapolis, IN) implanted with an estradiol pellet subcutaneously. For Colo205 xenografts, 5×10^6 Colo205 cells in HBSS were subcutaneously injected into Charles River nu/nu mice (Hollister, CA). When the mean tumor size reached $\sim 200 \text{ mm}^3$, the mice were randomly grouped into 7 groups of 8 mice (BT474M1) or 10 mice (Colo205). The bH1-44 treated groups were compared with groups treated with anti-VEGF (B20-4.1) (14), Herceptin or the combination (Herceptin + anti-VEGF). The anti-VEGF antibody B20-4.1 is a surrogate of bevacizumab for the xenograft experiment as it blocks human VEGF produced by the xenograft tumor and stromal murine VEGF, both of which may contribute to tumor angiogenesis (14). Bevacizumab has activity against human

VEGF only. bH1-44 was selected for binding to murine VEGF in addition to human VEGF (Table S9 , Fig. S2). Antibodies were administered intraperitoneally once a week. The tumor sizes were measured twice a week. Volumes were calculated as $V = 0.5ab^2$ (a is the longest dimension of the tumor and b perpendicular to a). The statistical evaluation used one-way analysis followed by two-tailed student *t* tests. Adjustment of the alpha level due to multiple comparisons (Bonferroni) did not alter the significance of our conclusions. Partial responses (PR) were defined as a response of 50-99% reduction in tumor volume compared to V0 (initial volume). In all groups, antibody was present in the serum at high concentrations, indicating pharmacokinetics similar to other IgGs (Table S10). Serum samples were collected 7 days after the first and third treatment. The concentration of human antibody was determined using ELISA. Donkey anti human IgG Fc was immobilized on an immuno plate. Dilutions of serum and an antibody standard were incubated on the plate for 2 h. Bound antibody was detected by Horseradish Peroxidase conjugated goat anti-human IgG Fc followed by absorbance reading of added substrate. Sample concentrations were determined using a 4-parameter algorithm.

Construction of bH1-44 double mutants

The mutations I29A and Y32A of the LC, and R50A and R58A of the HC were chosen based on the shotgun Ala scanning mutagenesis of bH1 and bH1-44 (Fig. 2A and S8). The scanning indicated that these mutations would disrupt binding to either VEGF or HER2 without affecting the other antigen-binding specificity. A phagemid that display bH1-44 Fab on phage was used as a template for Kunkel mutagenesis to introduce Alanine residues at positions 29 and 32 (LC), or 50 and 58 (HC). The binding

specificities of the double mutants were confirmed by phage ELISA. The two double mutants were expressed as IgG protein. The ability of the double mutants to bind and block VEGF and HER2 was assessed by incubating biotinylated VEGF or HER2 with IgG followed by capture immobilized to anti-VEGF antibody or Herceptin (Fig. S9).

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Supplementary online material (SOM) for
**“Variants of therapeutic antibody Herceptin
that interact with HER2 and at the antigen
binding site”**

Figures and Tables

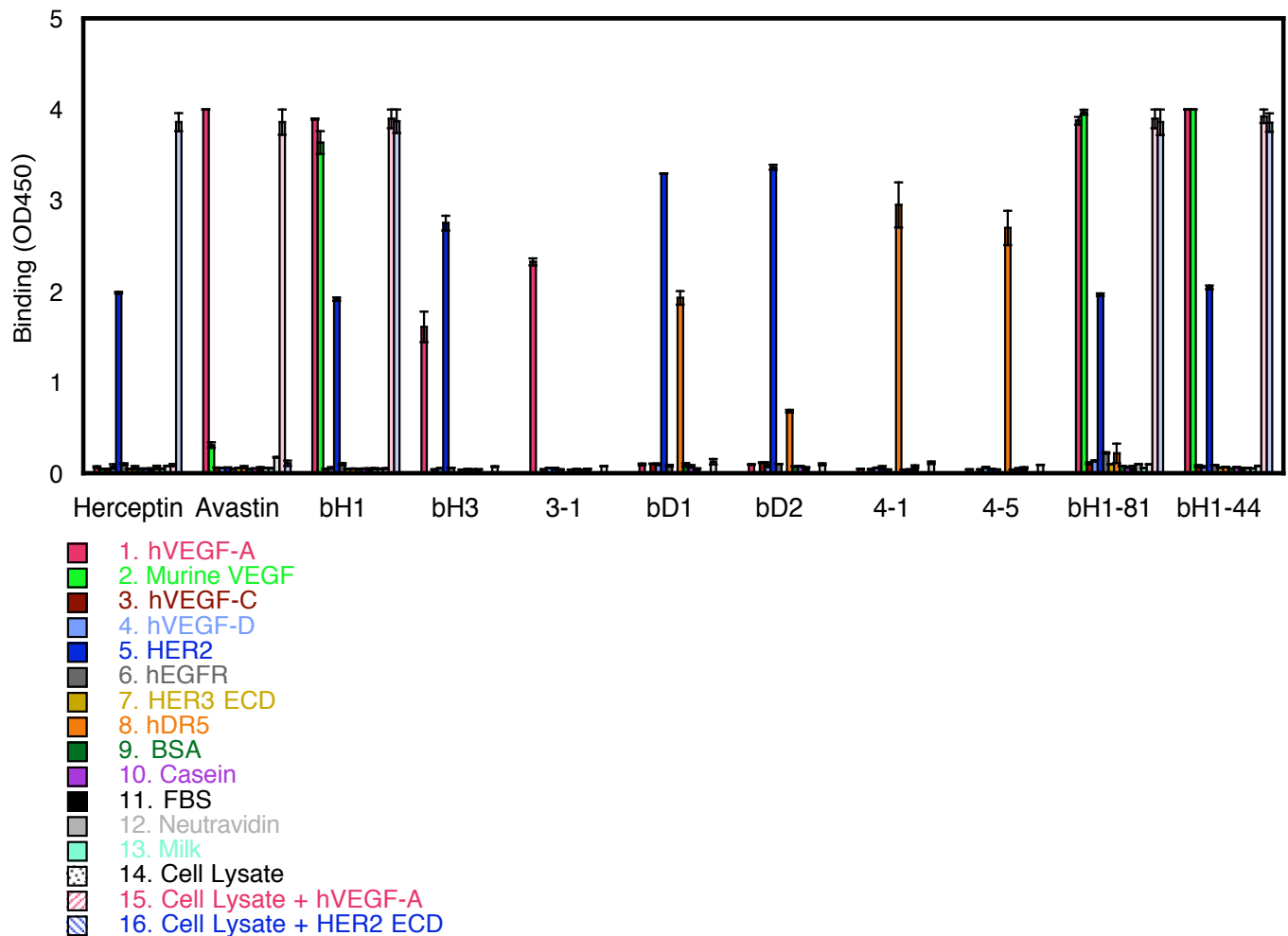


Figure S1. Binding specificity of the antibodies derived from the LC library.

The IgGs were assayed by ELISA for binding to various immobilized purified proteins (including the cognate antigens) or cell lysates. Immobilized proteins were incubated with 15 ug/mL hIgG for an hour. Bound IgG was detected spectrophotometrically (optical density at 450 nm, y-axis). The proteins included in the assay were: 1. vascular endothelial growth factor A (VEGF, pink), 2. murine vascular endothelial growth factor (Murine VEGF, light green), 3. vascular endothelial growth factor C, (hVEGF-C, brown), 4. vascular endothelial growth factor D, (hVEGF-D, light blue), 5. HER2 extracellular domain (HER2 ECD, dark blue), 6. epidermal growth factor receptor extracellular domain (hEGFR, dark gray), 7. ErbB3/HER3 extracellular domain (HER3 ECD, beige), 8. human death receptor 5 (hDR5, orange), 9. bovine serum albumine (BSA, dark green), 10. casein (purple), 11. fetal bovine serum (FBS, black), 12. neutravidin (light gray), 13. 5% milk (cyan), 14. mouse fibroblast cell lysate (black stripes), and mouse fibroblast cell lysate spiked with 15. hVEGF-A (pink stripes) or 16. HER2 ECD (blue stripes). Error bars represent the standard error means (SEM) of duplicates. The antibodies bH3, 3-1, bD1, bD2, 4-1, and 4-5 were not tested for binding to samples 2, 7, 12, 13, 15 and 16. The cell lysates (14, 15, 16) were immobilized at a concentration of approximately 20-40 ug/mL spiked with 20 ug/mL VEGF₁₀₉ (15) or 20 ug/mL HER2 ECD (16).

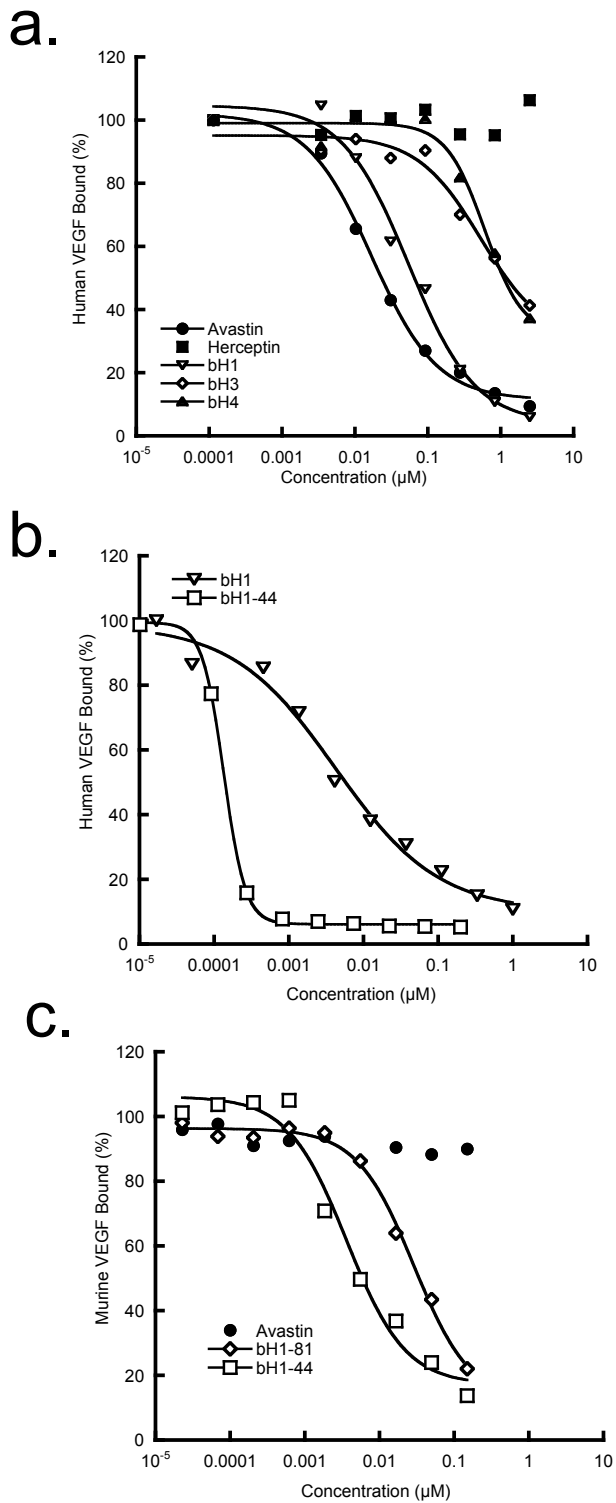


Figure S2. Anti-VEGF antibodies block VEGF binding to VEGF receptors.

Biotinylated human VEGF₁₆₅ (a, b) or murine VEGF₁₆₄ (c) were incubated with increasing concentrations of IgG (x-axis), then captured on immobilized human VEGFR2-ECD Fc fusion (a, c) or VEGFR1-Fc (b) and detected with horseradish peroxidase-conjugated streptavidin and added substrate (normalized % OD450nm, y-axis).

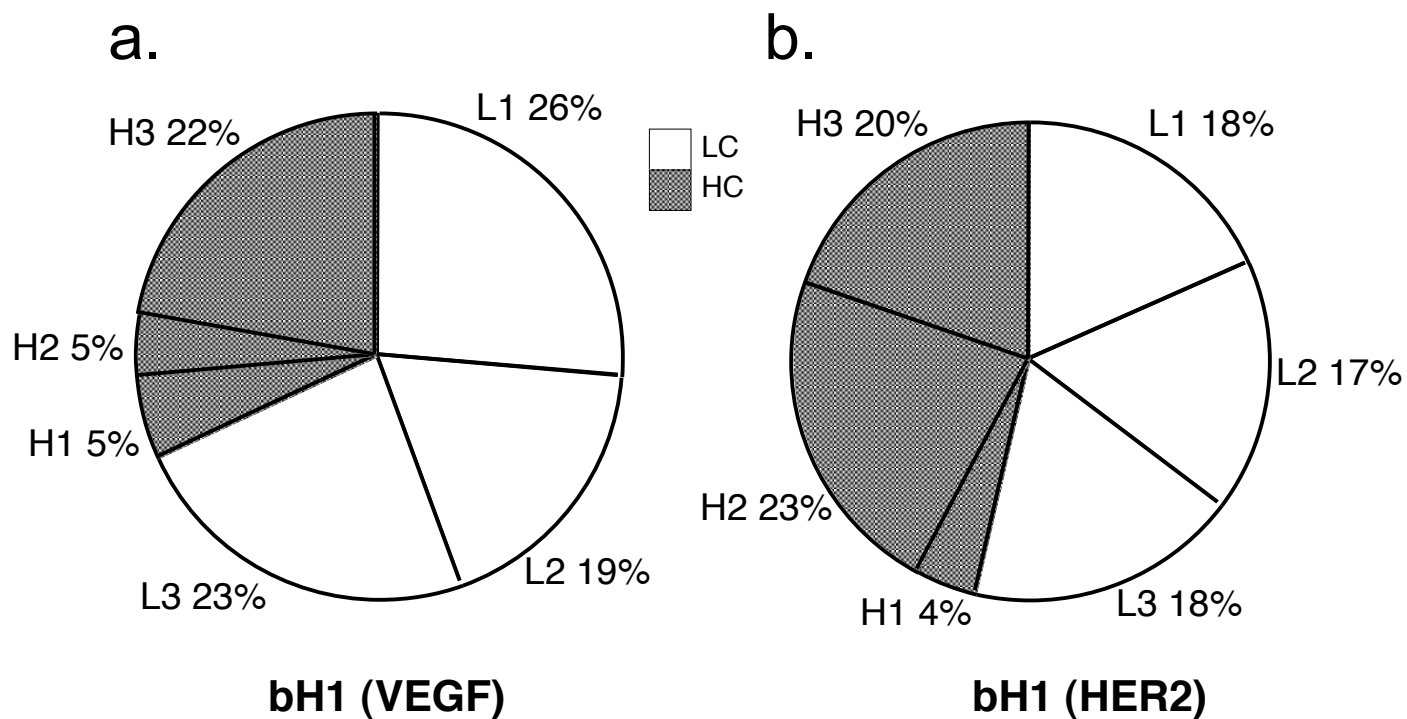


Figure S3. Individual CDR contributions to the structural paratope.

The pie-charts indicate the proportion of buried surface area between bH1 and VEGF (a) or HER2 (b) contributed by each CDR. The paratope size is 730 Å² for VEGF and 690 Å² for HER2. Heavy chain CDRs are colored gray and light chain CDRs are colored white.

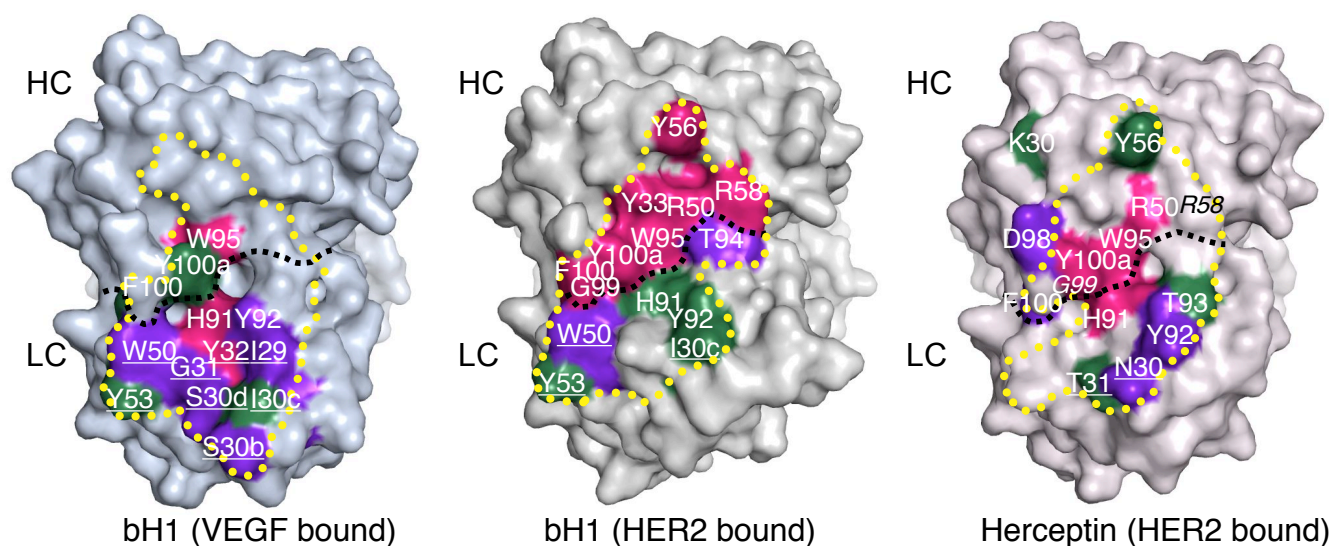


Figure S4. The functional paratopes of bH1 determined by alanine scanning mutagenesis are mapped on the Fab surface and compared to Herceptin. The residues are colored based on their functional importance as indicated by alanine mutation (pink, $\Delta\Delta G_{wt/mu} \geq 1.5$ kcal/mol; purple, $\Delta\Delta G_{wt/mu} \geq 1$ kcal/mol; green, $\Delta\Delta G_{wt/mu} \geq 0.5$ kcal/mol). Mutation of Arg 58 and Gly99 of Herceptin HC was not investigated (*italic*). The residues that differ between bH1 and Herceptin are underlined. The yellow dotted line outline the contact area as in Fig. 1B. The black dotted line separates the HC and LC.

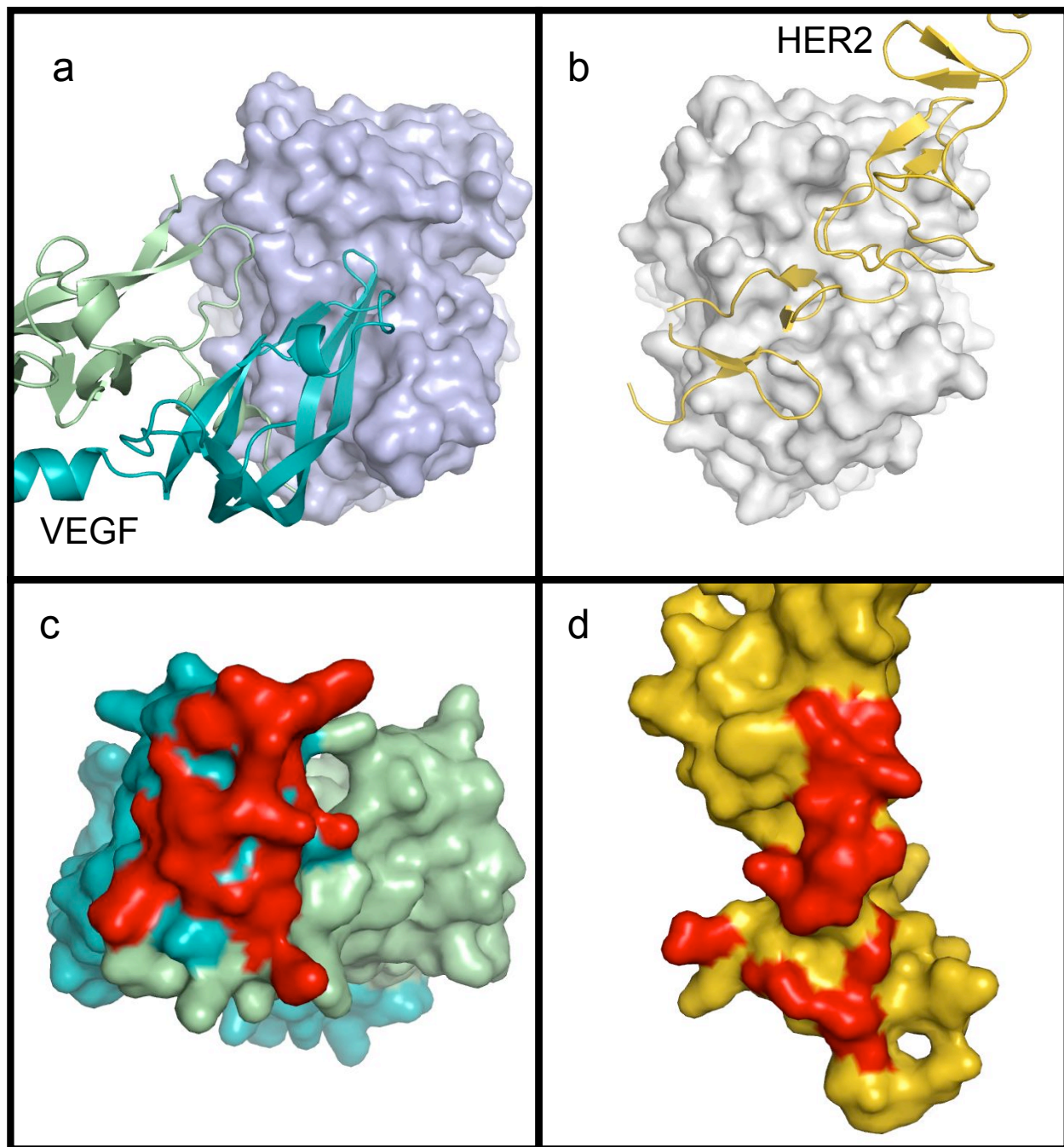


Figure S5. The two antigens of bH1 are distinct. Close-up of the bH1 (blue) /VEGF (green) (A) and bH1 (gray) /HER2 (gold) (B) binding interface to illustrate the structural differences between VEGF and HER2 in the regions of antibody binding. Surface representations of VEGF (C) and HER2-ECD (D) are shown in the same orientation relative to bH1 Fab. The residues in contact with bH1 Fab (closer than 4.5 Å) are highlighted in red. There is no apparent similarity between the two epitopes for bH1 in terms of chemical composition or topology.

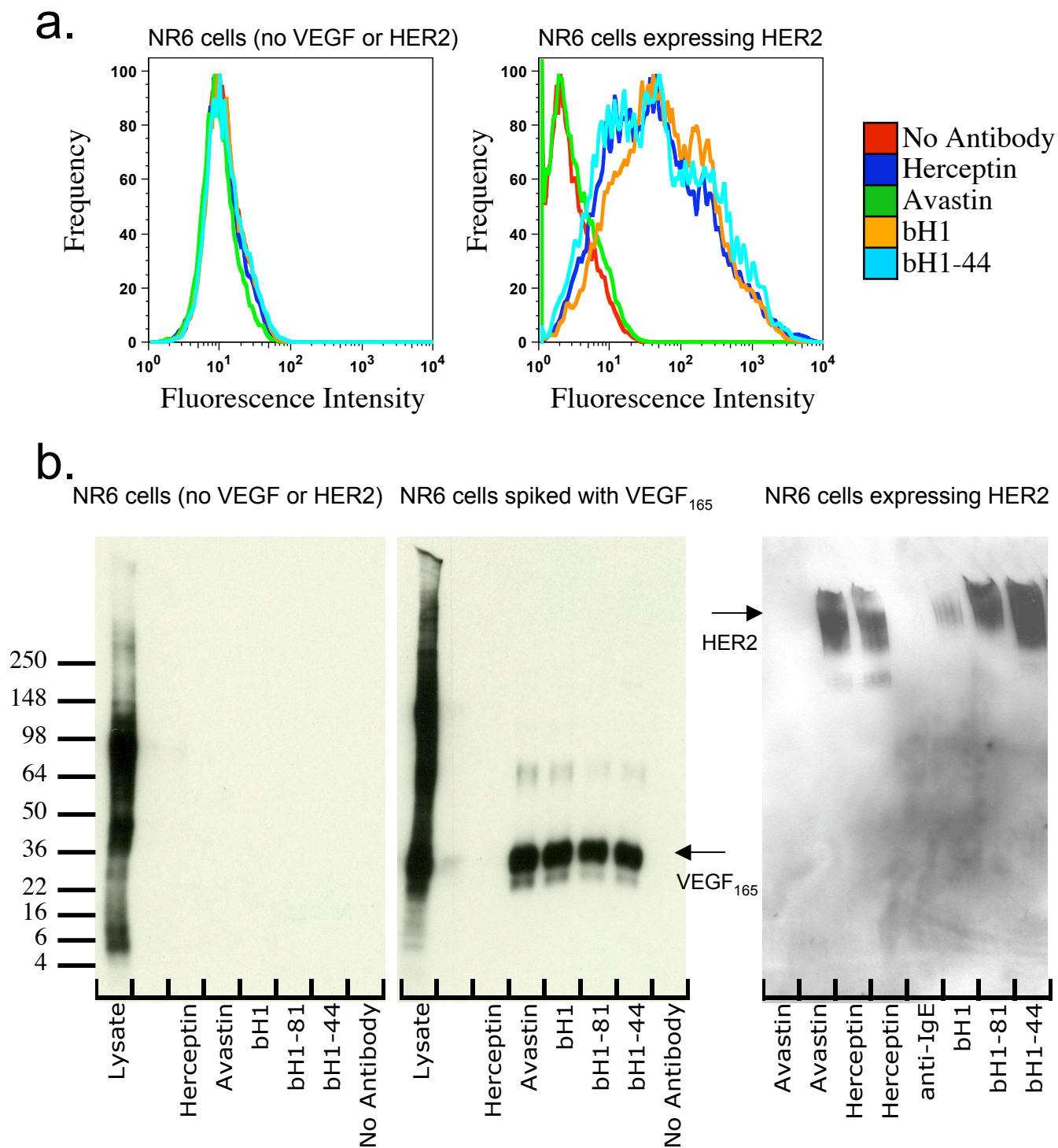


Figure S6. The specific binding of bH1 and bH1-44. (a) NR6 or HER2-expressing NR6 cells were incubated with 15 $\mu\text{g/mL}$ indicated antibodies (as human IgG) and then PE conjugated goat-anti-human IgG. The binding of the IgG to the cells was analyzed using a FACS Calibur flow cytometer. (b) NR6 cells were surface labeled with amine reactive biotinylation and solubilized with detergent. Lysates of $\sim 2.5 \times 10^5$ NR6 cells with or without spiked biotinylated VEGF₁₆₅, or HER2-expressing NR6 cells were incubated with 15 $\mu\text{g/mL}$ antibody (100nM) and immunoprecipitated with protein A-sepharose beads. The eluted proteins were separated by SDS-PAGE on a 4-20 % Tris-Glycine gel. Lysates of $\sim 25,000$ cells were loaded in the control lysate lane. Captured proteins were detected by Western blotting using streptavidin-HRP.

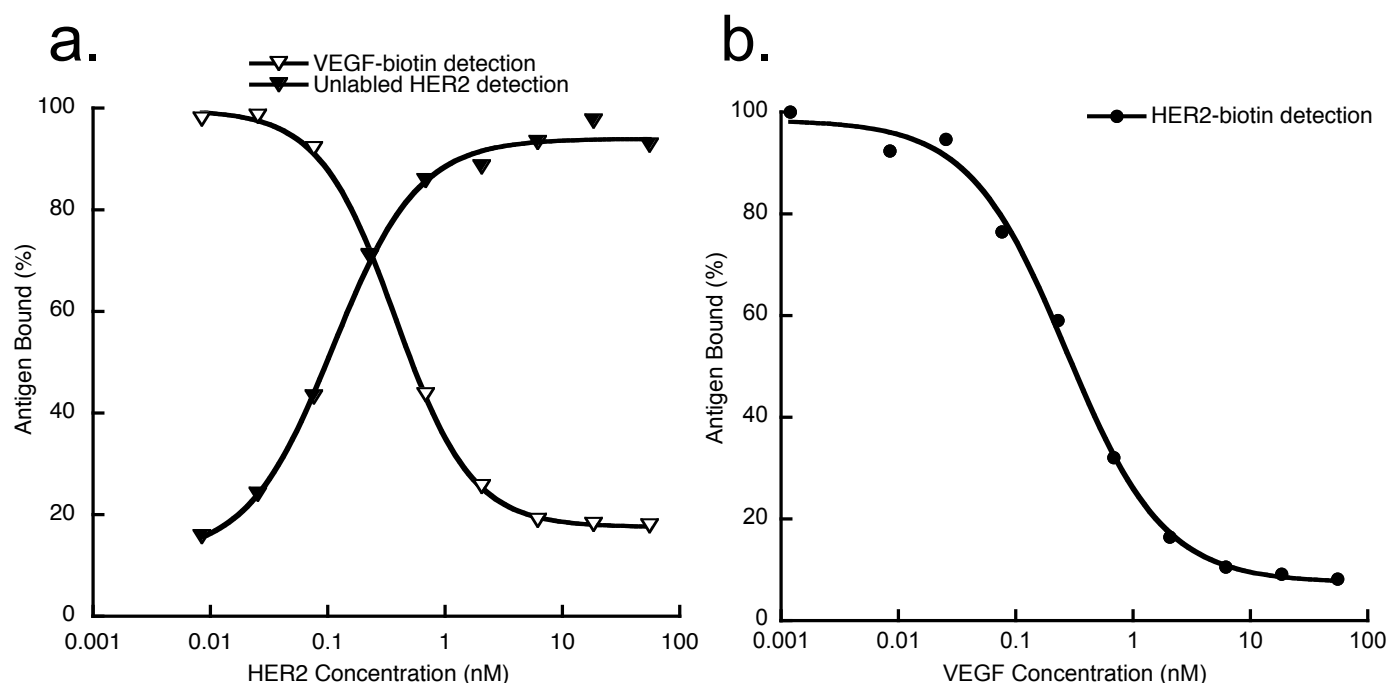


Figure S7. Competition of VEGF and HER2 for binding bH1-44 IgG in solution. (a) bH1-44 IgG at a concentration of 0.1 nM was incubated with biotinylated human VEGF₁₆₅ and increasing concentrations of HER2 ECD. Anti-human Fc coated ELISA wells captured bH1-44 IgG with bound biotin-VEGF, which was detected with streptavidin-HRP. HER2 ECD bound to captured bH1-44 was detected using a murine anti-HER2 antibody binding a non-overlapping epitope on HER2 followed by an HRP-conjugated goat anti-mouse IgG. The IC₅₀ of HER2 competing for bH1-44 is 0.8 nM (b) bH1-44 IgG (0.2 nM) was incubated with biotinylated HER2 and increasing concentrations of human VEGF₁₆₅. bH1-44 was captured by immobilized anti-human Fc and bH1-44-bound biotin-HER2 detected with streptavidin-HRP. The IC₅₀ of VEGF competing for binding bH1-44 is 0.4 nM.

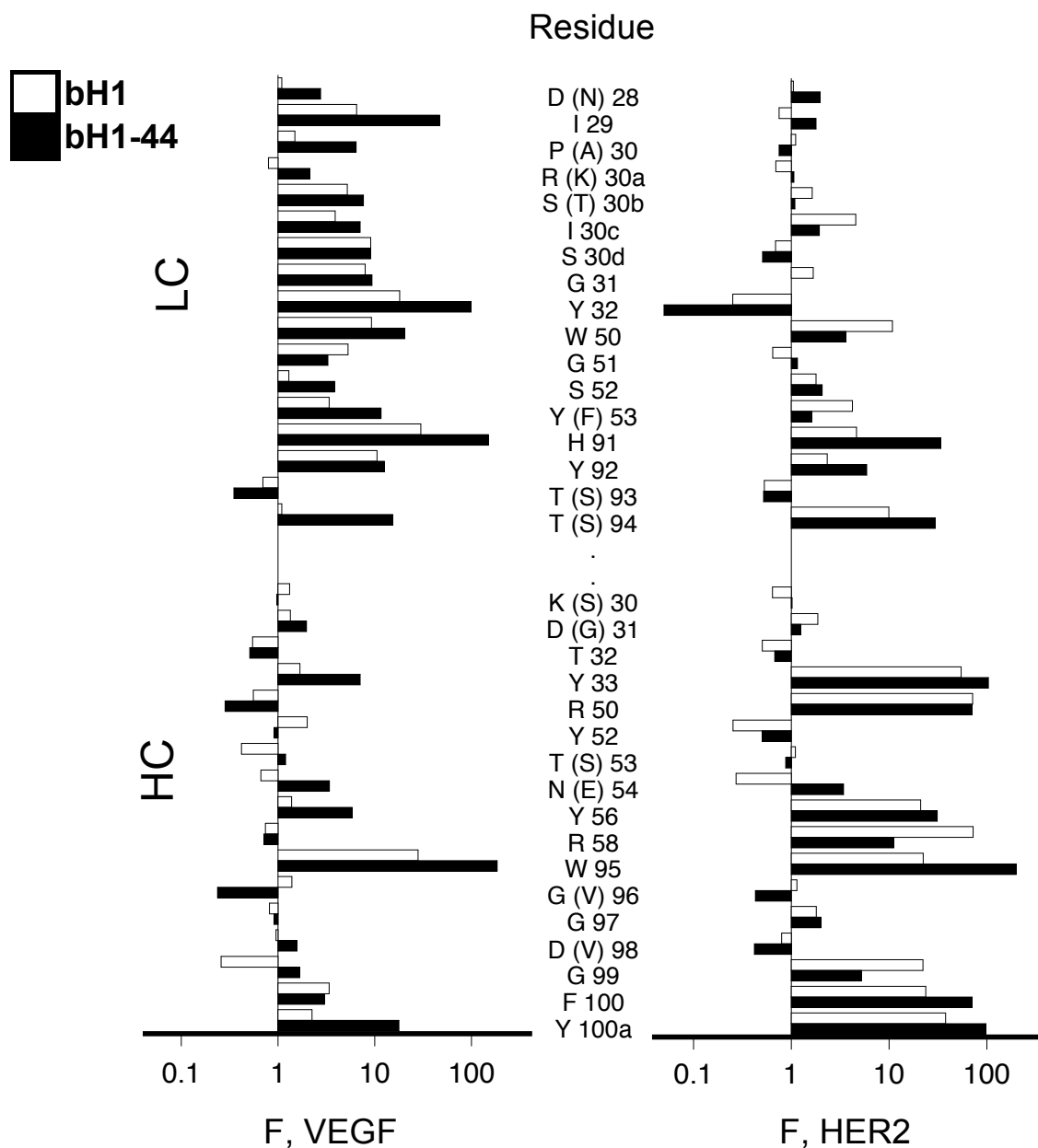


Figure S8. Alanine scanning of bH1-44 shows a similar antigen-binding site as the parent antibody bH1. Alanine scanning mutagenesis analysis was performed as described in Table S7. F values represent the relative contribution of each scanned residue to antigen binding. F values were determined for bH1-44 binding to VEGF and HER2 (black bars), and compared to the F values of bH1 (white bars). The amino acids in parenthesis denote bH1-44 residues that differ from bH1.

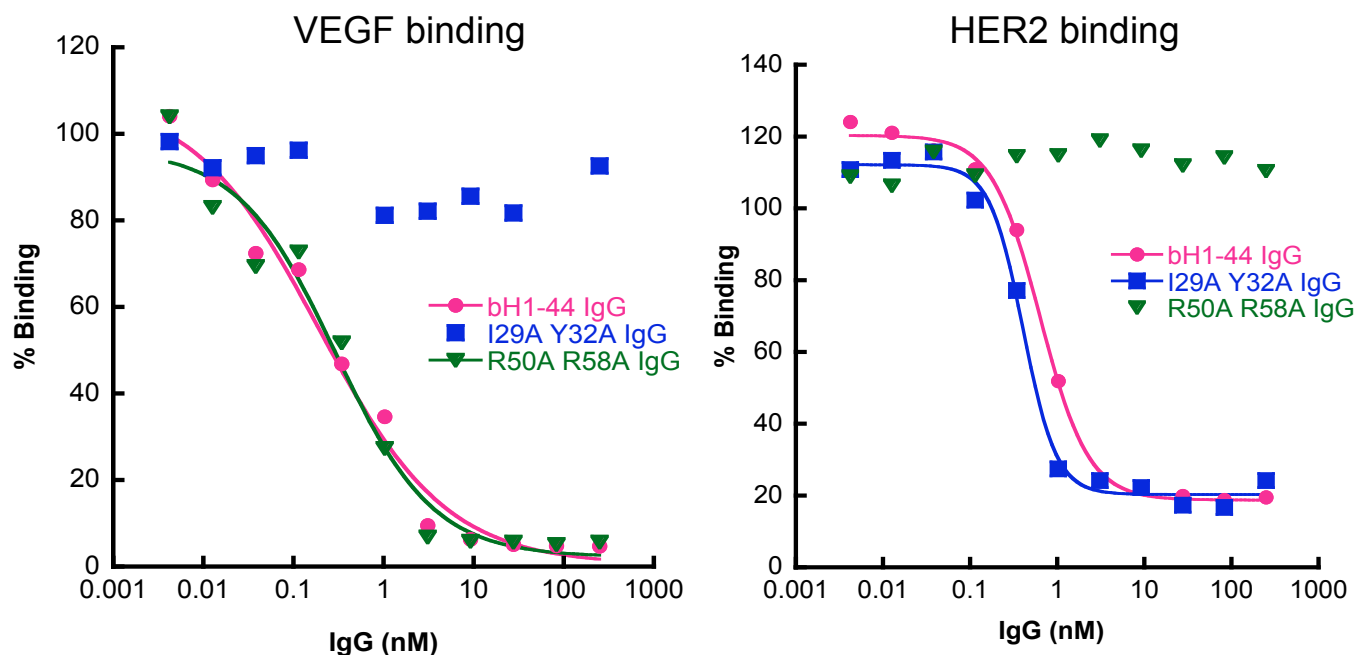


Figure S9. Mutation of two residues to alanine converts dual specific bH1-44 to essentially mono-specific. The ELISA binding assays show the potency of bH1-44 IgG, and the two double mutants binding to the biotinylated VEGF₁₀₉ (left) or HER2-ECD (right) and competing with the immobilized anti-VEGF antibody or Herceptin, respectively. The I29A/Y32A LC mutant has lost binding of VEGF while maintaining similar affinity for HER2 as bH1-44. The R50A/R58A HC mutant has lost affinity for HER2, but retain VEGF binding.

LC CDR	Position	Natural Diversity										Design Diversity										Template (Herceptin) Sequence	
		Prevalence (%)										Prevalence (%)											
CDR1	28	S	G	D	N	T	R				D	E	G	A	V	N	Y	H	X		D		
		71	12	7	4	3	2				39	10	7	7	7	6	6	6	<2				
	29	I	V	L							I	V									V		
		43	42	12							50	50											
	30	S	L	R	G	N	V	T	D		S	L	R	G	A	V	T	P	X		N		
		55	17	6	5	4	4	2	2		9	9	9	6	6	6	6	6	3				
	30a-e	S	N	Y	G	K	H	D			S	L	R	G	A	V	T	P	X		*		
		34	23	10	9	8	6	5			9	9	9	6	6	6	6	6	3				
	31	S	N	T	R	D	I	K			S	L	R	G	A	V	T	P	X		T		
		44	32	11	3	2	2	2			9	9	9	6	6	6	6	6	3				
	32	Y	N	W	F	S	D	R			Y	W	G	R	N	S	D	T	A	H	P	A	
		68	8	6	5	4	3	2			31	8	8	8	6	6	6	6	6	6	6		
	33	L	V	F	X						V	L										V	
		94	3	1	2						50	50											
CDR2	50	G	A	D	W	K	L	E	S		W	G	S	L	R	A	V	T	P	X	S		
		25	22	19	10	8	6	3	2		16	12	7	7	7	5	5	5	5	2			
	51	A	V	G	T						A	G										A	
		79	7	6	5						50	50											
	52	S									S											S	
		95									100												
	53	S	N	T	K	R	I				S	Y	X									F	
		36	28	26	3	2	2				44	43	<2										
CDR3	91	Y	S	R	A	G	H	F	L	D		H	Y	S	A	D	R	G	W			H	
		52	12	10	7	4	3	2	2	2		20	15	15	15	15	7	7	7				
	92	G	Y	N	S	D	L	T	H	I		G	S	D	R	A	V	Y	L	C	N	X	Y
		22	22	15	12	7	6	4	3	2		11	9	8	7	7	6	6	5	5	5	<5	
	93	S	N	Q	T	H	G	D	R		G	S	D	R	A	V	Y	L	C	N	X	T	
		46	21	7	6	4	3	3	2		11	9	8	7	7	6	6	5	5	5	<5		
	93a-b	W	S	T	P	L	Y	V	A	N		G	D	S	Y	R	A	V	C	N	X		*
		28	25	17	11	6	4	2	2	2		12	10	9	7	7	7	6	6	5	<5		
	94	T	P	S	W	Y	L	F	A	V	G	T	S	A	G								T
		19	19	18	12	10	7	5	3	2	1	25	25	25	25								

Table S1. Natural and designed diversity of light chain CDRs.

To design the diversity of LC CDRs, twelve positions were chosen: 28, 29, 30, 31, 32 of CDR1, 50, 51, 53 of CDR2 and 91, 92, 93, 94 of CDR3. Some positions (30, 31, 50, 92, 93) were fully randomized while other positions allowed limited diversity to mimic natural antibodies. The natural diversity of these light chain CDR residues was calculated from the alignment of approximately 1500 human kappa light chain sequences in the Kabat database and indicated as the frequency of amino acid types. For library construction, tailored degenerate codons were designed to encode a large proportion of the natural amino acid types at each position. Some positions were biased for the Herceptin template sequence. X denotes additional amino acids designed to occur at low frequencies. The length variation of natural LC CDRs was also reflected in the library and was constructed by inserting 0-5 residues between residues 30 and 31 (30a-e) and 0-2 residues between 93 and 94 (93a-b).

		Positions				
CDR-L1	28	29	30	31	32	33
	G ₇₀ A ₇₀ C ₇₀ *	RTT	NNK	NNK	TAC	STA
	G ₇₀ A ₇₀ C ₇₀ *	RTT	NNK	NNK	DGG	STA
	G ₇₀ A ₇₀ C ₇₀ *	RTT	NNK	NNK	NMT	STA
CDR-L2	50	51	52	53		
	NNK	GST	TCC	NNK		
	TGG	GST	TCC	NNK		
	KGG	GST	TCC	TMT		
	NNK	GST	TCC	TMT		
CDR-L3	91	92	93	94		
	CAT	NNK	NNK	RST		
	KMT	XYZ [†]	XYZ [†]	RST		
	DGG	XYZ [†]	XYZ [†]	RST		

Table S2. Light Chain Library oligo-nucleotide design.

For CDR-L1, each length was a mixture of three oligonucleotides at 1:1:2 ratios. For CDR-L2, four oligonucleotides were mixed 1:1:2:10. Three oligonucleotides for CDR-L3 were mixed 1:3:1. * G₇₀A₇₀C₇₀ allows 70% of the designated nucleotide and 10% each of the other three to encode approximately 50% Glu and 50% of the other amino acids.

[†]XYZ is a variation of NNK that has unequal portions of A/G/T/C nucleotides to reduce the prevalence of aliphatic (hydrophobic) amino acids. X denotes 19% A, 38% G, 26% T and 17% C. Y denotes 34% A, 31% G, 17% T and 18% C. Z denotes 24% G and 76% C.

Target	Positive Clones (%)	Unique Clones	dual specific Clones
VEGF	77	41	30
DR5	85	5	2
Fc	63	31	1

Table S3. Light chain library selection summary.

By screening of 95 random clones at the end of four rounds of library selection on each of the secondary antigens, the fractions of positive clones for each antigen were determined. The number of unique clones as determined by sequence analysis and the number of unique clones that maintain HER2 binding (dual specific clones) are listed. These clones show minimum background binding signals to irrelevant antigens, such as bovine serum albumin.

a.

	CDR-L1										CDR-L2					CDR-L3					Mutations/ Insertions		
	28	29	30	30a	30b	30c	30d	30e	31	32	33	50	51	52	53	91	92	93	93a	93b	94		
hu4D5	D	V	N						T	A	V	S	A	S	F	H	Y	T					
anti-VEGF	D	I	G	G					G	S	V	W	G	S	F	H	Y	T			T	7	
	D	I	H	S					G	S	V	W	G	S	Y	H	Y	T			T	8	
	D	V	F						T	S	V	D	A	S	Y	R	Y	I	W		A	8	
	D	I	W						R	A	V	P	A	S	N	G	Y	Y	I		A	9	
	D	I	W						R	W	V	A	A	S	S	H	A	G			A	9	
	Y	V	W						Q	Y	V	H	A	S	S	G	Y	W	V		A	10	
	D	I	P	S					G	A	V	G	A	S	S	G	Y	W	V		A	11	
	D	I	W						I	W	L	A	G	S	S	H	D	Q			A	11	
	E	V	Y						S	Y	V	P	A	S	S	G	F	W	I		G	11	
	N	V	W						D	W	V	P	A	S	S	G	W	Y	I		A	11	
	N	V	W						S	H	V	G	G	S	S	R	L	W	F		T	11	
	W	V	P	S					H	T	V	L	G	S	Y	S	D	Y			T	11	
	Y	V	Y	S					T	T	V	N	G	S	S	A	S	A			A	11	
	E	I	F	P					L	Y	V	L	G	S	S	W	D				G	11	
	N	I	F						S	H	V	L	P	G	S	Y	A	F	W	V		S	13
	Q	I	W						R	H	L	T	G	S	S	S	Y	W	V		A	13	
	Y	I	W						N	Y	V	G	G	S	S	S	W	W	A		G	13	
anti-DR5	S	V	S						S	A	V	S	A	S	S	Y	S	S			S	8	
	V	V	S						M	T	V	G	A	S	S	Y	G	S	Y		S	11	
	N	V	G						R	P	V	G	G	S	S	Y	G	S	F		G	12	
	R	I	N	S					H	T	V	W	G	S	H	Y	S	N	R		T	12	
	D	I	W	N	R				R	A	L	E	G	S	S	G	G	S	Y	S	14		
	D	I	W	N	R				R	A	L	K	G	S	S	G	G	S	Y	S	14		
anti-Fc	E	V	L						T	S	V	S	A	S	F	H	Y	T			T	3	
	K	I	Q						A	Y	V	S	A	S	F	H	Y	T			T	5	
	N	I	V	V	R				P	Y	V	S	A	S	F	H	Y	T			T	7	
	D	V	G	G	G				S	G	V	G	G	S	S	H	Y	T			T	8	
	D	I	G						A	G	L	S	A	S	F	S	E	S	R		S	10	
	D	I	S						R	Y	L	S	A	S	F	Y	G	W	R	R	T	10	
	D	V	G	G					L	G	L	S	A	S	S	G	G	A	D		T	10	
	D	V	N						R	Y	V	A	G	S	Y	G	I	D	L	A	T	10	
	D	V	S	R					Y	D	L	F	A	S	S	S	G	Y	H		T	11	
	V	V	R						Q	H	V	R	A	S	S	S	D	A	S		A	11	
	D	V	H	R					D	S	V	W	G	S	Q	W	T	W	A	D	T	12	
	D	V	H	P	S				P	R	V	L	G	S	S	A	N	V	D		T	12	
	E	I	S	R					Y	A	V	W	A	S	S	G	V	Y	N		A	12	
	N	V	P	R					W	S	L	W	A	S	S	W	V	T	H	E	T	12	
	P	V	F	R					R	G	L	S	A	S	S	G	L	R	H		G	12	
	A	V	P	R					Y	G	V	W	G	S	D	S	G	W	S		A	13	
	D	I	G	L					H	A	L	W	G	S	S	W	D	G	K		I	13	
D	I	R	G	Q				R	G	V	S	A	S	S	G	S	G	S		S	T	13	
D	V	S	G					R	G	L	D	A	S	S	G	A	A	Y		A	13		
E	I	V						F	S	V	G	G	S	S	G	D	S	K		S	13		
N	V	P	L					N	D	V	H	G	S	S	W	A	H	Y		G	13		
V	I	A	R					Y	D	L	W	A	S	S	A	G	A	R		A	14		
D	I	P	R	H				F	R	L	G	G	S	S	G	A	W	S	E	T	15		
Y	I	P	R					D	A	L	W	G	S	Y	W	A	S	W		D	A	15	
N	V	S	D	S	L	Q		N	S	V	L	G	S	S	G	L	D	L				16	
N	V	S	R	V	S	W	F	E	T	L	G	G	S	Y	W	F	T	W		A	G	17	

b.

	CDR-L1										CDR-L2					CDR-L3					Mutation s/Insertio	
	28	29	30	30a	30b	30c	30d	30e	31	32	33	50	51	52	53	91	92	93	93a	93b	94	ns. -
	hu4D5	D	V	N					T	A	V	S	A	S	F	H	Y	T				
anti-HER2/VEGF	D	V	W						K	W	V	A	A	S	S	H	Y	T			T	5
	D	I	K	N					G	S	V	W	A	S	F	H	Y	T			T	6
	D	I	L	G					G	S	V	W	A	S	F	H	Y	T			T	6
	D	I	M	S					G	S	V	W	A	S	F	H	Y	T			T	6
	D	I	R	A					G	S	V	W	A	S	F	H	Y	T			T	6
	D	I	R	G					G	S	V	W	A	S	F	H	Y	T			T	6
	D	V	R	Q					G	S	V	W	G	S	F	H	Y	T			T	6
	D	I	A	A					G	S	V	W	A	S	Y	H	Y	T			T	7
	D	I	A	G					G	S	V	W	A	S	Y	H	Y	T			T	7
	D	I	A	H					G	S	V	W	A	S	Y	H	Y	T			T	7
	D	I	A	K					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	G	A					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	G	A					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	G	G					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	G	L					G	S	V	W	A	S	Y	H	Y	T			T	7
	D	I	G	M					G	S	V	W	A	S	Y	H	Y	T			T	7
	D	I	K	H					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	L	A					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	L	G					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	L	I					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	L	T					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	M	L					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	Q	I					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	R	I					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	R	M					G	S	V	W	A	S	Y	H	Y	T			T	7
	D	I	R	Q					G	S	V	W	A	S	Y	H	Y	T			T	7
	D	I	R	T					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	R	V					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	S	M					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	S	R					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	S	V					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	V	S					G	S	V	W	G	S	F	H	Y	T			T	7
	D	I	W						H	W	V	A	G	S	S	H	Y	T			T	7
	N	I	A	Q					G	S	V	W	A	S	F	H	Y	T			T	7
	D	I	A	M					G	S	L	W	G	S	F	H	Y	T			T	8
	D	I	A	F					G	S	V	W	G	S	Y	H	Y	T			T	8
	D	I	A	R					G	S	V	W	G	S	M	H	Y	T			T	8
	D	I	A	S					G	S	V	W	G	S	L	H	Y	T			T	8
	D	I	A	S					G	S	V	W	G	S	S	H	Y	T			T	8
	D	I	G	S					G	S	V	W	G	S	Y	H	Y	T			T	8
	D	I	I	G					G	S	V	W	G	S	Y	H	Y	T			T	8
	D	I	K	A					G	S	V	W	G	S	Y	H	Y	T			T	8
	D	I	K	F					G	S	V	W	G	S	Y	H	Y	T			T	8
	D	I	K	L					G	S	V	W	G	S	L	H	Y	T			T	8
	D	I	K	L					G	S	V	W	G	S	M	H	Y	T			T	8
	D	I	K	S					G	S	V	W	G	S	T	H	Y	T			T	8
	D	I	K	V					G	S	V	W	G	S	Y	H	Y	T			T	8
D	I	K	W					G	S	V	W	G	S	T	H	Y	T			T	8	
D	I	L	K					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	L	S					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	Q	R					G	S	V	W	G	S	C	H	Y	T			T	8	
D	I	Q	S					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	Q	T					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	R	E					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	R	F					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	R	G					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	R	L					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	R	M					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	R	R					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	R	R					G	S	V	W	G	S	A	H	Y	T			T	8	
D	I	R	S					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	R	S					G	S	V	W	G	S	T	H	Y	T			T	8	
D	I	R	S					G	S	V	W	G	S	N	H	Y	T			T	8	
D	I	Q	S					G	S	V	W	G	S	E	H	Y	T			T	8	
D	I	R	S					G	S	V	W	G	S	S	H	Y	T			T	8	
D	I	R	V					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	S	S					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	T	M					G	S	V	W	G	S	L	H	Y	T			T	8	
D	I	Y	M					G	S	V	W	G	S	Y	H	Y	T			T	8	
D	I	A	T					G	S	L	W	G	S	Y	H	Y	T			T	9	
D	I	K	S					G	S	L	W	G	S	Y	H	Y	T			T	9	
D	I	R	G					G	S	V	W	G	S	Y	Y	T			T	9		
G	I	R	T					G	S	V	W	G	S	Y	H	Y	T			T	9	
N	I	A	M					G	S	V	W	G	S	Y	H	Y	T			T	9	
N	I	R	S					G	S	V	W	G	S	Y	H	Y	T			T	9	
N	I	R	T					G	S	V	W	G	S	Y	H	Y	T			T	9	
N	I	Y	A					G	S	L	W	G	S	Y	H	Y	T			T	10	
N	I	Y	S					G	S	L	W	G	S	Y	H	Y	T			T	10	
D	I	P	R	S	I	S		G	S	V	W	G	S	Y	H	Y	T			T	11	
anti-HER2/DR5 anti-HER2/Ec	N	I	R	N	G			G	G	L	S	A	S	F	H	Y	T			T	8	
	N	O	V	S				K	H	V	W	G	S	Y	S	Y	S			G	10	
	Q	Y	S	K				Y	D	V	W	G	S	S	G	F	R	S		S	13	

	bH1 Fab/hVEGF complex	bH1 Fab/HER2-ECD complex
Data Collection Statistics		
Space group	C222 ₁	P2 ₁ 2 ₁ 2 ₁
Unit Cell (Å)	a=100.6, b=198.0, c=77.7	a=62.3, b=115.1, c=208.2
Beamline, wavelength	ALS 5.0.1	ALS 5.0.1
Resolution (Å)	50.0-2.6	50.0-2.9
Rsym ^{*a}	0.090 (0.66)	0.095 (0.66)
Number of Observations	151689	192951
Unique Reflections	24705	34149
Completeness (%) [*]	99.8 (100)	100 (100)
Iσ(I) [*]	16.0 (3.0)	18.5 (2.6)
Refinement Statistics		
Content of asymmetric unit	1/2 VEGF dimer, 1 Fab	1 Her2-ECD monomer, 1 Fab
Resolution (Å)	30.0-2.6	30.0-2.9
Reflections used	22977	32277
R Factor ^b , Rfree	0.19, 0.25	0.22, 0.28
RMS Deviation Bonds (Å)	0.011	0.010
RMS Deviation Angles (°)	1.3	1.3
Ramachandran Statistics		
Favoured Regions (%)	96.5%	89.9%
Allowed Regions (%)	99.4%	97.9%
Outliers (%)	0.6%	2.1%
Number of Residues	528	1017
Numbers of Waters	49	0
Number of Sugars	0	2
Number of Ligands/Ions	1 (Glycerol)	1 (MES)

Table S5. Crystallographic statistics

Rsym^a = $\sum |I - \langle I \rangle| / \sum I$. $\langle I \rangle$ is the average intensity of symmetry-related observations of a unique reflection

R Factor^b = $\sum |F_0 - F_c| / \sum F_0$. Rfree is calculated as R except for 5% of the reflections excluded from all refinement

*Values in parenthesis denote values of the highest resolution shell.

bH1 residue	Area buried by HER2 (%)	HER2 residues contacting bH1	Area buried by VEGF (%)	VEGF residues contacting bH1
Heavy Chain	Y33	48 E558 F573	87	H86
	R50	97 E558 D560 F573	35	H86
	Y52		30	H86
	Y56	42 P557 E558		
	R58	50 E558 Q561		
	W95	100 P572 F573	61	H86 Q87
	G99	93 D570 P579 K593	75	K48 I83 Q89
	Y100a	80 D570 P571 P572 F573	88	I83 K84 P85 H86 Q87 G88 Q89
Light Chain	S28		59	I91 G92 E93
	I29		77	R82 H90 I91 G92
	S30		69	H90 I91
	G31		85	G88 Q89 H90 I91
	Y32	97 D570 P571 A600 C601 Q602	89	Q89 H90
	W50	62 K593 P603	59	F17 M81 Q89
	Y53	44 P603 C604 P605	74	F17 M18 I91
	H91	90 P571 P572	81	G88 Q89
	Y92	55 K569 P571 P572	76	Y45 K84 G88 Q89 H90
	T93		61	K84 Q87 G88
	T94	68 D560 P572	55	H86 Q87

Table S6. List of structural contacts of the complex of bH1 Fab/HER2 ECD and bH1 Fab/VEGF₁₀₉. The table lists bH1 residues with > 25% of the total surface area buried upon HER2 or VEGF binding. The VEGF and HER2 residues within 4.5 Å of the bH1 residues are listed. The surface area of each residue that is buried upon complex formation was calculated using IMOL based on the coordinates of the crystal structures 3BDY, 3BE1 and 1N8Z (PDB).

a.

	Antigen selection (VEGF)				Display Selection (anti-gD)				F _{wt/mut} values				ΔΔG _{wt/mut} (kcal/mol)			
	wt/m1	wt/m2	wt/m3	wt/m4	wt/m1	wt/m2	wt/m3	wt/m4	F _{wt/m1}	F _{wt/m2}	F _{wt/m3}	F _{wt/m4}	ΔΔG _{wt/m1}	ΔΔG _{wt/m2}	ΔΔG _{wt/m3}	ΔΔG _{wt/m4}
CDR-L1	Q27			1.4				1.0				1.4 ^E			0.2 ^E	
	D28	1.8		3.5	1.7			1.3	1.1 ^A			2.7 ^E	0 ^A			0.6 ^E
	I29	12.8	17.0	1.9	1.4	2.0	3.0	1.0	1.2	6.5 ^A	5.7 ^T	1.9 ^V	1.2 ^L	1.1 ^A	1.0 ^T	0.1 ^L
	P30	1.7		3.8	1.2			1.3	1.5 ^A			2.9 ^A	0.2 ^A			0.6 ^A
	R30a	1.9	4.1	1.9	0.8	2.4	0.9	0.9	1.9	0.8 ^A	4.5 ^G	2.1 ^P	0.4 ^K	-0.2 ^A	0.9 ^G	0.4 ^P
	S30b**	5.5		0.7	1.1			1.1	5.2 ^A			0.6 ^T	1.0 ^A			-0.3 ^T
	I30c***	12.7	7.6	1.0	23.8	3.2	2.8	1.0	1.0	3.9 ^A	2.7 ^T	1.0 ^V	23.2 ^L	0.8 ^A	0.6 ^T	0 ^V
	S30d**	13.2		16.9	1.4			1.3	9.1 ^A			13.0 ^T	1.3 ^A			1.9 ^T
	G31***	16.0		35.5	2.0			2.9	8.0 ^A			12.2 ^A	1.2 ^A			1.5 ^A
	Y32***	26.0	78.0	26.0	23.0	1.4	1.9	1.1	0.8	18.1 ^A	41.2 ^D	23.1 ^S	1.7 ^A	2.2 ^D	1.9 ^S	>2.0 ^F
CDR-L2	W50**	39.0	26.0	39.0	20.4	4.2	1.2	1.7	1.8	9.3 ^A	21.7 ^G	23.2 ^S	11.6 ^L	1.3 ^A	1.8 ^G	1.9 ^S
	G51	16.0		20.4	3.0				2.0	5.3 ^A			10.2 ^A	1.0 ^A		1.4 ^A
	S52	2.0		6.5	1.6				1.6	1.3 ^A			4.0 ^A	0.1 ^A		0.8 ^A
	Y53**	9.0	63.0	4.5	0.1	2.7	2.0	1.1	0.8	3.4 ^A	31.5 ^D	4.2 ^S	0.1 ^F	0.7 ^A	2.0 ^D	0.8 ^S
CDR-L3	H91***	80.0	40.0	40.0	>150	2.7	2.1	2.1	1.9	30 ^A	19.2 ^D	19.2 ^P	>80 ^N	2.0 ^A	1.7 ^D	1.7 ^P
	Y92***	25.0	12.5	75.0	1.0	2.4	1.7	0.7	0.5	10.6 ^A	7.2 ^D	104.5 ^S	2.0 ^F	1.4 ^A	1.2 ^D	2.8 ^S
	T93**	0.7		0.9	1.0				1.5	0.7 ^A			0.6 ^S	-0.3 ^A		-0.3 ^S
	T94**	0.7		0.6	0.6				1.3	1.1 ^A			0.4 ^S	0 ^A		-0.5 ^S
CDR-H1	K30	4.4	1.9	3.0	1.5	3.3	1.7	1.9	1.1	1.3 ^A	1.2 ^E	1.6 ^T	1.3 ^R	0.2 ^A	0.1 ^E	0.3 ^T
	D31	2.2		0.6	1.6				0.9	1.3 ^A			0.7 ^E	0.2 ^A		-0.2 ^E
	T32	0.8		2.1	1.5				0.6	0.5 ^A			3.3 ^S	-0.4 ^A		0.7 ^S
	Y33***	2.5	0.3	0.2	5.9	1.5	1.3	1.5	2.5	1.7 ^A	0.2 ^D	0.1 ^S	2.4 ^F	0.3 ^A	-1.0 ^D	-1.2 ^S
CDR-H2	R50*	0.6	0.6	11.0	1.6	1.1	0.5	9.0	1.0	0.6 ^A	1.0 ^G	1.2 ^P	1.6 ^K	-0.3 ^A	0 ^G	0.1 ^P
	Y52*	4.4	74.0	9.3	1.2	2.2	1.6	1.6	0.9	2.0 ^A	45.5 ^D	5.7 ^S	1.3 ^F	0.4 ^A	2.3 ^D	1.0 ^S
	T53	0.4		0.7	0.9				0.6	0.4 ^A			1.2 ^S	-0.5 ^A		0.1 ^S
	N54	0.6	0.2	0.6	0.7	1.0	1.1	1.9	1.0	0.7 ^A	0.2 ^D	0.3 ^T	0.7 ^D	-0.2 ^A	-1.0 ^D	-0.7 ^T
	Y56	5.3	6.4	4.9	0.8	3.8	3.1	1.9	0.8	1.4 ^A	2.0 ^D	2.6 ^S	1.1 ^F	0.2 ^A	0.4 ^D	0.6 ^S
	R58	0.8	0.5	24.0	1.2	1.1	0.8	2.4	2.1	0.7 ^A	0.7 ^G	9.9 ^P	0.6 ^K	-0.2 ^A	-0.2 ^G	1.4 ^P
CDR-H3	W95**	>102	>102	1.6	>96	3.6	1.1	0.7	0.5	>28 ^A	>91 ^G	2.3 ^S	>209 ^L	>2.0 ^A	>2.7 ^G	0.5 ^S
	G96	1.9		5.3	1.3				1.4	1.4 ^A			3.8 ^A	0.2 ^A		0.8 ^A
	G97	1.4		4.9	1.7				1.2	0.8 ^A			4.0 ^A	-0.1 ^A		0.8 ^A
	D98	1.0		1.6	1.0				0.9	1.0 ^A			1.7 ^E	0 ^A		0.3 ^E
	G99***	0.3		0.5	1.2				1.9	0.3 ^A			0.3 ^A	-0.8 ^A		-0.8 ^A
	F100	11.5	23.0	1.0	8.6	3.4	2.2	1.6	2.5	3.4 ^A	10.5 ^S	0.6 ^V	3.4 ^T	0.7 ^A	1.4 ^S	-0.3 ^V
	Y100a***	4.8	57.0	1.9	0.3	2.1	2.4	1.4	0.5	2.3 ^A	24.2 ^D	1.3 ^S	0.6 ^F	0.5 ^A	1.9 ^D	0.2 ^S
																-0.3 ^F

b.

	Antigen Selection (HER2)				Display Selection (anti-gD)				F _{wt/mut} values				ΔΔG _{wt/mut} (kcal/mol)			
	wt/m1	wt/m2	wt/m3	wt/m4	wt/m1	wt/m2	wt/m3	wt/m4	F _{wt/m1}	F _{wt/m2}	F _{wt/m3}	F _{wt/m4}	ΔΔG _{wt/m1}	ΔΔG _{wt/m2}	ΔΔG _{wt/m3}	ΔΔG _{wt/m4}
CDR-L1	Q27			1.6				1.0				1.6 ^E			0.3 ^E	
	D28	1.8		1.7	1.7			1.3	1.1 ^A			1.3 ^E	0 ^A			0.2 ^E
	I29	1.5	2.0	0.8	1.3	2.0	3.0	1.0	1.2	0.8 ^A	0.7 ^T	0.8 ^V	1.1 ^L	-0.2 ^A	-0.2 ^T	0.1 ^L
	P30	1.3		0.8	1.2			1.3	1.1 ^A			0.6 ^A	0.1 ^A			-0.3 ^A
	R30a	1.7	1.8	1.7	1.4	2.4	0.9	0.9	1.9	0.7 ^A	1.9 ^G	1.8 ^P	0.7 ^K	-0.2 ^A	0.4 ^G	0.3 ^P
	S30b	1.7		1.7	1.1			1.1	1.6 ^A			1.6 ^T	0.3 ^A			0.3 ^T
	I30c	14.8	11.8	2.0	1.3	3.2	2.8	1.0	1.0	4.6 ^A	4.2 ^T	2.0 ^V	1.2 ^L	0.9 ^A	0.9 ^T	0.4 ^V
	S30d	1.0		2.2	1.4			1.3	0.7 ^A			1.7 ^T	-0.2 ^A			0.3 ^T
	G31*	3.4		5.1	2.0			2.9	1.7 ^A			1.8 ^A	0.3 ^A			0.3 ^A
	Y32***	0.4	12.0	0.2	1.8	1.4	1.9	1.1	0.8	0.3 ^A	6.3 ^D	0.2 ^S	2.1 ^F	-0.8 ^A	1.1 ^D	-1.0 ^S
CDR-L2	W50**	45.5	22.8	>98	17.0	4.2	1.2	1.7	1.8	10.8 ^A	19 ^G	>58 ^S	9.6 ^L	1.4 ^A	1.7 ^G	>2.4 ^S
	G51	1.9		1.7	3.0				2.0	0.6 ^A			0.9 ^A	-0.3 ^A		-0.1 ^A
	S52	2.9		3.0	1.6				1.6	1.8 ^A			1.9 ^A	0.3 ^A		0.4 ^A
	Y53*	11.3	39.5	8.8	0.6	2.7	2.0	1.1	0.8	4.2 ^A	19.8 ^D	8.1 ^S	0.8 ^F	0.9 ^A	1.8 ^D	1.2 ^S
CDR-L3	H91***	12.4	1.0	>98	2.8	2.7	2.1	2.1	1.9	4.7 ^A	0.5 ^D	>47 ^P	1.5 ^N	0.9 ^A	-0.4 ^D	>2.3 ^P
	Y92**	5.5	38.5	81.0	1.8	2.4	1.7	0.7	0.5	2.3 ^A	22.2 ^D	112.9 ^S	3.5 ^F	0.5 ^A	1.8 ^D	2.8 ^S
	T93	0.5		0.7	1.0				1.5	0.5 ^A			0.5 ^S	-0.4 ^A		-0.4 ^S
	T94**	6.5		1.4	0.6				1.3	10 ^A			1.1 ^S	1.4 ^A		0.1 ^S
CDR-H1	K30	2.1	1.7	2.1	1.1	3.3	1.7	1.9	1.1	0.6 ^A	1.0 ^E	1.1 ^T	1.0 ^R	-0.3 ^A	0 ^E	0.1 ^T
	D31	3.1		0.7	1.6				0.9	1.9 ^A			0.7 ^E	0.4 ^A		-0.2 ^E
	T32	0.8		0.7	1.5				0.6	0.5 ^A			1.2 ^S	-0.4 ^A		0.1 ^S
	Y33*	>81	>81	>81	47.0	1.5	1.3	1.5	2.5	>55 ^A	>62 ^D	>52 ^S	19.1 ^F	>2.4 ^A	>2.4 ^D	>2.3 ^S
CDR-H2	R50***	>81	>81	>81	95.0	1.1	0.5	9.0	1.0	>72 ^A	>153 ^G	>9 ^P	96.8 ^K	>2.5 ^A	>3.0 ^G	>1.3 ^P
	Y52	0.6	2.7	0.5	0.6	2.2	1.6	1.6	0.9	0.3 ^A	1.6 ^G	0.3 ^S	0.7 ^F	-0.8 ^A	0.3 ^G	-0.7 ^S
	T53	1.0		0.7	0.9				0.6	1.1 ^A			1.2 ^S	0.1 ^A		0.1 ^S
	N54	0.3	0.5	0.5	1.0	1.0	1.1	1.9	1.0	0.3 ^A	0.5 ^D	0.2 ^T	1.0 ^D	-0.8 ^A	-0.5 ^D	-0.9 ^T
	Y56*	>81	>81	>81	1.2	3.8	3.1	1.9	0.8	>21 ^A	>26 ^D	>42 ^S	1.6 ^F	>1.8 ^A	>1.9 ^D	>2.2 ^S
	R58**	>81	80.0	>81	>96	1.1	0.8	2.4	2.1	>73 ^A	104.8 ^G	>33 ^P	>46 ^K	>2.5 ^A	2.8 ^G	>2.1 ^P
CDR-H3	W95***	>81	>81	>81	>96	3.6	1.1	0.7	0.5	>22 ^A	>73 ^G	>117 ^S	>209 ^L	>1.8 ^A	>2.5 ^G	>2.8 ^S
	G96	1.5		0.6	1.3				1.4	1.1 ^A			0.4 ^A			-0.5 ^A
	G97	3.1		3.8	1.7				1.2	1.8 ^A			3.0 ^A	0.3 ^A		0.7 ^A
	D98	0.8		0.4	1.0				0.9	0.8 ^A			0.4 ^E	-0.1 ^A		-0.5 ^E
	G99***	26.0		18.0	1.2				1.9	22.3 ^A			9.5 ^A	1.8 ^A		1.3 ^A
	F100	>81	>81	39.5	95.0	3.4	2.2	1.6	2.5	>24 ^A	>37 ^S	24.2 ^V	38.0 ^V	>1.9 ^A	>2.1 ^S	1.9 ^V
	Y100a***	80.0	>81	>81	6.3	2.1	2.4	1.4	0.5	38 ^A	>34 ^D	>56.7 ^S	13.8 ^F	2.2 ^A	>2.1 ^D	>2.4 ^S
																1.6 ^F

Table S7. Shotgun alanine- and homolog-scanning of bH1 Fab for binding to VEGF and HER2.

The effects of mutation to alanine (m1), or additional mutations (m2, m3; due to limitations of shotgun codons), or to a homologous amino acid (m4) are calculated as the ratio of occurrence of wild type (wt) and mutants (mut), wt/mut, for VEGF (a) or HER2 (b) binding clones. When the wt

Shape complementarity in antibody/antigen complexes		
Antibody	Antigen	Sc*
Herceptin	HER2	0.75
bH1	HER2	0.72
bH1	VEGF	0.68
bH1 (VEGF-bound conformation)	HER2	0.40
bH1 (HER2-bound conformation)	VEGF	0.44

* Sc=Median Shape Complementarity Statistic

Table S8. The different surface conformations of bH1 for binding HER2 and VEGF. The shape complementarity (represented as Sc) between the antibody and the antigen was determined as described (*Lawrence MC, Colman PM. J Mol Biol. 234, 946-50, 1993*). The high shape complementarity in the bH1/VEGF and bH1/HER2 complexes, similar to the complementarity between Herceptin and HER2, are in the range of reported antibody-antigen complexes (Sc ~ 0.64-0.68. *Above reference*). Superposition of HER2 with bH1 in its VEGF-bound conformation or VEGF with bH1 in its HER2-bound form reveals little shape complementarity in each complex. The low Sc values are similar to the complementarity observed when juxtaposing an antibody with an unrelated antigen. (Sc~0.35. *Above reference*). The results demonstrate the extent to which bH1 rearranges to accommodate the two different antigens.

Antibody	Light Chain																		Kd (nM)								
	CDR-L1									CDR-L2						CDR-L3						hVEGF		mVEGF		HER2	
	28	29	30	30a	30b	30c	30d	31	32	50	51	52	53	54	55	91	92	93	94	95	96	Fab	IgG	Fab	IgG	Fab	IgG
bH1	D	I	P	R	S	I	S	G	Y	W	G	S	Y	L	Y	H	Y	T	T	P	P	300	250	>1000	>1000	26	21
bH1-81	N		A	K	T								F					S	S			58	41	ND	150	6	7
bH1-44	N		A	K	T								F					S	S			3	16	33	36	0.2	1

Antibody	Heavy Chain																									
	CDR-H1						CDR-H2										CDR-H3									
	28	29	30	31	32	33	50	51	52	52a	53	54	55	56	57	58	95	96	97	98	99	100	100a	100b	101	102
bH1	N	I	K	D	T	Y	R	I	Y	P	T	N	G	Y	T	R	W	G	G	D	G	F	Y	A	M	D
bH1-81																										
bH-44			S	G							S	E						V		V						

Table S9. Variants of bH1 with improved affinity

The table summarizes the CDR sequences and the affinities (as determined by surface plasmon resonance) of the clones with improved binding affinity as Fab or IgG . The bH1 variants were isolated in two steps by selection and screening of phage-displayed libraries, which were generated by mutating the HC and LC CDRs of bH1 based on the information of the functional paratopes of bH1 for VEGF and HER2 described in Table S7. The improved clone bH1-81 was isolated by affinity-based selections of the described LC homolog shotgun scan library. In the second step, the highest affinity clone bH1-44 was isolated from a library constructed by randomizing residues of bH1-81. Randomized positions are shown in bold. The monovalent affinities of the antibodies for human VEGF₈₋₁₀₉, murine VEGF₁₋₁₀₈ and HER2 ECD were measured by BIAcore as described in Methods. The table shows representative dissociation constants (K_D) for each binding interaction based on three independent measurements. The bH1 variants bind the full length protein (VEGF₁₋₁₆₅) and VEGF₈₋₁₀₉ with similar affinity in solution competition experiments. ND= Not determined. bH1-44 was selected for improved binding to murine VEGF in addition to human VEGF, in order to enable the assessment of its *in vivo* activity in inhibiting tumor xenograft growth. Both tumor-produced VEGF (human) and stromal VEGF (murine, from mouse) may contribute to tumor growth.

Group	Antibody Concentration (µg/ml)	
	day 7	day 21
Control IgG 10 mg/kg	65 +/- 10	n/a
Herceptin 10 mg/kg	83 +/- 38	n/a
anti-VEGF 10 mg/kg	20 +/- 6	36 +/- 10
anti-VEGF+Herceptin 10+10 mg/kg	41 +/- 20	63 +/- 31
bH1-44 10 mg/kg	30 +/- 9	46 +/- 25
bH1-44 20 mg/kg	37 +/- 7	34 +/- 15

Table S10. Antibody levels circulating in mouse.

The antibody concentrations in the serum from Colo205 tumor bearing mice 7 days after the first and third treatment (day 7 and 21) were measured by ELISA. Antibody was present at high levels in serum from all treatment groups (Mean +/- SEM, N=5). The concentrations represented the low (trough) levels before additional treatment cycle and are typical and expected based on the human IgG pharmacokinetic behavior in mice. Similar levels were observed from BT474M1 tumor bearing mice. Data for the control and Herceptin groups were not available (n/a) on day 21 as these groups were terminated. The serum levels of bH1-44 and anti-VEGF treated groups are lower than those of Herceptin-treated groups, likely due to the cross-reactivity of bH1-44 and anti-VEGF (B20-4.1) with murine VEGF as previously described (S14).

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