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(54) Title: VEGF-BINDING MOLECULES

(57) Abstract: VEGF-binding molecules, preferably VEGF-binding immunoglobulin single variable domains like VHHs anddomain antibodies, pharmaceutical compositions containing same and their use in the treatment of diseases that are associated with VEGF- mediated effects on angiogenesis.Nucleic acids encoding VEGF-binding molecules, host cells and methods for preparing same.

VEGF-BINDING MOLECULES

FIELD OF THE INVENTION

The invention relates to the field of human therapy, in particular cancer therapy
5 and agents and compositions useful in such therapy.

BACKGROUND OF THE INVENTION

As described in e.g. US 2008/0014196 and WO2008101985, angiogenesis is implicated in the pathogenesis of a number of disorders, including solid tumors and metastasis as well as eye diseases. One of the most important pro-
10 angiogenic factors is vascular endothelial growth factor (VEGF), also termed VEGF-A or vascular permeability factor (VPF). VEGF belongs to a gene family that includes placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and VEGF-F. Alternative splicing of mRNA of a single gene of human VEGF results in at least six isoforms (VEGF121, VEGF145, VEGF165,
15 VEGF183, VEGF189, and VEGF206), VEGF165 being the most abundant isoform.

Two VEGF tyrosine kinase receptors (VEGFR) have been identified that interact with VEGF, i.e. VEGFR-1 (also known as Flt-1) and VEGFR-2 (also known as KDR or FIK-1). VEGFR-1 has the highest affinity for VEGF, while
20 VEGFR- 2 has a somewhat lower affinity for VEGF. Ferrara (Endocrine Rev. 2004, 25: 581-611) provide a detailed description of VEGF, the interaction with its receptors and its function in normal and pathological processes can be found in Hoeben *et al*. Pharmacol. Rev. 2004, 56: 549-580.

VEGF has been reported to be a pivotal regulator of both normal and abnormal
25 angiogenesis (Ferrara and Davis-Smyth, Endocrine Rev. 1997, 18: 4-25;

Ferrara J. MoL Med. 1999, 77: 527-543). Compared to other growth factors that contribute to the processes of vascular formation, VEGF is unique in its high specificity for endothelial cells within the vascular system.

VEGF mRNA is overexpressed by the majority of human tumors. In the case of tumor growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia, and for providing nourishment for the growth and metastasis of the tumor (Folkman *et al.*, 1989, Nature 339 -58), which allows the tumor cells to acquire a growth advantage compared to the normal cells. Therefore, anti-angiogenesis therapies have become an important treatment option for several types of tumors. These therapies have focused on blocking the VEGF pathway (Ferrara *et al.*, Nat Rev Drug Discov. 2004 May; 3(5): 391-400.

VEGF is also involved in eye diseases. The concentration of VEGF in eye fluids is highly correlated with the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies. Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by age-related macular degeneration (AMD). Up-regulation of VEGF has also been observed in various inflammatory disorders. VEGF has been implicated in the pathogenesis of RA, an inflammatory disease in which angiogenesis plays a significant role.

The elucidation of VEGF and its role in angiogenesis and different processes has provided a potential new target of therapeutic intervention. The function of VEGF has been inhibited by small molecules that block or prevent activation of VEGF receptor tyrosine kinases (Schlaeppi and Wood, 1999, Cancer Metastasis Rev., 18: 473-481) and consequently interfere with the VEGF receptor signal transduction pathway. Cytotoxic conjugates containing bacterial or plant toxins can inhibit the stimulating effect of VEGF on tumor

angiogenesis. VEGF-DT385 toxin conjugates (diphtheria toxin domains fused or chemically conjugated to VEGF165), for example, efficiently inhibit tumor growth in vivo. Tumor growth inhibition could also be achieved by delivering a Flk-1 mutant or soluble VEGF receptors by a retrovirus.

5 VEGF-neutralizing antibodies, such as A4.6.I and MV833, have been developed to block VEGF from binding to its receptors and have shown preclinical antitumor activity (Kim *et al.* Nature 1993, 362: 841-844; Folkman Nat. Med. 1995, 1: 27-31; Presta *et al.* Cancer Res. 1997, 57: 4593-4599; Kanai *et al*. Int. J. Cancer 1998, 77: 933-936; Ferrara and Alitalo Nat. Med.
10 1999, 5: 1359-1364; 320, 340. For a review of therapeutic anti-VEGF approaches trials, see Campochiaro and Hackett (Oncogene 2003, 22: 6537-6548).

Most clinical experience has been obtained with A4.6.1, also called bevacizumab (Avastin®; Genentech, San Francisco, CA).

15 WO2008101985 describes immunoglobulin single variable domains from camelides (VHHs or “Nanobodies®, as defined herein) that bind to VEGF, and their use in the treatment of conditions and diseases characterized by excessive and/or pathological angiogenesis or neovascularization.

It has been an object of the present invention to provide novel improved
20 VEGF-binding molecules.

It has been a further object of the invention to provide methods for the prevention, treatment, alleviation and/or diagnosis of such diseases, disorders or conditions, involving the use and/or administration of such agents and compositions. In particular, it is has been an object of the invention to provide
25 such pharmacologically active agents, compositions and/or methods that provide advantages compared to the agents, compositions and/or methods currently used and/or known in the art. These advantages include improved

therapeutic and/or pharmacological properties and/or other advantageous properties, e.g. for manufacturing purposes, especially as compared to conventional anti-VEGF antibodies as those described above, or fragments thereof.

5 More in particular, it has been an object of the invention to provide novel VEGF-binding molecules, and, specifically, VEGF-binding molecules that bind to mammalian VEGF and, especially, human VEGF, wherein such molecules or polypeptides are suitable for the therapeutic and diagnostic purposes as described herein. It has been a further object of the invention to provide
10 immunoglobulin single variable domains that specifically bind to VEGF.

BRIEF SUMMARY OF THE INVENTION

According to a first aspect, there are provided VEGF-binding molecules, preferably VEGF-binding immunoglobulin single variable domains like VHHs and domain antibodies.

15 In another aspect, the invention relates to nucleic acids encoding VEGF-binding molecules as well as host cells containing such nucleic acids.

The invention further relates to a product or composition containing or comprising at least one VEGF-binding molecule of the invention and optionally one or more further components of such compositions.

20 The invention further relates to methods for preparing or generating the VEGF-binding molecules, nucleic acids, host cells, products and compositions described herein.

The invention further relates to applications and uses of the VEGF-binding molecules, nucleic acids, host cells, products and compositions described
25 herein, as well as to methods for the prevention and/or treatment for diseases associated with VEGF-mediated effects on angiogenesis.

These and other aspects, embodiments, advantages and applications of the invention will become clear from the further description hereinbelow.

DEFINITIONS

Unless indicated or defined otherwise, all terms used have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to the standard handbooks, such as Sambrook *et al*, "Molecular Cloning: A Laboratory Manual" (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory Press (1989); Lewin, "Genes IV", Oxford University Press, New York, (1990), and Roitt *et al.*, "Immunology" (2nd Ed.), Gower Medical Publishing, London, New York (1989), as well as to the general background art cited herein; Furthermore, unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail can be performed and have been performed in a manner known *per se*, as will be clear to the skilled person. Reference is for example again made to the standard handbooks, to the general background art referred to above and to the further references cited therein.

Unless indicated otherwise, the terms "*immunoglobulin*" and "*immunoglobulin sequence*" - whether used herein to refer to a *heavy chain antibody* or to a *conventional 4-chain antibody* - are used as general terms to include both the full-size antibody, the individual chains thereof, as well as all parts, domains or fragments thereof (including but not limited to antigen-binding domains or fragments such as VHH domains or VH/VL domains, respectively). In addition, the term "sequence" as used herein (for example in terms like "immunoglobulin sequence", "antibody sequence", "(single) variable domain sequence", "VHH sequence" or "protein sequence"), should generally be understood to include both the relevant amino acid sequence as well as nucleic acid sequences or nucleotide sequences encoding the same, unless the context requires a more limited interpretation.

The term "*domain*" (of a polypeptide or protein) as used herein refers to a folded protein structure which has the ability to retain its tertiary structure independently of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins, and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain.

The term "*immunoglobulin domain*" as used herein refers to a globular region of an antibody chain (such as e.g. a chain of a conventional 4-chain antibody or of a heavy chain antibody), or to a polypeptide that essentially consists of such a globular region. Immunoglobulin domains are characterized in that they retain the immunoglobulin fold characteristic of antibody molecules, which consists of a 2-layer sandwich of about 7 antiparallel beta-strands arranged in two beta-sheets, optionally stabilized by a conserved disulphide bond.

The term "*immunoglobulin variable domain*" as used herein means an immunoglobulin domain essentially consisting of four "framework regions" which are referred to in the art and hereinbelow as "framework region 1" or "FR1"; as "framework region 2" or"FR2"; as "framework region 3" or "FR3"; and as "framework region 4" or "FR4", respectively; which framework regions are interrupted by three "complementarity determining regions" or "CDRs", which are referred to in the art and hereinbelow as "complementarity determining region 1"or "CDR1"; as "complementarity determining region 2" or "CDR2"; and as "complementarity determining region 3" or "CDR3", respectively. Thus, the general structure or sequence of an immunoglobulin variable domain can be indicated as follows: FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. It is the immunoglobulin variable domain(s) that confer specificity to an antibody for the antigen by carrying the antigen-binding site.

The term "*immunoglobulin single variable domain*" as used herein means an immunoglobulin variable domain which is capable of specifically binding to an

epitope of the antigen without pairing with an additional variable immunoglobulin domain. One example of immunoglobulin single variable domains in the meaning of the present invention are "*domain antibodies*", such as the immunoglobulin single variable domains VH and VL (VH domains and VL domains). Another example of immunoglobulin single variable domains are "*VHH domains*" (or simply "VHHs") from camelids, as defined hereinafter.

In view of the above definition, the antigen-binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab fragment, a F(ab')2 fragment, an Fv fragment such as a disulphide linked Fv or a scFv fragment, or a diabody (all known in the art) derived from such conventional 4-chain antibody, would normally not be regarded as an immunoglobulin single variable domain, as, in these cases, binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associating) immunoglobulin domains such as light and heavy chain variable domains, i.e. by a VH-VL pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

"*VHH domains*", also known as VHHs, V_HH domains, VHH antibody fragments, and VHH antibodies, have originally been described as the antigen binding immunoglobulin (variable) domain of "heavy chain antibodies" (i.e. of "antibodies devoid of light chains"; Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R.: "Naturally occurring antibodies devoid of light chains"; Nature 363, 446-448 (1993)). The term "VHH domain" has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_H domains" or "VH domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as

"V_L domains" or "VL domains"). VHH domains can specifically bind to an epitope without an additional antigen binding domain (as opposed to VH or VL domains in a conventional 4-chain antibody, in which case the epitope is recognized by a VL domain together with a VH domain). VHH domains are small, robust and efficient antigen recognition units formed by a single immunoglobulin domain.

In the context of the present invention, the terms VHH domain, VHH, V_HH domain, VHH antibody fragment, VHH antibody, as well as "Nanobody®" and "Nanobody® domain" ("Nanobody" being a trademark of the company Ablynx N.V.; Ghent; Belgium) are used interchangeably and are representatives of immunoglobulin single variable domains (having the structure FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 and specifically binding to an epitope without requiring the presence of a second immunoglobulin variable domain), and which are distinguished from VH domains by the so-called "hallmark residues", as defined in e.g. WO2009/109635, Fig. 1.

The amino acid residues of a immunoglobulin single variable domain, e.g. a VHH, are numbered according to the general numbering for V_H domains given by Kabat et al. ("Sequence of proteins of immunological interest", US Public Health Services, NIH Bethesda, MD, Publication No. 91), as applied to VHH domains from Camelids, as shown e.g. in Figure 2 of Riechmann and Muyldermans, J. Immunol. Methods 231, 25-38 (1999). According to this numbering,

- FR1 comprises the amino acid residues at positions 1-30,
- CDR1 comprises the amino acid residues at positions 31-35,
- FR2 comprises the amino acids at positions 36-49,
- CDR2 comprises the amino acid residues at positions 50-65,
- FR3 comprises the amino acid residues at positions 66-94,

- CDR3 comprises the amino acid residues at positions 95-102, and
- FR4 comprises the amino acid residues at positions 103-113.

However, it should be noted that - as is well known in the art for V_H domains and for VHH domains - the total number of amino acid residues in each of the CDRs may vary and may not correspond to the total number of amino acid residues indicated by the Kabat numbering (that is, one or more positions according to the Kabat numbering may not be occupied in the actual sequence, or the actual sequence may contain more amino acid residues than the number allowed for by the Kabat numbering). This means that, generally, the numbering according to Kabat may or may not correspond to the actual numbering of the amino acid residues in the actual sequence.

Alternative methods for numbering the amino acid residues of V_H domains, which methods can also be applied in an analogous manner to VHH domains, are known in the art. However, in the present description, claims and figures, the numbering according to Kabat and applied to VHH domains as described above will be followed, unless indicated otherwise.

The total number of amino acid residues in a VHH domain will usually be in the range of from 110 to 120, often between 112 and 115. It should however be noted that smaller and longer sequences may also be suitable for the purposes described herein.

Methods of obtaining VHHs that bind to a specific antigen or epitope have been described earlier, e.g. in WO2006/040153 and WO2006/122786. As also described therein in detail, VHH domains derived from camelids can be "humanized" (also termed “sequence-optimized” herein, “sequence-optimizing” may, in addition to humanization, encompass an additional modification of the sequence by one or more mutations that furnish the VHH with improved properties, such as the removal of potential post translational modification

sites) by replacing one or more amino acid residues in the amino acid sequence of the original VHH sequence by one or more of the amino acid residues that occur at the corresponding position(s) in a VH domain from a conventional 4-chain antibody from a human being. A humanized VHH domain can contain one or more fully human framework region sequences, and, in an even more specific embodiment, can contain human framework region sequences derived from DP-29, DP-47, DP-51, or parts thereof, optionally combined with JH sequences, such as JH5.

Domain antibodies, also known as "Dab"s and "dAbs" (the terms "Domain Antibodies" and "dAbs" being used as trademarks by the GlaxoSmithKline group of companies) have been described in e.g. Ward, E.S., *et al.*: "Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli"; Nature 341: 544-546 (1989); Holt, L.J. *et al.*: "Domain antibodies: proteins for therapy"; TRENDS in Biotechnology 21(11): 484-490 (2003); and WO2003/002609.

Domain antibodies essentially correspond to the VH or VL domains of antibodies from non-camelid mammals, in particular human 4-chain antibodies. In order to bind an epitope as a single antigen binding domain, i.e. without being paired with a VL or VH domain, respectively, specific selection for such antigen binding properties is required, e.g. by using libraries of human single VH or VL domain sequences.

Domain antibodies have, like VHHs, a molecular weight of approximately 13 to approximately 16 kDa and, if derived from fully human sequences, do not require humanization for e.g. therapeutical use in humans. As in the case of VHH domains, they are well expressed also in prokaryotic expression systems, providing a significant reduction in overall manufacturing cost.

Furthermore, it will also be clear to the skilled person that it is possible to "graft" one or more of the CDR's mentioned above onto other "scaffolds", including but not limited to human scaffolds or non-immunoglobulin scaffolds. Suitable scaffolds and techniques for such CDR grafting are known in the art.

5 The terms "*epitope*" and "*antigenic determinant*", which can be used interchangeably, refer to the part of a macromolecule, such as a polypeptide, that is recognized by antigen-binding molecules, such as conventional antibodies or the polypeptides of the invention, and more particularly by the antigen-binding site of said molecules. Epitopes define the minimum binding 10 site for an immunoglobulin, and thus represent the target of specificity of an immunoglobulin.

A polypeptide (such as an immunoglobulin, an antibody, an immunoglobulin single variable domain of the invention, or generally an antigen-binding molecule or a fragment thereof) that can "*bind to*" or "*specifically bind to*", that 15 "*has affinity for*" and/or that "*has specificity for*" a certain epitope, antigen or protein (or for at least one part, fragment or epitope thereof) is said to be "*against*" or "*directed against*" said epitope, antigen or protein or is a "*binding*" molecule with respect to such epitope, antigen or protein. In this context, a VEGF-binding molecule may also be referred to as “VEGF-neutralizing.

20 Generally, the term "*specificity*" refers to the number of different types of antigens or epitopes to which a particular antigen-binding molecule or antigen-binding protein (such as an immunoglobulin single variable domain of the invention) molecule can bind. The specificity of an antigen-binding molecule can be determined based on its affinity and/or avidity. The affinity, represented 25 by the equilibrium constant for the dissociation of an antigen with an antigen-binding protein (KD), is a measure for the binding strength between an epitope and an antigen-binding site on the antigen-binding protein: the lesser the value of the KD, the stronger the binding strength between an epitope and the

antigen-binding molecule (alternatively, the affinity can also be expressed as the affinity constant (KA), which is 1/KD). As will be clear to the skilled person (for example on the basis of the further disclosure herein), affinity can be determined in a manner known per se, depending on the specific antigen of interest. Avidity is the measure of the strength of binding between an antigen-binding molecule (such as an immunoglobulin, an antibody, an immunoglobulin single variable domain or a polypeptides containing it and the pertinent antigen. Avidity is related to both the affinity between an epitope and its antigen binding site on the antigen-binding molecule and the number of pertinent binding sites present on the antigen-binding molecule.

The part of an antigen-binding molecule that recognizes the epitope is called a *paratope*.

Unless indicated otherwise, the term “VEGF-binding molecule” includes anti-VEGF antibodies, anti-VEGF antibody fragments, “anti-VEGF antibody-like molecules” and conjugates with any of these. Antibodies include, but are not limited to, monoclonal and chimerized monoclonal antibodies. The term „antibody“ encompasses complete immunoglobulins, like monoclonal antibodies produced by recombinant expression in host cells, as well as VEGF-binding antibody fragments or “antibody-like molecules”, including single-chain antibodies and linear antibodies, so-called “SMIPs” (“Small Modular Immunopharmaceuticals”), as e.g described in WO02/056910. Anti-VEGF antibody-like molecules include immunoglobulin single variable domains, as defined herein. Other examples for antibody-like molecules are immunoglobulin super family antibodies (IgSF), or CDR-grafted molecules.

“*VEGF-binding molecule”* refers to both monovalent VEGF-binding molecules (i.e. molecules that bind to one epitope of VEGF) as well as to bi- or multivalent binding molecules (i.e. binding molecules that bind to more than one epitope, e.g. “biparatopic” molecules as defined hereinbelow).

VEGF-binding molecules containing more than one VEGF-binding immunoglobulin single variable domain are also termed “formatted” VEGF-binding molecules, they may, in addition to the VEGF-binding immunoglobulin single variable domains, comprise linkers and/or moieties with effector functions, e.g. half-life-extending moieties like albumin-binding immunoglobulin single variable domains, and/or a fusion partner like serum albumin and/or an attached polymer like PEG.

The term "*biparatopic VEGF-binding molecule”* or "*biparatopic immunoglobulin single variable domain”* as used herein shall mean a VEGF-binding molecule comprising a first immunoglobulin single variable domain and a second immunoglobulin single variable domain as herein defined, wherein the two molecules bind to two different, i.e. non-overlapping epitopes of the VEGF antigen. The biparatopic polypeptides according to the invention are composed of immunoglobulin single variable domains which have different specificities with respect to the epitope. The part of an antigen-binding molecule (such as an antibody or an immunoglobulin single variable domain of the invention) that recognizes the epitope is called a *paratope*.

A formatted VEGF-binding molecule may, albeit less preferred, also comprise two identical VEGF-binding immunoglobulin single variable domains or two different immunoglobulin single variable domains that recognize the same or overlapping epitopes. In this case, the two immunoglobulin single variable domains may bind to the same or an overlapping epitope in each of the two monomers that form the VEGF dimer.

Typically, the VEGF-binding molecules of the invention will bind with a dissociation constant (K_D) of 10E-5 to 10E-14 moles/liter (M) or less, and preferably 10E-7 to 10E-14 moles/liter (M) or less, more preferably 10E-8 to 10E-14 moles/liter, and even more preferably 10E-11 to 10E-13 (as measured in a Biacore or in a KinExA assay), and/or with an association constant (K_A) of

at least 10E7 ME-1 , preferably at least 10E8 ME-1, more preferably at least 10E9 ME-1, such as at least 10E11 ME-1. Any K_D value greater than 10E-4 M is generally considered to indicate non-specific binding. Preferably, a polypeptide of the invention will bind to the desired antigen, i.e. VEGF, with a K_D less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. Specific binding of an antigen-binding protein to an antigen or epitope can be determined in any suitable manner known per se, including, for example, the assays described herein, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known per se in the art.

Amino acid residues will be indicated according to the standard three-letter or one-letter amino acid code, as generally known and agreed upon in the art. When comparing two amino acid sequences, the term "*amino acid difference*" refers to insertions, deletions or substitutions of the indicated number of amino acid residues at a position of the reference sequence, compared to a second sequence. In case of substitution(s), such substitution(s) will preferably be conservative amino acid substitution(s), which means that an amino acid residue is replaced with another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO98/49185, wherein conservative amino acid substitutions preferably are substitutions in which one amino acid within the following groups (i) - (v) is substituted by another amino acid residue within the same group: (i) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (ii) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (iii) polar, positively charged residues: His, Arg and Lys; (iv) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (v) aromatic residues: Phe, Tyr and Trp.

Particularly preferred conservative amino acid substitutions are as follows: Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into
5 Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser;Trp into Tyr; Tyr into Trp or into Phe; Val into Ile or into Leu.

A polypeptide or nucleic acid molecule is considered to be "*(in) essentially isolated (form)*" - for example, when compared to its native biological source
10 and/or the reaction medium or cultivation medium from which it has been obtained - when it has been separated from at least one other component with which it is usually associated in said source or medium, such as another protein/polypeptide, another nucleic acid, another biological component or macromolecule or at least one contaminant, impurity or minor component. In
15 particular, a polypeptide or nucleic acid molecule is considered "essentially isolated" when it has been purified at least 2-fold, in particular at least 10- fold, more in particular at least 100-fold, and up to 1000-fold or more. A polypeptide or nucleic acid molecule that is "in essentially isolated form" is preferably essentially homogeneous, as determined using a suitable technique, such as a
20 suitable chromatographical technique, such as polyacrylamide gel electrophoresis.

"*Sequence identity*" between two VEGF-binding molecule sequences indicates the percentage of amino acids that are identical between the sequences. It may be calculated or determined as described in paragraph f) on pages 49
25 and 50 of WO08/020079. "Sequence similarity" indicates the percentage of amino acids that either are identical or that represent conservative amino acid substitutions.

Alternative methods for numbering the amino acid residues of V_H domains, which methods can also be applied in an analogous manner to VHH domains, are known in the art. However, in the present description, claims and figures, the numbering according to Kabat and applied to VHH domains as described above will be followed, unless indicated otherwise.

An "affinity-matured" VEGF-binding molecule, in particular a VHH or a domain antibody, has one or more alterations in one or more CDRs which result in an improved affinity for VEGF, as compared to the respective parent VEGF-binding molecule. Afffinity-matured VEGF-binding molecules of the invention may be prepared by methods known in the art, for example, as described by Marks *et al.*, 1992, Biotechnology 10:779-783, or Barbas, *et al.*, 1994, Proc. Nat. Acad. Sci, USA 91: 3809-3813.; Shier *et al.*, 1995, Gene 169:147-155; Yelton *et al.*, 1995, Immunol. 155: 1994-2004; Jackson *et al.*, 1995, J. Immunol. 154(7):3310-9; and Hawkins *et al.*, 1992, J. Mol. Biol. 226(3): 889 896; KS Johnson and RE Hawkins, "Affinity maturation of antibodies using phage display", Oxford University Press 1996.

For the present invention, an "amino acid sequences of SEQ ID NO: x": includes, if not otherwise stated, an amino acid sequence that is 100% identical with the sequence shown in the respective SEQ ID NO: x;

a) amino acid sequences that have at least 80% amino acid identity with the sequence shown in the respective SEQ ID NO: x;

b) amino acid sequences that have 3, 2, or 1 amino acid differences with the sequence shown in the respective SEQ ID NO: x.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer to be treated with a VEGF-binding molecule of the invention, include but are not limited to carcinoma, lymphoma,

blastoma, sarcoma, and leukemia. More particular examples of such cancers, as suggested for treatment with VEGF antagonists in US 2008/0014196, include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, gastric cancer, melanoma, and various types of head and neck cancer. Dysregulation of angiogenesis can lead to many disorders that can be treated by compositions and methods of the invention. These disorders include both non-neoplastic and neoplastic conditions. Neoplasties include but are not limited those described above.

Non-neoplastic disorders include, but are not limited to, as suggested for treatment with VEGF antagonists in US 2008/0014196, undesired or aberrant hypertrophy, arthritis, rheumatoid arthritis (RA), psoriasis, psoriatic plaques, sarcoidosis, atherosclerosis, atherosclerotic plaques, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, arteriovenous malformations (AVM), meningioma, hemangioma, angiofibroma, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, acute lung injury/ ARDS, sepsis, primary pulmonary hypertension, malignant pulmonary effusions, cerebral edema (e.g., associated with acute stroke/ closed head injury/ trauma), synovial inflammation, pannus formation in RA, myositis

ossificans, hypertropic bone formation, osteoarthritis (OA), refractory ascites, polycystic ovarian disease, endometriosis, 3rd spacing of fluid diseases (pancreatitis, compartment syndrome, burns, bowel disease), uterine fibroids, premature labor, chronic inflammation such as IBD (Crohn's disease and
5 ulcerative colitis), renal allograft rejection, inflammatory bowel disease, nephrotic syndrome, undesired or aberrant tissue mass growth (non-cancer), hemophilic joints, hypertrophic scars, inhibition of hair growth, Osier-Weber syndrome, pyogenic granuloma retrolental fibroplasias, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, preeclampsia, ascites,
10 pericardial effusion (such as that associated with pericarditis), and pleural effusion.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention relates to a VEGF-binding molecule comprising at least a variable domain with four framework regions and three
15 complementarity determining regions CDR1, CDR2 and CDR3, respectively, wherein said CDR3 has the amino acid sequence Ser Arg Ala Tyr Xaa Ser Xaa Arg Leu Arg Leu Xaa Xaa Thr Tyr Xaa Tyr as shown in SEQ ID NO: 1, wherein

Xaa at position 5 is Gly or Ala;
20 Xaa at position 7 is Ser or Gly;
Xaa at position 12 is Gly, Ala or Pro;
Xaa at position 13 is Asp or Gly;
Xaa at position 16 is Asp or Glu; and

wherein said VEGF-binding molecule is capable of blocking the interaction of
25 human recombinant VEGF165 with the human recombinant VEGFR-2 with an inhibition rate of ≥60%.

According to preferred embodiments, Xaa at position 5 is Gly, Xaa at position 7 is Ser, Xaa at position 12 is Ala, and Xaa at position 13 is Asp.

In particular, said CDR3 has a sequence selected from

SEQ ID NO: 2 SRAYGSSRLRLGDTYDY,
5 SEQ ID NO: 3 SRAYGSSRLRLADTYDY;
SEQ ID NO: 4 SRAYGSSRLRLADTYEY;
SEQ ID NO: 5 SRAYGSGRLRLADTYDY;
SEQ ID NO: 6 SRAYASSRLRLADTYDY;
SEQ ID NO: 7 SRAYGSSRLRLPDTYDY;
10 SEQ ID NO: 8 SRAYGSSRLRLPGTYDY.

According to certain embodiments, a VEGF-binding molecule comprises one or more immunoglobulin single variable domains each containing

a. a CDR3 with an amino acid sequence selected from a first group of sequences shown in SEQ ID NO: 2 to 8;

15 b. a CDR1 and a CDR2 with an amino acid sequences that is contained, as indicated in Table 3, in a sequence selected from a second group of amino acid sequences shown in SEQ ID NOs: 9 to 46, wherein said second sequence contains the respective CDR3 selected according to a).

20 According to preferred embodiments, the immunoglobulin single variable domains are VHHs.

According to specific embodiments, the VHHs have amino acid sequences selected from sequences shown in SEQ ID NOs: 9 - 46.

According to another specific embodiment, the VHHs have amino acid
25 sequences selected from SEQ ID NOs: 15, SEQ ID NO: 18 and SEQ ID NO: 25.

The invention also relates to VEGF-binding molecules that have been obtained by affinity maturation and/or sequence optimization of an above-defined VHH, e.g. to a VHH that has been obtained by sequence optimization of a VHH having an amino acid sequence shown in SEQ ID NO: 18. Examples are VHHs having amino acid sequences selected from sequences shown in SEQ ID NOs: 47 – 57.

According to certain embodiments, a VEGF-binding molecule of the invention may be formatted, as herein defined, e.g. it may be biparatopic or comprise two identical immunoglobulin single variable domains. Such VEGF-binding molecules may comprise two or more VHHs, which are

a) identical VHHs that are capable of blocking the interaction between recombinant human VEGF and the recombinant human VEGFR-2 with an inhibition rate of ≥ 60% or

b) different VHHs that bind to non-overlapping epitopes of VEGF, wherein at least one VHH is capable of blocking the interaction between recombinant human VEGF and the recombinant human VEGFR-2 with an inhibition rate of ≥ 60% and wherein at least one VHH binds is capable of blocking said interaction with an inhibition rate of ≤ 60 %.

The percentage of blocking said interaction at an inhibition rate of ≥ 60% or ≤ 60 %, respectively, refers to an inhibition rate as determined by an Amplified Luminescent Proximity Homogeneous Assay (AlphaScreen®), a competition ELISA, a plasmon resonance (SPR) based assay (Biacore®) as used in the Examples.

In the following, the ability of VHHs according to a) is also termed “receptor-blocking”, while the ability of VHHs according to b) is also termed “non-receptor-blocking”.

Preferably, the receptor-blocking VHHs have an inhibition rate of ≥ 80%, more preferably ≥ 90%; the most preferred VHHs being complete receptor blockers, i.e. have an inhibition rate of 100 %.

A VEGF-binding may contain two or more identical VHHs a) selected from VHHs having amino acid sequences shown in SEQ ID NOs: 9 – 46 or VHHs that have been obtained by affinity maturation and/or sequence optimization of such VHH. The VHH may be selected from VHHs having the amino acid shown in SEQ ID NO: 18 or SEQ ID NO: 47 – 57.

According to preferred embodiments, a formatted VEGF-binding molecule comprises two VHHs each having the amino acid sequence shown in SEQ ID NO: 57.

In formatted VEGF-binding molecules comprising two different VHHs

a) said one or more VHHs with an inhibition rate of ≥ 60% are selected from

    i. VHHs having an amino acid sequence selected from amino acid sequences shown in SEQ ID NOs: 9 – 46 or

    ii. VHHs that have been obtained by affinity maturation and/or sequence optimization of such VHHs, and wherein

b) said one or more VHHs with an inhibition rate of ≤ 60 % are selected from

    i. SEQ ID NOs: 58 – 124 or

    ii. VHHs that have been obtained by affinity maturation and/or sequence optimization of such VHH.

According to preferred embodiments, two VHHs are contained in polypeptides with amino acid sequences shown in SEQ ID NOs: 128 – 168, separated by linker sequences as indicated in Table 15.

In a preferred VEGF-binding molecule VHH a) i. has an amino acid sequence shown in SEQ ID NO: 18 and VHH b) i. has an amino acid sequence shown in SEQ ID NO: 64.

In other preferred VEGF-binding molecules VHHs according to a) ii. are selected from VHHs having an amino acid sequence shown in SEQ ID NOs: 47 – 57 and VHHs according to b) ii. are selected from VHHs having an amino acid sequence shown in SEQ ID NOs: 125 – 127 .

Particularly preferred is a biparatopic VEGF-binding molecule comprising two VHHs, one of them having the amino acid shown in SEQ ID NO: 57 and one of them having the amino acid shown in SEQ ID NO: 127.

The VEGF-binding molecules with improved properties in view of therapeutic application, e.g. enhanced affinity or decreased immunogenicity, may be obtained from individual VEGF-binding molecules of the invention by techniques known in the art, such as affinity maturation (for example, starting from synthetic, random or naturally occurring immunoglobulin sequences), CDR grafting, humanizing, combining fragments derived from different immunoglobulin sequences, PCR assembly using overlapping primers, and similar techniques for engineering immunoglobulin sequences well known to the skilled person; or any suitable combination of any of the foregoing, also termed “sequence optimization”, as described herein. Reference is, for example, made to standard handbooks, as well as to the further description and Examples.

If appropriate, a VEGF-binding molecule of the invention with increased affinity may be obtained by affinity-maturation of another VEGF-binding molecule, the

latter representing, with respect to the affinity-matured molecule, the “parent” VEGF-binding molecule.

Immunoglobulin single variable domains, e.g. VHHs and domain antibodies, according to the preferred embodiments of the invention, have a number of unique structural characteristics and functional properties which makes them highly advantageous for use in therapy as functional antigen-binding molecules. In particular, and without being limited thereto, VHH domains (which have been "designed" by nature to functionally bind to an antigen without pairing with a light chain variable domain) can function as single, relatively small, functional antigen-binding structural units.

Due to their unique properties, immunoglobulin single variable domains, as defined herein, like VHHs or VHs (or VLs) - either alone or as part of a larger polypeptide, e.g. a biparatopic molecule - offer a number of significant advantages:

- only a single domain is required to bind an antigen with high affinity and with high selectivity, so that there is no need to have two separate domains present, nor to assure that these two domains are present in the right spacial conformation and configuration (i.e. through the use of especially designed linkers, as with scFv's);
- immunoglobulin single variable domains can be expressed from a single nucleic acid molecule and do not require any post-translational modification (like glycosylation;
- immunoglobulin single variable domains can easily be engineered into multivalent and multispecific formats (as further discussed herein);

- immunoglobulin single variable domains have high specificity and affinity for their target, low inherent toxicity and can be administered via alternative routes than infusion or injection;
- immunoglobulin single variable domains are highly stable to heat, pH, 5 proteases and other denaturing agents or conditions and, thus, may be prepared, stored or transported without the use of refrigeration equipments;
- immunoglobulin single variable domains are easy and relatively inexpensive to prepare, both on small scale and on a manufacturing 10 scale. For example, immunoglobulin single variable domains can be produced using microbial fermentation (e.g. as further described below) and do not require the use of mammalian expression systems, as with for example conventional antibodies;
- immunoglobulin single variable domains are relatively small 15 (approximately 15 kDa, or 10 times smaller than a conventional IgG) compared to conventional 4-chain antibodies and antigen-binding fragments thereof, and therefore show high(er) penetration into tissues (including but not limited to solid tumors and other dense tissues) and can be administered in higher doses than such conventional 4-chain 20 antibodies and antigen-binding fragments thereof;
- VHHs have specific so-called “cavity-binding properties” (inter alia due to their extended CDR3 loop, compared to VH domains from 4-chain antibodies) and can therefore also access targets and epitopes not accessible to conventional 4-chain antibodies and antigen-binding 25 fragments thereof;
- VHHs have the particular advantage that they are highly soluble and very stable and do not have a tendency to aggregate (as with the

mouse-derived antigen-binding domains described by Ward *et al.*, Nature 341: 544-546 (1989)).

The immunoglobulin single variable domains of the invention are not limited with respect to a specific biological source from which they have been
5 obtained or to a specific method of preparation. For example, obtaining VHHs may include the following steps:

(1) isolating the VHH domain of a naturally occurring heavy chain antibody; or screening a library comprising heavy chain antibodies or VHHs and isolating VHHs therefrom;

10 (2) expressing a nucleic acid molecule encoding a VHH with the naturally occurring sequence;

(3) "humanizing" (as described herein) a VHH, optionally after affinity maturation, with a naturally occurring sequence or expressing a nucleic acid encoding such humanized VHH;

15 (4) "camelizing" (as described below) a immunoglobulin single variable heavy domain from a naturally occurring antibody from an animal species, in particular a species of mammal, such as from a human being, or expressing a nucleic acid molecule encoding such camelized domain;

(5) "camelizing" a VH, or expressing a nucleic acid molecule encoding such a
20 camelized VH;

(6) using techniques for preparing synthetically or semi-synthetically proteins, polypeptides or other amino acid sequences;

(7) preparing a nucleic acid molecule encoding a VHH domain using techniques for nucleic acid synthesis, followed by expression of the nucleic
25 acid thus obtained;

(8) subjecting heavy chain antibodies or VHHs to affinity maturation, to mutagenesis (e.g. random mutagenesis or site-directed mutagenesis) and/or any other technique(s) in order to increase the affinity and/or specificity of the VHH; and/or

5 (9) combinations or selections of the foregoing steps.

Suitable methods and techniques for performing the above-described steps are known in the art and will be clear to the skilled person. By way of example, methods of obtaining VHH domains binding to a specific antigen or epitope have been described in WO2006/040153 and WO2006/122786.

10 According to specific embodiments, the immunoglobulin single variable domains of the invention or present in the polypeptides of the invention are VHH domains with an amino acid sequence that essentially corresponds to the amino acid sequence of a naturally occurring VHH domain, but that has been "humanized" or “sequence-optimized” (optionally after affinity-maturation), i.e.
15 by replacing one or more amino acid residues in the amino acid sequence of said naturally occurring VHH sequence by one or more of the amino acid residues that occur at the corresponding position(s) in a variable heavy domain of a conventional 4-chain antibody from a human being. This can be performed using methods known in the art, which can by routinely used by the skilled
20 person.

A humanized VHH domain may contain one or more fully human framework region sequences, and, in an even more specific embodiment, may contain human framework region sequences derived from the human germline Vh3 sequences DP-29, DP-47, DP-51, or parts thereof, or be highly homologous
25 thereto, optionally combined with JH sequences, such as JH5. Thus, a humanization protocol may comprise the replacement of any of the VHH residues with the corresponding framework 1, 2 and 3 (FRl, FR2 and FR3)

residues of germline VH genes such as DP 47, DP 29 and DP 51) either alone or in combination. Suitable framework regions (FR) of the immunoglobulin single variable domains of the invention can be selected from those as set out e.g. in WO 2006/004678 and specifically, include the so-called "KERE" and
5 "GLEW" classes. Examples are immunoglobulin single variable domains having the amino acid sequence G-L-E-W at about positions 44 to 47, and their respective humanized counterparts. A humanized VHH domain may contain one or more fully human framework region sequences.

In VHHs of the invention that start with EVQ, the N-terminal E may be replaced
10 by a D (which is often a result of sequence-optimization) or it may be missing (as for expression of the VHH in *E.coli).* For formatted VEGF-binding molecules, this usually applies only to the VHH that is situated N-terminally.

A preferred, but non-limiting humanizing substitution for VHH domains belonging to the 103 P,R,S-group and/or the GLEW-group (as defined below)
15 is 108Q to 108L. Methods for humanizing immunoglobulin single variable domains are known in the art.

According to another embodiment, the immunoglobulin single variable domain is a domain antibody, as defined herein.

In yet another embodiment, the representatives of the class of VEGF-binding
20 immunoglobulin single variable domains of the invention have amino acid sequences that correspond to the amino acid sequence of a naturally occurring VH domain that has been "camelized", i.e. by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring variable heavy chain from a conventional 4-chain antibody by one or more
25 amino acid residues that occur at the corresponding position(s) in a VHH domain of a heavy chain antibody. This can be performed in a manner known per se, which will be clear to the skilled person, and reference is additionally

be made to WO 94/04678. Such camelization may preferentially occur at amino acid positions which are present at the VH-VL interface and at the so-called Camelidae Hallmark residues (see for example also WO 94/04678). A detailled description of such "humanization" and "camelization" techniques and preferred framework region sequences consistent therewith can additionally be taken from e.g. pp. 46 and pp. 98 of WO 2006/040153 and pp. 107 of WO 2006/122786.

The VEGF-binding molecules of the invention, e.g. immunoglobulin single variable domains, have specificity for VEGF in that they comprise one or more immunoglobulin single variable domains specifically binding to one or more epitopes within the VEGF molecule.

Specific binding of an VEGF-binding molecule to its antigen VEGF can be determined in any suitable manner known per se, including, for example, the assays described herein, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA and ELISA) and sandwich competition assays, and the different variants thereof known per se in the art.

With regard to the antigen VEGF, a VEGF-binding molecule of the invention, e.g. an immunoglobulin single variable domain, is not limited with regard to the species. Thus, the immunoglobulin single variable domains of the invention preferably bind to human VEGF, if intended for therapeutic purposes in humans. However, immunoglobulin single variable domains that bind to VEGF from another mammalian species are also within the scope of the invention. An immunoglobulin single variable domain of the invention binding to one species form of VEGF may cross-react with VEGF, which has a different sequence than the human one, from one or more other species. For example, immunoglobulin single variable domains of the invention binding to human VEGF may exhibit cross reactivity with VEGF from one or more other species

of primates and/or with VEGF from one or more species of animals that are used in animal models for diseases, for example monkey, mouse, rat, rabbit, pig, dog, and in particular in animal models for diseases and disorders associated with VEGF-mediated effects on angiogenesis (such as the species and animal models mentioned herein). Immunoglobulin single variable domains of the invention that show such cross-reactivity are advantageous in a research and/or drug development, since it allows the immunoglobulin single variable domains of the invention to be tested in acknowledged disease models such as monkeys, in particular Cynomolgus or Rhesus, or mice and rats.

Preferably, in view of cross-reactivity with one or more VEGF molecules from species other than human that is/are intended for use as an animal model during development of a therapeutic VEGF antagonist, a VEGF-binding molecule recognizes an epitope in a region of the VEGF of interest that has a high degree of identity with human VEGF.

An immunoglobulin single variable domain of the invention recognizes an epitope which is, totally or in part, located in a region of VEGF that is relevant for binding to its receptor, in particular to VEGFR-2, which has been shown to be the receptor whose activation is causally involved in the neovascularisation of tumors. According to preferred aspects, immunoglobulin single variable domains of the invention block VEGF receptor activation, in particular VEGFR-2 activation, at least partially, preferably substantially and most preferably totally.

As described above, the ability of a VEGF-binding molecule to block the interaction between VEGF and its receptors, in particular the VEGFR-2, can be determined by an Amplified Luminescent Proximity Homogeneous Assay (AlphaScreen®), a competition ELISA, or a plasmon resonance (SPR) based assay (Biacore®), as described in the Examples.

Preferably, an immunoglobulin single variable domain of the invention binds to VEGF with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM (as determined by Surface Plasmon Resonance analysis, as described in Example 5.7).

5 Preferably, the immunoglobulin single variable domains of the invention have IC_{50} values, as measured in a competition ELISA assay as described in Example 5.1. in the range of 10^{-6} to 10^{-10} moles/litre or less, more preferably in the range of 10^{-8} to 10^{-10} moles/litre or less and even more preferably in the range of 10^{-9} to 10^{-10} moles/litre or less.

10 According to a non-limiting but preferred embodiment of the invention, VEGF-binding immunoglobulin single variable domains of the invention bind to VEGF with an dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter (M) or less, and preferably 10^{-7} to 10^{-12} moles/liter (M) or less and more preferably 10^{-8} to 10^{-12} moles/liter (M), and/or with an association constant (K_A) of at least 10^{7} M^{-1},
15 preferably at least 10^{8} M^{-1}, more preferably at least 10^{9} M^{-1}, such as at least 10^{12} M^{-1}; and in particular with a K_D less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. The K_D and K_A values of the immunoglobulin single variable domain of the invention against VEGF can be determined.

20 Biparatopic VEGF-binding molecules comprising two or more immunoglobulin single variable domains essentially consist of or comprise (i) a first immunoglobulin single variable domain specifically binding to a first epitope of VEGF and (ii) a second immunoglobulin single variable domain specifically binding to a second epitope of VEGF, wherein the first epitope of VEGF and
25 the second epitope of VEGF are not identical epitopes. In other words, such polypeptide of the invention comprises or essentially consist of two or more immunoglobulin single variable domains that are directed against at least two non-overlapping epitopes present in VEGF, wherein said immunoglobulin

single variable domains are linked to each other in such a way that they are capable of simultaneously binding VEGF. In this sense, the polypeptide of the invention can also be regarded as a “bivalent” or "multivalent" immunoglobulin construct, and especially as a "multivalent immunoglobulin single variable domain construct", in that the polypeptide contains at least two binding sites for VEGF. (Such constructs are also termed “formatted” VEGF binding molecules, e.g. “formatted” VHHs).

Such VEGF-binding molecule of the invention includes (at least) two anti-VEGF immunoglobulin single variable domains, wherein (the) two immunoglobulin single variable domains are preferably directed against non-overlapping epitopes within the VEGF molecule. Thus, these two immunoglobulin single variable domains will have a different antigen specificity and therefore different CDR sequences. For this reason, such polypeptides of the invention will herein also be named "biparatopic polypeptides", or "biparatopic domain antibody constructs" (if the immunoglobulin single variable domains consist or essentially consist of domain antibodies), or "biparatopic VHH constructs" (if the immunoglobulin single variable domains consist or essentially consist of VHHs), respectively, as the two immunoglobulin single variable domains will include two different paratopes.

If a polypeptide of the invention is a biparatopic molecule as defined herein, at least one of the immunoglobulin single variable domain components binds to an epitope such that the interaction between recombinant human VEGF and recombinant humen VEGFR-2 is blocked at an inhibition rate of ≥80%. As has been shown in experiments of the invention, certain formatted molecules contain two VHHs that both block the VEGFR2 receptor at an inhibition rate of ≥80%. Certain VHHs of the invention block the VEGFR-2 at an inhibition rate of 100%, i.e. they are complete blockers.

In both cases, additional sequences and moieties may be present within the VEGF-binding molecules of the invention, e.g. N-terminally, C-terminally, or located between the two immunoglobulin single variable domains, e.g. linker sequences and sequences providing for effector functions, as set out in more 5 detail herein.

According to another, albeit less preferred embodiment, a VEGF-binding molecule of the invention may include more than two anti-VEGF immunoglobulin single variable domains, i.e. three, four or even more anti-VEGF VHHs. In this case, at least two of the anti-VEGF immunoglobulin single 10 variable domains are directed against non-overlapping epitopes within the VEGF molecule, wherein any further immunoglobulin single variable domain may bind to any of the two non-overlapping epitopes and/or a further epitope present in the VEGF molecule.

According to the invention, the two or more immunoglobulin single variable 15 domains can be, independently of each other, VHHs or domain antibodies, and/or any other sort of immunoglobulin single variable domains, such as VL domains, as defined herein, provided that these immunoglobulin single variable domains will bind the antigen, i.e. VEGF.

According to a preferred embodiment, the first and the second immunoglobulin 20 single variable domains essentially consist of either VHH sequences or domain antibody sequences, as defined herein. According to a particularly preferred embodiment, the first and the second immunoglobulin single variable domains essentially consist of VHH sequences.

According to certain embodiments of the invention, the at least two 25 immunoglobulin single variable domains present in a VEGF-binding molecule of the invention can be connected with each other directly (i.e. without use of a linker) or via a linker. The linker is preferably a linker peptide and will be

selected so as to allow binding of the at least two different immunoglobulin single variable domains to each of their at least two non-overlapping epitopes of VEGF, either within one and the same VEGF molecule, or within two different molecules.

5 Suitable linkers will *inter alia* depend on the epitopes and, specifically, the distance between the epitopes on VEGF to which the immunoglobulin single variable domains bind, and will be clear to the skilled person based on the disclosure herein, optionally after some limited degree of routine experimentation.

10 Also, when the two or more immunoglobulin single variable domains that bind to VEGF are VHHs or domain antibodies, they may be linked to each other via a third VHH or antibody, respectively (in such VEGF-binding molecules, the two or more immunoglobulin single variable domains may be linked directly to said third immunoglobulin single variable domain or via suitable linkers). Such 15 a third VHH or domain antibody may for example be a VHH or domain antibody that provides for an increased half-life. For example, the latter VHH or domain antibody may be a domain antibody or VHH that is capable of binding to a (human) serum protein such as (human) serum albumin or (human) transferrin.

20 Alternatively, the two or more immunoglobulin single variable domains that bind to VEGF may be linked in series (either directly or via a suitable linker) and the third VHH or domain antibody (which may provide for increased half-life) may be connected directly or via a linker to one of these two or more aforementioned immunoglobulin sequences.

25 Suitable linkers are described herein in connection with specific polypeptides of the invention and may - for example and without limitation - comprise an amino acid sequence, which amino acid sequence preferably has a length of 9

or more amino acids, more preferably at least 17 amino acids, such as about 20 to 40 amino acids. However, the upper limit is not critical but is chosen for reasons of convenience regarding e.g. biopharmaceutical production of such polypeptides.

5 The linker sequence may be a naturally occurring sequence or a non-naturally occurring sequence. If used for therapeutic purposes, the linker is preferably non-immunogenic in the subject to which the VEGF-binding molecule of the invention is administered.

One useful group of linker sequences are linkers derived from the hinge region
10 of heavy chain antibodies as described in WO96/34103 and WO94/04678.

Other examples are poly-alanine linker sequences such as Ala- Ala- Ala.

Further preferred examples of linker sequences are Gly/Ser linkers of different length such as $(gly_x ser_y)_z$ linkers, including $(gly_4ser)_3$, $(gly_4ser)_4$, (gly_4ser), (gly_3ser), gly_3, and $(gly_3ser_2)_3$.

15 Some non-limiting examples of linkers are contained in VEGF-binding molecules of the invention shown in Table 15 (SEQ ID NOs 128 – 168), e.g. the linkers

GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (35GS; SEQ ID NO: 169);

20 GGGGSGGGS (9GS; SEQ ID NO: 170);

GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (40GS; SEQ ID NO: 171).

If a formatted VEGF-binding molecule of the invention is modified by the attachment of a polymer, for example of a polyethylene glycol PEG
25 (polyethylene glycol) moiety, the linker sequence preferably includes an amino

acid residue, such as a cysteine or a lysine, allowing such modification, e.g. PEGylation, in the linker region.

Examples of linkers useful for for PEGylation are:

GGGGCGGGS (“GS9,C5”, SEQ ID NO: 172);

5 GGGGCGGGGSGGGGSGGGGSGGGGS (“GS25,C5, SEQ ID NO:173)

GGGSGGGGSGGGGCGGGGSGGGGSGGG ("GS27,C14", SEQ ID NO:174),

GGGGSGGGGSGGGGCGGGGSGGGGSGGGGSGGGGS ("GS35,C15", SEQ ID NO:175), and

GGGGCGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (“GS35,C5”, SEQ
10 ID NO:176).

Furthermore, the linker may also be a poly(ethylene glycol) moiety, as shown in e.g. WO2004/081026.

In another embodiment, the at least two VEGF-binding immunoglobulin single variable domains are linked to each other via another moiety (optionally via
15 one or two linkers), such as another polypeptide which, in a preferred but non-limiting embodiment, may be a further immunoglobulin single variable domain as described above. Such moiety may either be essentially inactive or may have a biological effect such as improving the desired properties of the polypeptide or may confer one or more additional desired properties to the
20 polypeptide. For example, and without limitation, the moiety may improve the half-life of the protein or polypeptide, and/or may reduce its immunogenicity or improve any other desired property.

According to a preferred embodiment, a VEGF-binding molecule of the invention includes, especially when intended for use or used as a therapeutic
25 agent, a moiety which extends the half-life of the polypeptide of the invention in

serum or other body fluids of a patient. The term "half-life" is defined as the time it takes for the serum concentration of the (modified) polypeptide to reduce by 50%, *in vivo,* for example due to degradation of the polypeptide and/or clearance and/or sequestration by natural mechanisms.

5 More specifically, such half-life extending moiety can be covalently linked to or fused to an immunoglobulin single variable domain and may be, without limitation, an Fc portion, an albumin moiety, a fragment of an albumin moiety, an albumin binding moiety, such as an anti-albumin immunoglobulin single variable domain, a transferrin binding moiety, such as an anti-transferrin
10 immunoglobulin single variable domain, a polyoxyalkylene molecule, such as a polyethylene glycol molecule, an albumin binding peptide or a hydroxyethyl starch (HES) derivative.

In another embodiment, the VEGF-binding molecule of the invention comprises a moiety which binds to an antigen found in blood, such as serum
15 albumin, serum immunoglobulins, thyroxine-binding protein, fibrinogen or transferrin, thereby conferring an increased half-life *in vivo* to the resulting polypeptide of the invention. According to a specifically preferred embodiment, such moiety is an albumin-binding immunoglobulin and, especially preferred, an albumin-binding immunoglobulin single variable domain such as an
20 albumin-binding VHH domain.

If intended for use in humans, such albumin-binding immunoglobulin single variable domain preferably binds to human serum albumin and preferably is a humanized albumin-binding VHH domain.

Immunoglobulin single variable domains binding to human serum albumin are
25 known in the art and are described in further detail in e.g. WO2006/122786. Specifically, useful albumin binding VHHs are ALB 1 and its humanized

counterpart, ALB 8 (WO2009/095489). Other albumin binding VHH domains mentioned in the above patent publication may, however, be used as well.

A specifically useful albumin binding VHH domain is ALB8 which consists of or contains the amino acid sequence shown in SEQ ID NO: 177.

5 According to a further embodiment of the invention, the two immunoglobulin single variable domains, in preferably VHHs, may be fused to a serum albumin molecule, such as described e.g. in WO01/79271 and WO03/59934. As e.g. described in WO01/79271, the fusion protein may be obtained by conventional recombinant technology: a DNA molecule coding for serum albumin, or a 10 fragment thereof, is joined to the DNA coding for the VEGF-binding molecule, the obtained construct is inserted into a plasmid suitable for expression in the selected host cell, e.g. a yeast cell like Pichia pastoris or a bacterial cell, and the host cell is then transfected with the fused nucleotide sequence and grown under suitable conditions. The sequence of a useful HSA is shown in SEQ ID 15 NO: 178:

According to another embodiment, a half-life extending modification of a polypeptide of the invention (such modification also reducing immunogenicity of the polypeptide) comprises attachment of a suitable pharmacologically acceptable polymer, such as straight or branched chain poly(ethylene glycol) 20 (PEG) or derivatives thereof (such as methoxypoly(ethylene glycol) or mPEG). Generally, any suitable form of PEGylation can be used, such as the PEGylation used in the art for antibodies and antibody fragments (including but not limited to domain antibodies and scFv's); reference is made, for example, to: Chapman, Nat. Biotechnol., 54, 531-545 (2002); Veronese and Harris, 25 Adv. Drug Deliv. Rev. 54, 453-456 (2003); Harris and Chess, Nat. Rev. Drug. Discov. 2 (2003); and WO2004/060965.

Various reagents for PEGylation of polypeptides are also commercially available, for example from Nektar Therapeutics, USA, or NOF Corporation, Japan, such as the Sunbright® EA Series, SH Series, MA Series, CA Series, and ME Series, such as Sunbright® ME-100MA, Sunbright® ME-200MA, and 5 Sunbright® ME-400MA.

Preferably, site-directed PEGylation is used, in particular via a cysteine-residue (see for example Yang *et al.*, Protein Engineering 16, 761-770 (2003)). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in a polypeptide of the invention, a polypeptide of the invention 10 may be modified so as to suitably introduce one or more cysteine residues for attachment of PEG, or an amino acid sequence comprising one or more cysteine residues for attachment of PEG may be fused to the N- and/or C-terminus of a polypeptide of the invention, all using techniques of protein engineering known per se to the skilled person.

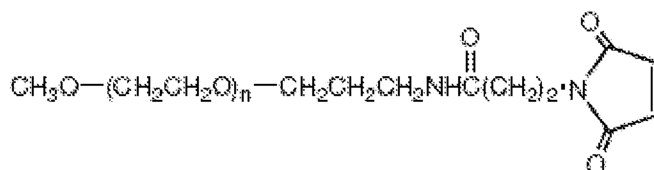
15 Preferably, for the polypeptides of the invention, a PEG is used with a molecular weight of more than 5 kDa, such as more than 10 kDa and less than 200 kDa, such as less than 100 kDa; for example in the range of 20 kDa to 80 kDa.

With regard to PEGylation, its should be noted that generally, the invention 20 also encompasses any biparatopic VEGF-binding molecule that has been PEGylated at one or more amino acid positions, preferably in such a way that said PEGylation either (1) increases the half-life in vivo; (2) reduces immunogenicity; (3) provides one or more further beneficial properties known per se for PEGylation; (4) does not essentially affect the affinity of the 25 polypeptide for VEGF (e.g. does not reduce said affinity by more than 50 %, and more preferably not by more than 10%, as determined by a suitable assay described in the art); and/or (4) does not affect any of the other desired properties of the VEGF-binding molecules of the invention. Suitable

PEG-groups and methods for attaching them, either specifically or non-specifically, will be clear to the skilled person. Various reagents for PEGylation of polypeptides are also commercially available, for example from Nektar Therapeutics, USA, or NOF Corporation, Japan, such as the Sunbright® EA
5 Series, SH Series, MA Series, CA Series, and ME Series, such as Sunbright® ME-100MA, Sunbright® ME-200MA, and Sunbright® ME-400MA.

According to an especially preferred embodiment of the invention, a PEGylated polypeptide of the invention includes one PEG moiety of linear PEG having a molecular weight of 40 kDa or 60 kDa, wherein the PEG moiety
10 is attached to the polypeptide in a linker region and, specifially, at a Cys residue at position 5 of a GS9-linker peptide as shown in SEQ ID NO: 172, at position 14 of a GS27-linker peptide as shown in SEQ ID NO:174, or at position 15 of a GS35-linker peptide as shown in SEQ ID NO:175, or at position 5 of a 35GS-linker peptide as shown in SEQ ID NO:176.

15 A VEGF-binding molecule of the invention may be PEGylated with one of the PEG reagents as mentioned above, such as "Sunbright® ME-400MA", as shown in the following chemical formula:



20 In another aspect, the invention relates to nucleic acid molecules that encode VEGF-binding molecules of the invention. Such nucleic acid molecules will also be referred to herein as "nucleic acids of the invention" and may also be in the form of a genetic construct, as defined herein. A nucleic acid of the invention may be genomic DNA, cDNA or synthetic DNA (such as DNA with a
25 codon usage that has been specifically adapted for expression in the intended

host cell or host organism). According to one embodiment of the invention, the nucleic acid of the invention is in essentially isolated form, as defined hereabove.

The nucleic acid of the invention may also be in the form of, may be present in and/or may be part of a vector, such as for example a plasmid, cosmid or YAC. The vector may especially be an expression vector, i.e. a vector that can provide for expression of the VEGF-binding molecule *in vitro* and/or *in vivo* (i.e. in a suitable host cell, host organism and/or expression system). Such expression vector generally comprises at least one nucleic acid of the invention that is operably linked to one or more suitable regulatory elements, such as promoter(s), enhancer(s), terminator(s), and the like. Such elements and their selection in view of expression of a specific sequence in a specific host are common knowledge of the skilled person. Specific examples of regulatory elements and other elements useful or necessary for expressing VEGF-binding molecules of the invention, such as promoters, enhancers, terminators, integration factors, selection markers, leader sequences, reporter genes, and the like, are disclosed e.g. on pp. 131 to 133 of WO2006/040153.

The nucleic acids of the invention may be prepared or obtained in a manner known *per se* (e.g. by automated DNA synthesis and/or recombinant DNA technology), based on the information on the amino acid sequences for the polypeptides of the invention given herein, and/or can be isolated from a suitable natural source.

In another aspect, the invention relates to host cells that express or that are capable of expressing one or more a VEGF-binding molecule of the invention; and/or that contain a nucleic acid of the invention. According to a particularly preferred embodiment, said host cells are bacterial cells; other useful cells are yeast cells, fungal cells or mammalian cells.

Suitable bacterial cells include cells from gram-negative bacterial strains such as strains of *Escherichia coli*, *Proteus*, and *Pseudomonas*, and gram-positive bacterial strains such as strains of *Bacillus*, *Streptomyces*, *Staphylococcus*, and *Lactococcus.* Suitable fungal cell include cells from species of *Trichoderma*, *Neurospora*, and *Aspergillus*. Suitable yeast cells include cells from species of *Saccharomyces* (for example *Saccharomyces cerevisiae),* *Schizosaccharomyces* (for example *Schizosaccharomyces pombe*), *Pichia* (for example *Pichia pastoris* and *Pichia methanolica*), and *Hansenula*.

Suitable mammalian cells include for example CHO cells, BHK cells, HeLa cells, COS cells, and the like. However, amphibian cells, insect cells, plant cells, and any other cells used in the art for the expression of heterologous proteins can be used as well.

The invention further provides methods of manufacturing a VEGF-binding molecule of the invention, such methods generally comprising the steps of:
- culturing host cells comprising a nucleic acid capable of encoding a VEGF-binding molecule under conditions that allow expression of the VEGF-binding molecule of the invention; and
- recovering or isolating the polypeptide expressed by the host cells from the culture; and
- optionally further purifying and/or modifying and/or formulating the VEGF-binding molecule of the invention.

For production on an industrial scale, preferred host organisms include strains of *E. coli*, *Pichia pastoris*, and *S. cerevisiae* that are suitable for large scale expression, production and fermentation, and in particular for large scale pharmaceutical expression, production and fermentation.

The choice of the specific expression system depends in part on the requirement for certain post-translational modifications, more specifically

glycosylation. The production of a VEGF-binding molecule of the invention for which glycosylation is desired or required would necessitate the use of mammalian expression hosts that have the ability to glycosylate the expressed protein. In this respect, it will be clear to the skilled person that the
5 glycosylation pattern obtained (i.e. the kind, number and position of residues attached) will depend on the cell or cell line that is used for the expression.

VEGF-binding molecules of the invention may be produced in a cell as set out above either intracellullarly (e.g. in the cytosol, in the periplasma or in inclusion bodies) and then isolated from the host cells and optionally further purified; or
10 they can be produced extracellularly (e.g. in the medium in which the host cells are cultured) and then isolated from the culture medium and optionally further purified.

Methods and reagents used for the recombinant production of polypeptides, such as specific suitable expression vectors, transformation or transfection
15 methods, selection markers, methods of induction of protein expression, culture conditions, and the like, are known in the art. Similarly, protein isolation and purification techniques useful in a method of manufacture of a polypeptide of the invention are well known to the skilled person.

In a further aspect, the invention relates to a peptide having an amino acid
20 sequence of a CDR3 contained in an anti-VEGF-VHH having an amino acid sequence selected from sequences shown in SEQ ID NOs: 9 to 57 or SEQ ID NOs: 58 - 127, respectively, and a nucleic acid molecule encoding same.

These peptides correspond to CDR3s derived from the VHHs of the invention. They, in particular the nucleic acid molecules encoding them, are useful for
25 CDR grafting in order to replace a CDR3 in an immunoglobulin chain, or for insertion into a non-immunoglobulin scaffold, e.g. a protease inhibitor, DNA-binding protein, cytochrome b562, a helix-bundle protein, a disulfide-bridged

peptide, a lipocalin or an anticalin, thus conferring target-binding properties to such scaffold. The method of CDR-grafting is well known in the art and has been widely used, e.g. for humanizing antibodies (which usually comprises grafting the CDRs from a rodent antibody onto the Fv frameworks of a human 5 antibody).

In order to obtain an immunoglobulin or a non-immunoglobulin scaffold containing a CDR3 of the invention, the DNA encoding such molecule may be obtained according to standard methods of molecular biology, e.g. by gene synthesis, by oligonucleotide annealing or by means of overlapping PCR 10 fragments, as e.g. described by Daugherty *et al.*, 1991, Nucleic Acids Research, Vol. 19, 9, 2471 – 2476. A method for inserting a VHH CDR3 into a non-immunoglobulin scaffold has been described by Nicaise *et al.*, 2004, Protein Science, 13, 1882 – 1891.

The invention further relates to a product or composition containing or 15 comprising at least one VEGF-binding molecule of the invention and optionally one or more further components of such compositions known *per se*, i.e. depending on the intended use of the composition.

For pharmaceutical use, a VEGF-binding molecule of the invention may be formulated as a pharmaceutical preparation or composition comprising at least 20 one VEGF-binding molecule of the invention and at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally one or more further pharmaceutically active polypeptides and/or compounds. By means of non-limiting examples, such a formulation may be in a form suitable for oral administration, for parenteral administration (such as by intravenous, 25 intramuscular or subcutaneous injection or intravenous infusion), for topical administration, for administration by inhalation, by a skin patch, by an implant, by a suppository, etc. Such suitable administration forms - which may be solid, semi-solid or liquid, depending on the manner of administration - as well as

methods and carriers for use in the preparation thereof, will be clear to the skilled person, and are further described herein.

Thus, in a further aspect, the invention relates to a pharmaceutical composition that contains at least one VEGF-binding molecule, in particular one immunoglobulin single variable domain, of the invention and at least one suitable carrier, diluent or excipient (i.e. suitable for pharmaceutical use), and optionally one or more further active substances.

The VEGF-binding molecules of the invention may be formulated and administered in any suitable manner known per se: Reference, in particular for the immunoglobulin single variable domains, is for example made to WO04/041862, WO04/041863, WO04/041865, WO04/041867 and WO08/020079, as well as to the standard handbooks, such as Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Company, USA (1990), Remington, the Science and Practice of Pharmacy, 21th Edition, Lippincott Williams and Wilkins (2005); or the Handbook of Therapeutic Antibodies (S. Dubel, Ed.), Wiley, Weinheim, 2007 (see for example pages 252-255).

For example, an immunoglobulin single variable domain of the invention may be formulated and administered in any manner known per se for conventional antibodies and antibody fragments (including ScFv's and diabodies) and other pharmaceutically active proteins. Such formulations and methods for preparing the same will be clear to the skilled person, and for example include preparations suitable for parenteral administration (for example intravenous, intraperitoneal, subcutaneous, intramuscular, intraluminal, intra-arterial or intrathecal administration) or for topical (i.e. transdermal or intradermal) administration.

Preparations for parenteral administration may for example be sterile solutions, suspensions, dispersions or emulsions that are suitable for infusion or

injection. Suitable carriers or diluents for such preparations for example include, without limitation, sterile water and pharmaceutically acceptable aqueous buffers and solutions such as physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution; water oils; glycerol; ethanol; glycols such as propylene glycol or as well as mineral oils, animal oils and vegetable oils, for example peanut oil, soybean oil, as well as suitable mixtures thereof. Usually, aqueous solutions or suspensions will be preferred.

Thus, the VEGF-binding molecule of the invention may be systemically administered, e.g., orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. For oral therapeutic administration, the VEGF-binding molecule of the invention may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of the VEGF-binding molecule of the invention. Their percentage in the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of the VEGF-binding molecule of the invention in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, pills, capsules, and the like may also contain binders, excipients, disintegrating agents, lubricants and sweetening or flavouring agents, for example those mentioned on pages 143-144 of WO08/020079. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may

be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the VEGF-binding molecules of the invention, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in
5 preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the VEGF-binding molecules of the invention may be incorporated into sustained-release preparations and devices.

Preparations and formulations for oral administration may also be provided
10 with an enteric coating that will allow the constructs of the invention to resist the gastric environment and pass into the intestines. More generally, preparations and formulations for oral administration may be suitably formulated for delivery into any desired part of the gastrointestinal tract. In addition, suitable suppositories may be used for delivery into the
15 gastrointestinal tract.

The VEGF-binding molecules of the invention may also be administered intravenously or intraperitoneally by infusion or injection, as further described on pages 144 and 145 of WO08/020079.

For topical administration of the VEGF-binding molecules of the invention, it
20 will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid, as further described on page 145 of WO08/020079.

Generally, the concentration of the VEGF-binding molecules of the invention in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%,
25 preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the VEGF-binding molecules of the invention required for use in treatment will vary not only with the particular VEGF-binding molecule selected, but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician. Also, the dosage of the VEGF-binding molecules of the invention varies depending on the target cell, tumor, tissue, graft, or organ.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

An administration regimen may include long-term, daily treatment. By “long-term” is meant at least two weeks and preferably, several weeks, months, or years of duration. Necessary modifications in this dosage range may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. See Remington’s Pharmaceutical Sciences (Martin, E.W., ed. 4), Mack Publishing Co., Easton, PA. The dosage can also be adjusted by the individual physician in the event of any complication.

According to a further embodiment, the invention relates to the use of VEGF-binding molecules, e.g. immunoglobulin single variable domains, for therapeutic purposes, such as

- for the prevention, treatment and/or alleviation of a disorder, disease or condition, especially in a human being, that is associated with VEGF-mediated effects on angiogenesis or that can be prevented, treated or

alleviated by modulating the Notch signaling pathway with a VEGF-binding molecule,

- in a method of treatment of a patient in need of such therapy, such method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one VEGF-binding molecule of the invention, e.g. an immunoglobulin single variable domain, or a pharmaceutical composition containing same;

- for the preparation of a medicament for the prevention, treatment or alleviation of disorders, diseases or conditions associated with VEGF-mediated effects on angiogenesis;

- as an active ingredient in a pharmaceutical composition or medicament used for the above purposes.

According to a specific aspect, said disorder disorder, disease or condition is a cancer or cancerous disease, as defined herein.

According to another aspect, the disease is an eye disease associated with VEGF-mediated effects on angiogenesis or which can be treated or alleviated by modulating the Notch signaling pathway with a VEGF-binding molecule.

Depending on the cancerous disease to be treated, a VEGF-binding molecule of the invention may be used on its own or in combination with one or more additional therapeutic agents, in particular selected from chemotherapeutic agents like DNA damaging agents or therapeutically active compounds that inhibit angiogenesis, signal transduction pathways or mitotic checkpoints in cancer cells.

The additional therapeutic agent may be administered simultaneously with, optionally as a component of the same pharmaceutical preparation, or before or after administration of the VEGF-binding molecule.

In certain embodiments, the additional therapeutic agent may be, without limitation (and in the case of the receptors, including the respective ligands), one or more inhibitors selected from the group of inhibitors of EGFR, VEGFR, HER2-neu, Her3, AuroraA, AuroraB, PLK and PI3 kinase, FGFR, PDGFR, Raf,
5 KSP, PDK1, PTK2, IGF-R or IR.

Further examples of additional therapeutic agents are inhibitors of CDK, Akt, src/bcr abl, cKit, cMet/HGF, c-Myc, Flt3, HSP90, hedgehog antagonists, inhibitors of JAK/STAT, Mek, mTor, NFkappaB, the proteasome, Rho, an inhibitor of wnt signaling or an inhibitor of the ubiquitination pathway or another
10 inhibitor of the Notch signaling pathway.

Examples for Aurora inhibitors are, without limitation, PHA-739358, AZD-1152, AT 9283, CYC-116, R-763, VX-680, VX-667, MLN-8045, PF-3814735.

An example for a PLK inhibitor is GSK-461364.

Examples for raf inhibitors are BAY-73-4506 (also a VEGFR inhibitor),
15 PLX 4032, RAF-265 (also in addition a VEGFR inhibitor), sorafenib (also in addition a VEGFR inhibitor), and XL 281.

Examples for KSP inhibitors are ispinesib, ARRY-520, AZD-4877, CK-1122697, GSK 246053A, GSK-923295, MK-0731, and SB-743921.

Examples for a src and/or bcr-abl inhibitors are dasatinib, AZD-0530,
20 bosutinib, XL 228 (also an IGF-1R inhibitor), nilotinib (also a PDGFR and cKit inhibitor), imatinib (also a cKit inhibitor), and NS-187.

An example for a PDK1 inhibitor is BX-517.

An example for a Rho inhibitor is BA-210.

Examples for PI3 kinase inhibitors are PX-866, BEZ-235 (also an mTor inhibitor), XL 418 (also an Akt inhibitor), XL-147, and XL 765 (also an mTor inhibitor).

Examples for inhibitors of cMet or HGF are XL-184 (also an inhibitor of
5 VEGFR, cKit, Flt3), PF-2341066, MK-2461, XL-880 (also an inhibitor of VEGFR), MGCD-265 (also an inhibitor of VEGFR, Ron, Tie2), SU-11274, PHA-665752, AMG-102, and AV-299.

An example for a c-Myc inhibitor is CX-3543.

Examples for Flt3 inhibitors are AC-220 (also an inhibitor of cKit and PDGFR),
10 KW 2449, lestaurtinib (also an inhibitor of VEGFR, PDGFR, PKC), TG-101348 (also an inhibitor of JAK2), XL-999 (also an inhibitor of cKit, FGFR, PDGFR and VEGFR), sunitinib (also an inhibitor of PDGFR, VEGFR and cKit), and tandutinib (also an inhibitor of PDGFR, and cKit).

Examples for HSP90 inhibitors are tanespimycin, alvespimycin, IPI-504 and
15 CNF 2024.

Examples for JAK/STAT inhibitors are CYT-997 (also interacting with tubulin), TG 101348 (also an inhibitor of Flt3), and XL-019.

Examples for Mek inhibitors are ARRY-142886, PD-325901, AZD-8330, and XL 518.

20 Examples for mTor inhibitors are temsirolimus, AP-23573 (which also acts as a VEGF inhibitor), everolimus (a VEGF inhibitor in addition). XL-765 (also a PI3 kinase inhibitor), and BEZ-235 (also a PI3 kinase inhibitor).

Examples for Akt inhibitors are perifosine, GSK-690693, RX-0201, and triciribine.

Examples for cKit inhibitors are AB-1010, OSI-930 (also acts as a VEGFR inhibitor), AC-220 (also an inhibitor of Flt3 and PDGFR), tandutinib (also an inhibitor of Flt3 and PDGFR), axitinib (also an inhibitor of VEGFR and PDGFR), XL-999 (also an inhibitor of Flt3, PDGFR, VEGFR, FGFR), sunitinib (also an inhibitor of Flt3, PDGFR, VEGFR), and XL-820 (also acts as a VEGFR- and PDGFR inhibitor), imatinib (also a bcr-abl inhibitor), nilotinib (also an inhibitor of bcr-abl and PDGFR).

Examples for hedgehog antagonists are IPI-609 and CUR-61414.

Examples for CDK inhibitors are seliciclib, AT-7519, P-276, ZK-CDK (also inhibiting VEGFR2 and PDGFR), PD-332991, R-547, SNS-032, PHA-690509, and AG 024322.

Examples for proteasome inhibitors are bortezomib, carfilzomib, and NPI-0052 (also an inhibitor of NFkappaB).

An example for an NFkappaB pathway inhibitor is NPI-0052.

An example for an ubiquitination pathway inhibitor is HBX-41108.

In preferred embodiments, the additional therapeutic agent is an anti-angiogenic agent.

Examples for anti-angiogenic agents are inhibitors of the FGFR, PDGFR and VEGFR or the respective ligands (e.g VEGF inhibitors like pegaptanib or the anti-VEGF antibody bevacizumab), EGFL7 inhibitors, such as anti-EGFL7 MAb , angiopoietin1/2 inhibitors such as AMG386, and thalidomides, such agents being selected from, without limitation, bevacizumab, motesanib, CDP-791, SU-14813, telatinib, KRN-951, ZK-CDK (also an inhibitor of CDK), ABT-869, BMS-690514, RAF-265, IMC-KDR, IMC-18F1, IMiDs (immunomodulatory drugs), thalidomide derivative CC-4047, lenalidomide, ENMD 0995, IMC-D11, Ki 23057, brivanib, cediranib, XL-999 (also an inhibitor

of cKit and Flt3), 1B3, CP 868596, IMC 3G3, R-1530 (also an inhibitor of Flt3), sunitinib (also an inhibitor of cKit and Flt3), axitinib (also an inhibitor of cKit), lestaurtinib (also an inhibitor of Flt3 and PKC), vatalanib, tandutinib (also an inhibitor of Flt3 and cKit), pazopanib, GW 786034, PF-337210, IMC-1121B, AVE-0005, AG-13736, E-7080, CHIR 258, sorafenib tosylate (also an inhibitor of Raf), RAF-265 (also an inhibitor of Raf), vandetanib, CP-547632, OSI-930, AEE-788 (also an inhibitor of EGFR and Her2), BAY-57-9352 (also an inhibitor of Raf), BAY-73-4506 (also an inhibitor of Raf), XL 880 (also an inhibitor of cMet), XL-647 (also an inhibitor of EGFR and EphB4), XL 820 (also an inhibitor of cKit), and nilotinib (also an inhibitor of cKit and brc-abl).

The additional therapeutic agent may also be selected from EGFR inhibitors, it may be a small molecule EGFR inhibitor or an anti-EGFR antibody. Examples for anti-EGFR antibodies, without limitation, are cetuximab, panitumumab, matuzumab; an example for a small molecule EGFR inhibitor is gefitinib. Another example for an EGFR modulator is the EGF fusion toxin.

Among the EGFR and Her2 inhibitors useful for combination with the VEGF-binding molecule of the invention are lapatinib, gefitinib, erlotinib, cetuximab, trastuzumab, nimotuzumab, zalutumumab, vandetanib (also an inhibitor of VEGFR), pertuzumab, XL-647, HKI-272, BMS-599626 ARRY-334543, AV 412, mAB-806, BMS-690514, JNJ-26483327, AEE-788 (also an inhibitor of VEGFR), ARRY-333786, IMC-11F8, Zemab.

Other agents that may be advantageously combined in a therapy with the VEGF-binding molecule of the invention are tositumumab and ibritumomab tiuxetan (two radiolabelled anti-CD20 antibodies), alemtuzumab (an anti-CD52 antibody), denosumab, (an osteoclast differentiation factor ligand inhibitor), galiximab (a CD80 antagonist), ofatumumab (a CD20 inhibitor), zanolimumab (a CD4 antagonist), SGN40 (a CD40 ligand receptor modulator), rituximab (a

CD20 inhibitor), mapatumumab (a TRAIL-1 receptor agonist), REGN421(SAR153192) or OMP-21M18 (Dll4 inhibitors).

Other chemotherapeutic drugs that may be used in combination with the VEGF-binding molecules of the present invention are selected from, but not limited to hormones, hormonal analogues and antihormonals (e.g. tamoxifen, toremifene, raloxifene, fulvestrant, megestrol acetate, flutamide, nilutamide, bicalutamide, cyproterone acetate, finasteride, buserelin acetate, fludrocortisone, fluoxymesterone, medroxyprogesterone, octreotide, arzoxifene, pasireotide, vapreotide), aromatase inhibitors (e.g. anastrozole, letrozole, liarozole, exemestane, atamestane, formestane), LHRH agonists and antagonists (e.g. goserelin acetate, leuprolide, abarelix, cetrorelix, deslorelin, histrelin, triptorelin), antimetabolites (e.g. antifolates like methotrexate, pemetrexed, pyrimidine analogues like 5 fluorouracil, capecitabine, decitabine, nelarabine, and gemcitabine, purine and adenosine analogues such as mercaptopurine thioguanine, cladribine and pentostatin, cytarabine, fludarabine); antitumor antibiotics (e.g. anthracyclines like doxorubicin, daunorubicin, epirubicin and idarubicin, mitomycin-C, bleomycin dactinomycin, plicamycin, mitoxantrone, pixantrone, streptozocin); platinum derivatives (e.g. cisplatin, oxaliplatin, carboplatin, lobaplatin, satraplatin); alkylating agents (e.g. estramustine, meclorethamine, melphalan, chlorambucil, busulphan, dacarbazine, cyclophosphamide, ifosfamide, hydroxyurea, temozolomide, nitrosoureas such as carmustine and lomustine, thiotepa); antimitotic agents (e.g. vinca alkaloids like vinblastine, vindesine, vinorelbine, vinflunine and vincristine; and taxanes like paclitaxel, docetaxel and their formulations, larotaxel; simotaxel, and epothilones like ixabepilone, patupilone, ZK-EPO); topoisomerase inhibitors (e.g. epipodophyllotoxins like etoposide and etopophos, teniposide, amsacrine, topotecan, irinotecan) and miscellaneous chemotherapeutics such as amifostine, anagrelide, interferone alpha, procarbazine, mitotane, and porfimer, bexarotene, celecoxib.

The efficacy of VEGF-binding molecules of the invention or polypeptides, and of compositions comprising the same, can be tested using any suitable *in vitro* assay, cell- based assay, *in vivo* assay and/or animal model known per se, or any combination thereof, depending on the specific disease or disorder of
5 interest. Suitable assays and animal models will be clear to the skilled person, and for example include the assays described herein and used in the Examples below, e.g. a proliferation assay.

The data obtained in the experiments of the invention confirm that VEGF-binding molecules of the invention have properties that are superior to those of
10 VEGF-binding molecules of the prior art. Among such properties are complete inhibition of the VEGF165-VEGFR2 interaction and a low IC50, as can e.g. be taken from the ELISA data of Figure 1 and Table 5 as well as the IC_{50} (nM) values for VHHs in the AlphaScreen assay as shown in Figures 3, 17, 18 and Table 7; and the affinity K_D (nM) of purified VHHs on recombinant human
15 VEGF and mouse VEGF in Table 9, 10 and Figures 5-1 and 5-2. Also, as shown in Table 13, VEGF binders of the invention have high potency, i.e. in the subnanomolar range, in the HUVEC proliferation assay. This indicates that VEGF-binding molecules of the invention are promising candidates to have therapeutic efficacy in diseases and disorders associated with VEGF-mediated
20 effects on angiogenesis, such as cancer.

According to another embodiment of the invention, there is provided a method of diagnosing a disease by

a) contacting a sample with a VEGF-binding molecule of the invention as defined above, and

25 b) detecting binding of said VEGF-binding molecule to said sample, and

c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease or disorder associated with VEGF-mediated effects on angiogenesis.

For this and other uses, it may be useful to further modify a VEGF-binding molecule of the invention, such as by introduction of a functional group that is one part of a specific binding pair, such as the biotin-(strept)avidin binding pair. Such a functional group may be used to link the VEGF-binding molecule of the invention to another protein, polypeptide or chemical compound that is bound to the other half of the binding pair, i.e. through formation of the binding pair. For example, a VEGF-binding molecule of the invention may be conjugated to biotin, and linked to another protein, polypeptide, compound or carrier conjugated to avidin or streptavidin. For example, such a conjugated VEGF-binding molecule of the invention may be used as a reporter, for example in a diagnostic system where a detectable signal-producing agent is conjugated to avidin or streptavidin.

Brief description of the Figures:

Figure 1: Purified monovalent VHHs block the hVEGF165/hVEGFR2-Fc interaction (ELISA)

Figure 2: Purified monovalent VHHs block the hVEGF165/hVEGFR1-Fc interaction (ELISA)

Figure 3: Purified monovalent VHHs block the hVEGF165/hVEGFR2-Fc interaction (AlphaScreen)

Figure 4: Purified monovalent VHHs block the hVEGF165/hVEGFR1-Fc interaction (AlphaScreen)

Figures 5-1 and 5-2: Binding of monovalent VHHs to recombinant human and mouse VEGF (ELISA)

Figure 6: Binding of monovalent VHHs to human VEGF121

Figure 7-1 through 7-4: Purified VHHs do not bind to VEGFB, VEGFC, VEGFD and PlGF

Figure 8-1 and 8-2: Formatted VHHs block hVEGF165/hVEGFR2-Fc interaction (ELISA)

Figure 9-1 and 9-2: Formatted VHHs block hVEGF165/hVEGFR1-Fc interaction (ELISA)

Figure 10: Formatted VHHs block hVEGF165/hVEGFR2-Fc interaction (AlphaScreen)

Figure 11: Formatted VHHs block hVEGF165/hVEGFR1-Fc interaction (AlphaScreen)

Figure 12: Formatted VHHs block mVEGF164/mVEGFR2-Fc interaction (AlphaScreen)

Figure 13-1 and 13-2: Formatted VHHs bind to mouse and human VEGF

Figure 14-1 through 14-8: Formatted VHHs do not bind to VEGFB, VEGFC, VEGFD and PlGF

Figure 15: Formatted VHHs bind to VEGF121

Figure 16: Sequence alignment of VHH VEGFBII23B04 with human VH3/JH germline consensus sequence

Figure 17: VHH variants of VEGFBII23B04 block hVEGF165/hVEGFR2-Fc interaction (AlphaScreen)

Figure 18: Sequence-optimized clones of VEGFBII23B04 block the hVEGF165/hVEGFR2-Fc interaction (AlphaScreen)

Figure 19: Sequence alignment of VHH VEGFBII5B05 with human VH3/JH germline consensus sequence

Materials and methods:

a) Production and functionality testing of VEGF109

A cDNA encoding the receptor binding domain of human vascular endothelial growth factor isoform VEGF165 (GenBank: AAM03108.1; AA residues 27 - 135) is cloned into pET28a vector (Novagen, Madison, WI) and overexpressed in E.coli (BL21 Star DE3) as a His-tagged insoluble protein. Expression is induced by addition of 1 mM IPTG and allowed to continue for 4 hours at 37°C. Cells are harvested by centrifugation and lysed by sonication of the cell pellet. Inclusion bodies are isolated by centrifugation. After a washing step with 1% Triton X 100 (Sigma-Aldrich), proteins are solubilized using 7.5M guanidine hydrochloride and refolded by consecutive rounds of overnight dialysis using buffers with decreasing urea concentrations from 6M till 0M. The refolded protein is purified by ion exchange chromatography using a MonoQ5/50GL (Amersham BioSciences) column followed by gel filtration with a Superdex75 10/300 GL column (Amersheim BioSciences). The purity and homogeneity of the protein is confirmed by SDS-PAGE and Westen blot. In addition, binding activity to VEGFR1, VEGFR2 and Bevacizumab is monitored by ELISA. To this end, 1 µg/mL of recombinant human VEGF109 is immobilized overnight at 4°C in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Wells are blocked with a casein solution (1%). Serial dilutions of VEGFR1, VEGFR2 or Bevacizumab are added to the VEGF109 coated plate and binding is detected using alkaline phosphatase (AP) conjugated goat anti-human IgG, Fc specific (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) and a subsequent enzymatic reaction in the presence of the substrate PNPP (p-nitrophenylphosphate) (Sigma-Aldrich). VEGF109 could bind to VEGFR1, VEGFR2 and Bevacizumab, indicating that the produced VEGF109 is active.

b) KLH conjugation of VEGF165 and functionality testing of KLH-conjugated VEGF165

Recombinant human VEGF165 (R&D Systems, Minneapolis, MN, USA) is conjugated to mariculture keyhole limpet hemocyanin (mcKLH) using the
5 Imject Immunogen EDC kit with mcKLH (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Efficient conjugation of the polypeptide to mcKLH is confirmed by SDS-PAGE. Functionality of the conjugated protein is checked by ELISA: 2 µg/mL of KLH conjugated VEGF165 is immobilized overnight at 4°C in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany).
10 Wells are blocked with a casein solution (1%). Serial dilutions of VEGFR1 or VEGFR2 are added and binding is detected using a horseradish peroxidase (HRP)-conjugated goat anti-human IgG, Fc specific (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) and a subsequent enzymatic reaction in the presence of the substrate TMB (3,3',5,5'-
15 tetramentylbenzidine) (Pierce, Rockford, IL, USA). The KLH conjugated protein could still interact with VEGFR1, VEGFR2 and Bevacizumab, confirming that the relevant epitopes onVEGF165 are still accessible.

Example 1

Immunization with different VEGF formats induces a humoral immune
20 **response in llama**

1.1 Immunizations

After approval of the Ethical Committee of the faculty of Veterinary Medicine (University Ghent, Belgium), 4 llamas (designated No. 264, 265, 266, 267) are immunized according to standard protocols with 6 intramuscular injections
25 (100 or 50 µg/dose at weekly intervals) of recombinant human VEGF109. The first injection at day 0 is formulated in Complete Freund's Adjuvant (Difco, Detroit, MI, USA), while the subsequent injections are formulated in Incomplete

Freund's Adjuvant (Difco, Detroit, MI, USA). In addition, four llamas (designated No. 234, 235, 280 and 281) are immunized according to the following protocol: 5 intramuscular injections with KLH-conjugated human VEGH165 (100 or 50 µg/dose at biweekly intervals) followed by 4
5 intramuscular injections of human VEGF109 (first dose of 100 µg followed 2 weeks later with three 50 µg/dose at weekly interval).

1.2 Evaluation of VEGF-induced immune responses in llama

To monitor VEGF specific serum titers, an ELISA assay is set up in which 2 µg/mL of recombinant human VEGF165 or VEGF109 is immobilized
10 overnight at 4°C in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Wells are blocked with a casein solution (1%). After addition of serum dilutions, bound total IgG is detected using horseradish peroxidase (HRP)-conjugated goat anti-llama immunoglobulin (Bethyl Laboratories Inc., Montgomery, TX, USA) and a subsequent enzymatic reaction in the presence of the substrate
15 TMB (3,3',5,5'-tetramentylbenzidine) (Pierce, Rockford, IL, USA). For llamas 264, 265, 266 and 267, an additional ELISA is performed in which the isotype-specific responses against VEGF165 and VEGF109 are evaluated. Isotype specific responses are detected using mouse mAbs specifically recognizing conventional llama IgG1 and the heavy-chain only llama IgG2 and IgG3 [Daley
20 et al. (2005). Clin. Diagn. Lab. Imm. 12:380-386] followed by a rabbit anti-mouse-HRP conjugate (DAKO). ELISAs are developed using TMB as chromogenic substrate and absorbance is measured at 450nm. The serum titers for each llama are depicted in Table 1.

Table 1: Antibody-mediated specific serum response against VEGF165 and VEGF109

ELISA (recombinant protein solid phase coated)

		Recombinant human EGF165				Recombinant human VEGF109			
Llama	**Immunogen**	**Total IgG**	**IgG1**	**IgG2**	**IgG3**	**Total IgG**	**IgG1**	**IgG2**	**IgG3**
234	VEGF165-KLH + VEGF109	++	n/d	n/d	n/d	++	n/d	n/d	n/d
235	VEGF165-KLH + VEGF109	++	n/d	n/d	n/d	++	n/d	n/d	n/d
280	VEGF165-KLH + VEGF109	+	n/d	n/d	n/d	+	n/d	n/d	n/d
281	VEGF165-KLH + VEGF109	+	n/d	n/d	n/d	+	n/d	n/d	n/d
264	VEGF109	n/d	++	+	+	++	++	+	+
265	VEGF109	n/d	++	+	+	+	++	+	+
266	VEGF109	n/d	++	+	+/-	++	++	+	+/-
267	VEGF109	n/d	+/-	-	-	+/-	+/-	-	-

5 n/d, not determined

Example 2

Cloning of the heavy-chain only antibody fragment repertoires and preparation of phage

Following the final immunogen injection, immune tissues as the source of B-cells that produce the heavy-chain antibodies are collected from the immunized llamas. Typically, two 150-ml blood samples, collected 4 and 8 days after the last antigen injection, and one lymph node biopsy, collected 4 days after the last antigen injection are collected per animal. From the blood samples, peripheral blood mononuclear cells (PBMCs) are prepared using Ficoll-Hypaque according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). From the PBMCs and the lymph node biopsy, total RNA is extracted, which is used as starting material for RT-PCR to amplify the VHH encoding DNA segments, as described in WO05/044858. For each immunized llama, a library is constructed by pooling the total RNA isolated from all collected immune tissues of that animal. In short, the PCR-amplified VHH repertoire is cloned via specific restriction sites into a vector designed to facilitate phage display of the VHH library. The vector is derived from pUC119 and contains the LacZ promoter, a M13 phage gIII protein coding sequence, a resistance gene for ampicillin or carbenicillin, a multiple cloning site and a hybrid gIII-pelB leader sequence (pAX050). In frame with the VHH coding sequence, the vector encodes a C-terminal c-myc tag and a His6 tag. Phage are prepared according to standard protocols and stored after filter sterilization at 4ºC for further use.

Example 3

Selection of VEGF-specific VHHs via phage display

VHH phage libraries are used in different selection strategies applying a multiplicity of selection conditions. Variables include i) the VEGF protein format

(rhVEGF165, rhVEGF109 or rmVEGF164), ii) the antigen presentation method (solid phase: directly coated or via a biotin-tag onto Neutravidin-coated plates; solution phase: incubation in solution followed by capturing on Neutravidin-coated plates), iii) the antigen concentration and iv) the elution method (trypsin or competitive elution using VEGFR2). All selections are carried out in Maxisorp 96-well plates (Nunc, Wiesbaden, Germany).

Selections are performed as follows: Phage libraries are incubated at RT with variable concentrations of VEGF antigen, either in solution or immobilized on a solid support. After 2hrs of incubation and extensive washing, bound phage are eluted. In case trypsin is used for phage elution, the protease activity is immediately neutralized by addition of 0.8 mM protease inhibitor AEBSF. Phage outputs that show enrichment over background are used to infect *E. coli*. Infected E. *coli* cells are either used to prepare phage for the next selection round (phage rescue) or plated on agar plates (LB+amp+glucose$^{2\%}$) for analysis of individual VHH clones. In order to screen a selection output for specific binders, single colonies are picked from the agar plates and grown in 1 mL 96-deep-well plates. The lacZ-controlled VHH expression is induced by adding IPTG (0.1-1mM final). Periplasmic extracts (in a volume of ~ 80 µL) are prepared according to standard methods.

Example 4

Identification of VEGF-binding and VEGF receptor-blocking VHHs

Periplasmic extracts are tested for binding to human VEGF165 by ELISA. In brief, 2 µg/mL of recombinant human VEGF165 is immobilized overnight at 4°C in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Wells are blocked with a casein solution (1%). After addition of typically a 10-fold dilution of the periplasmic extracts, VHH binding is detected using a mouse anti-myc

(Roche) and an anti-mouse-HRP conjugate (DAKO). Clones showing ELISA signals of >3-fold above background are considered as VEGF binding VHHs.

In addition, periplasmic extracts are screened in a human VEGF165/human VEGFR2 AlphaScreen assay (Amplified Luminescent Proximity Homogeneous Assay) to assess the blocking capacity of the VHHs. Human VEGF165 is biotinylated using Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA). Human VEGFR2/Fc chimera (R&D Systems, Minneapolis, MN, USA) is captured using an anti-humanFc VHH which is coupled to acceptor beads according to the manufacturer's instructions (Perkin Elmer, Waltham, MA, US). To evaluate the neutralizing capacity of the VHHs, periplasmic extracts are diluted 1/25 in PBS buffer containing 0.03 % Tween 20 (Sigma-Aldrich) and preincubated with 0.4 nM biotinylated human VEGF165 for 15 minutes at room temperature (RT). To this mixture the acceptor beads (10µg/ml) and 0.4 nM VEGFR2-huFc are added and further incubated for 1 hour at RT in the dark. Subsequently donor beads (10µg/ml) are added followed by incubation of 1 hour at RT in the dark. Fluorescence is measured by reading plates on the Envision Multi label Plate reader (Perkin Elmer, Waltham, MA, USA) using an excitation wavelength of 680 nm and an emission wavelength between 520 nm and 620nm. Periplasmic extract containing irrelevant VHH is used as negative control. Periplasmic extracts containing anti-VEGF165 VHHs which are able to decrease the fluorescence signal with more than 60 % relative to the signal of the negative control are identified as a hit. All hits identified in the AlphaScreen are confirmed in a competition ELISA. To this end, 1 µg/mL of human VEGFR2 chimera (R&D Systems, Minneapolis, MN, USA) is coated in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Fivefold dilutions of the periplasmic extracts are incubated in the presence of a fixed concentration (4nM) of biotinylated human VEGF165 in PBS buffer containing 0.1 % casein and 0.05 % Tween 20 (Sigma-Aldrich). Binding of these VHH/bio-VEGF165 complexes to the human VEGFR2 chimera coated plate is detected using

horseradish peroxidase (HRP) conjugated extravidin (Sigma, St Louis, MO, USA). VHH sequence IDs and the corresponding AA sequences of VEGF-binding (non-receptor-blocking) VHHs and inhibitory (receptor-blocking) VHHs are listed in Table 2 and Table 3, respectively.

Table 2: Sequence IDs and AA sequences of monovalent “non-receptor-blocking” anti-VEGF VHHs (FR, framework; CDR, complementary determining region)

VHH ID/ SEQ ID NO:	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VEGFBII 01C02/58	EVQLVESGGG LVQAGGSLRL SCTASGGSFS	SYGMG	WFRQSPG KEREFVS	AISEYSNTY CSDSVRG	RFTISRDNTKNTV YLQMNSLTPDDTA IYYCAA	SPTILLTTEQWYK Y	WGQGTQ VTVSS
VEGFBII 01E07/59	EVQLVESGGG LVQAGDSLRL SCVATGRTFR	ASDMG	WFRQAPG KEREFVA	AINWSGLST FYTDSVKG	RFTISRDNDNGAL YLQMNTLKPEDTA VYSCAA	GRIPSSSRFSSPA AYAS	WGQGTQ VTVSS
VEGFBII 03D12/60	EVQLVESGGG LVQAGGSLRL SCTASTSIYT	ITVMA	WFRQAPG KEREFVA	AITWSAPTT YYADSVKG	RFTISRDNAKNTV YLRMNSLKPEDSA IYYCAA	DRFKGRSIVTPSD YRY	WGQGTQ VTVSS
VEGFBII 04B08/61	EVQLVESGGG LVQPGGSLRL SCAASGSAVG	DITVA	WYRQAPG IQRQLVA	TITPSGYTY YWDFVKG	RFTISRDNSKNIV YLQMNSLKPEDTA AYYCNT	QFY	WGQGTQ VTVSS
VEGFBII 05B02/62	EVQLVESGGG LVQAGGSLRL SCAASGRTFS	TDDVG	WFRQAPG KEREFVA	VIRWSTGGT YTSDSVKG	RFTLSRDNAKNTM YLQMNSLKPEDTA VYYCAA	RSRPLGAGAWYSG EKHYNY	WGQGTQ VTVSS
VEGFBII 05B03/63	EVQLVESGGG LAQAGDSLRL SCAASGRSFS	HYNMG	WFRQAPG KEREFVA	SIRGGGGST TYANSVKD	RFTISRENAKNTV YLQMNSLKPEDTA VYYCAA	TAFYRGPYDYDY	WGQGTQ VTVSS

VEGFBII 05B05/64	EVQLVESGGG LVQPGGSLRL SCVASGIRFM	SMA	WYRQAPG KHRELVA	RISSGGTTA YVDSVKG	RFTISRDNSKNTV YLQMNSLKAEDTA VYYCNT	FSSRPNP	WGAGTQ VTVSS
VEGFBII 06G02/65	EVQLVESGGG LVQPGGSLRL SCAASGNIFS	NNAMA	WYRQAPG KQRELVA	RISSGGGFT YYLDSVKG	RFTVSRDNAKNTV YLQMNSLKPEDTA VYYCNA	AYRTYNY	WGQGTQ VTVSS
VEGFBII 07A03/66	EVQLVESGGG LVQAGGSLRL SCAASTSIYS	ITVMA	WFRQAPG KESEFVA	AITWSAPSS YYADSVKG	RFTISRDNAKNTV YLQMNSLKPEDSA IYYCAA	DRFKGRSIVTRSD YKY	WGQGTQ VTVSS
VEGFBII 07A06/67	EVQLVESGGG LVQAGGSLRL SCAVSTSIYS	ISVMA	WFRQAPG KERAFVA	AITWSAPTT YYADSVKG	RFTISRDNAKNTV YLQTNSLKPEDSA IYYCAA	DRFKGRSIVTRSD YRY	WGQGTQ VTVSS
VEGFBII 07D08/68	EVQLVESGGG LVQTGGSLRL SCAASGRTFS	NYAMA	WFRQAPG KEREFVS	AINQRGSNT NYADSVKG	RFTISRDSAKNSV FLQMNSLKPEDTA VYYCAA	STWYGYSTYARRE EYRY	WGQGTQ VTVSS
VEGFBII 08D09/69	EVQLVESGGG LVQAGGSLRL SCAASGRSFS	DNVMG	WFRQAAG KEREFVA	HISRGGSRT EYAESVKG	RFTISRDNTKKTM YLQMNSLKPEDTA VYYCAA	SRSVALATARPYD Y	WGQGTQ VTVSS
VEGFBII 08E07/70	EVQLVESGGG LAQAGGSLRL SCTTSGLTFS	SYYMG	WFRQAPG KEREFVA	TISWNKIST IYTDSVKG	RFTVSRDNNKNTV YLQMNSLKPEDTA VYYCAA	DASRPTLRIPQY	WGQGTQ VTVSS
VEGFBII 08F06/71	EVQLVESGGG LVQPGGSLRL SCAASGSIVR	SDVMG	WYRQAPG KQRELVA	FIRSLGSTY YAGSVKG	RFTISRDDAANTV YLQMNNLKPEDTA VYYCNA	RFSGESY	WGQGTP VTVSS

VEGFBII 08F07/72	EVQLVESGGG LVQAGGSLRL SCAVSGSTFG	LYAMG	WFRQAPG REREFLS	AITWSAGDT QYADSVKG	RFTISRDNARNTV NLQMNGLKPEDTA VYYCAG	RQWGGTYYYHGSY AY	WGQGTQ VTVSS
VEGFBII 09A09/73	EVQLVESGGG LVQPGGSLRL SCVASGIRFM	SMA	WYRQAPG KHRELVA	RISSEGTTA YVDSVKG	RFTISRDNSKNTV YLQMNSLKAEDTA VYYCNT	FSSRPNP	WGAGTT VTVSS
VEGFBII 09A12/74	EVQLVESGGG LVQAGGSLRL SCAASGRTFS	TDDVG	WFRQAPG KEREFVA	VIRWSTGGT YTSDSVAG	RFTLSRDNAKNTM YLQMNSLKPEDTA VYYCAA	RSRPLGAGAWYTG ETRYDS	WGQGTQ VTVSS
VEGFBII 09D05/75	EVQLVESGGG LVQPGDSLRL SCAASGLSFS	RYGMG	WFRQAPG KEREFVI	AISEYDNVY TADSVRG	RFTISRDNSKSTV YLQMNSLKSEDTA VYYCAA	SPTILLSTDEWYK Y	WGRGTQ VTVSS
VEGFBII 09F05/76	EVQLVESGGG LVQAGGSLRL SCAASGRTFS	TDDVG	WFRQAPG KEREFVA	VIRWSTGGT YTSDSVKG	RFTLSRDNAKNTM YLQMNSLKPEDTA VYYCAA	RSRPLGAGAWYTG ETRYNY	WGQGTQ VTVSS
VEGFBII 10C07/77	EVQLVESGGG LVQAGGSLSL SCAASARAFS	NYAMG	WFRQVPG REREFVA	VITRSPSNT YYTDSVKG	RFTISRDNAKNIV YLQMNSLKPEDTA VYYCAA	HYWNSDSYTYTDS RWYNY	WGQGTQ VTVSS
VEGFBII 10E07/78	EVQLVESGGG LVQAGGSLRL SCAASGRTFS	NYAMG	WFRQAPG KERVLVA	DISSSGINT YVADAVKG	RFTISRDNAKNTV YLQMNSLKPEDTA VYYCAA	SAWWYSQMARDNY RY	WGQGTQ VTVSS
VEGFBII 10G04/79	EVQLVESGGG LVQAGGSLRL SCAASGDTLS	RYAMG	WFRQAPG KEREFVA	SINTSGKRT SYADSMKG	RFAVSRDNAKNTG YLQMNSLKLEDTA TYYCAA	DRFFGSDSNEPRA YRY	WGQGTQ VTVSS

VEGFBII 10G05/80	EVQLVESGGGLVQAGESLRLSCVASGITFS	NYNMG	WFRQAPGKEREFVA	TIRHHGYDTYYAESVKG	RFTISRDNAKNTVYLQMNSLKPEDTALYSCAK	KLFWDMDPKTGFSS	WGQGTQVTVSS
VEGFBII 11C08/81	EVQLVESGGGLVQAGGSLRLSCAASGRTLS	SYGLG	WFRQAPGKEREFVA	AIGWSGSSTYYADSVKG	RFTVSVDNAKNTVYLKMNSLEPEDTAVYYCAA	KVRNFNSDWDLLTSYNY	WGQGTQVTVSS
VEGFBII 11C11/82	EVQLVESGGGLVQAGGSLMLSCAASGRALS	SYAIG	WFRQAPGREREFVA	RISWSGANTYYADSVKG	RFTISRGNAKNTVYLQMNSLKPEDTAAYYCAA	QTTSKYDNYDARAYGY	WGQGTQVTVSS
VEGFBII 11D09/83	EEQLVESGGGLVQAGGSLMLSCAASGRALS	SYAIG	WFRQAPGREREFVA	RISWSGANTYYADSVKG	RFTISRGNAKNTVYLQMNSLKPEDTAAYYCAA	QTTSKYDNYDARAYGY	WGQGTQVTVSS
VEGFBII 11E04/84	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	SYAMG	WFRQAPGKEREFVA	TISQSGYSTYYADSVKG	RFTISRDNAKNTVNLQMNSLKPEDTAVYYCAA	DPFYSYGSPSPYRY	WGQGTQVTVSS
VEGFBII 11E05/85	EVQLVESGGGLVQPGGSLRLSCASSGRLFS	FSAMG	WFRQAPGKEREFVA	AFKWSGSTTYYADYVKG	RFTISTDNAKNILFLQMNSLKPEDTAIYYCAV	DRFYTGRYYSSDEYDY	WGQGTQVTVSS
VEGFBII 11F10/86	EVQLVESGGGLVQAGGSLRLSCAASTSIYS	ITVMA	WFRQAPGKEREFVA	AITWSAPSSYYADSVKG	RFTISRDNAKNTVYLQVNSLKPEDSAIYYCAA	DRFKGRSIVTRSDYRY	WGQGTQVTVSS
VEGFBII 11F12/87	EVQLVESGGGLVQSGGSLRLSCAASGRSFS	SLAMG	WFRQVPGKDREFVA	SISQSGITTSYADSVKS	RFTISRDSAKNTVYLQMNLLKPEDTAVYYCAT	SVFYSTALTRPVDYRY	WGQGTQVTVSS

VEGFBII 11G09/88	EVQLVESGGG LVQAGGSLRL SCAASTSIYS	ITVMA	WFRQAPG KEREFVA	AITWSAPTT YSADSVKG	RFTISRDNAKNTV YLQMNSLKPEDSA IYYCAA	DRFKGRSIVTRSD YRY	WGQGTQ VTVSS
VEGFBII 12A07/89	EVQLVESGGG LVQAGGSLRL SCSVTGRTFN	KYVMG	WFRQAPG NDREFVA	AITSRDGPT YYADSVKG	RFTISGDNTKNKI FLQMNSLMPEDTA VYYCAI	DEDLYHYSSYHFT RVDLYHY	WGQGTQ VTVSS
VEGFBII 12B01/90	EVQLVESGGG LVQPGGSLRL ACAASGFTLS	SSWMY	WVRQAPG KGLEWVS	RISPGGLFT YYVDSVKG	RFSVSTDNANNTL YLQMNSLKPEDTA LYSCAK	GGAPNYTP	RGRGTQ VTVSS
VEGFBII 12C04/91	EVQLVESGGG LVQPGGSLRL SCAASGSIVR	SDVMG	WYRQAPG KQRELVA	FIRSLGSTY YAGSVKG	RFTISRDNAANTV YLQMNNLKPEDTA VYYCNA	RFSGESY	WGQGTP VTVSS
VEGFBII 12E10/92	EVQLVESGGG LAQAGGSLRL SCTASGRTFN	NYVMG	WFRQAPG NEREFVA	AITSTNGPT YYADSVKG	RFTISGDNTKNKV FLQMDSLRPEDTA VYYCAI	DEDLYHYSSYHYT RVALYHY	WGQGTQ VTVSS
VEGFBII 12G04/93	EVQLVESGGG LVQSGDSLRL SCAVSGNTFG	LYAMG	WFRQAPG REREFVS	AITWSAGDT QYADSVKG	RFTISRDNARNTV NLQMNGLKPEDTA VYYCAG	RQWGGTYYYHGSY AW	WGQGTQ VTVSS
VEGFBII 16C03/94	EVQLVESEGG LVQAGGSLRL SCAASGRTFS	TDDVG	WFRQAPG KEREFVA	VIRWSTGGT YTSDSVKG	RFTLSRDNAKNTM YLQMNSLKPEDTA VYYCAA	RSRPLGAGAWYTG ENYYNY	WGQGTQ VTVSS
VEGFBII 16F11/95	EVQLVESGGG LVQAGGSLRL SCAASGRTSS	GYDMG	WFRQAPG KEREFVT	AITWSGGST YSPDSVKG	RFTISRDNAKNTV YLQMNNLTPEDTA VYYCAS	GRIWRSRDYDSEK YYDI	WGHGTQ VTVSS

VEGFBII 36C08/96	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	AYDMG	WFRQAPGKEREFVA	VISWTNSMTYYADSVKG	RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAV	DRRRTYSRWRFYTGVNDYDY	WGQGTQVTVSS
VEGFBII 37F09/97	EVQLVESGGGLVQTGGSLRLSCAASGRTFS	AYDMG	WFRQAPGKEREFVA	VISWSGGMTYYADSVQG	RFTISRDNAKSTVYLQMNSPKPEDTAVYYCAV	DRRRAYSRWRYYTGVNDYEF	WGQGTQVTVSS
VEGFBII 38A06/98	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	AYDMG	WFRQAPGKEREFVA	VISWSGGMTYYADSVKG	RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAV	DRRRLYSRWRYYTGVNDYDY	WGQGTQVTVSS
VEGFBII 39H11/99	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	AYDMG	WFRQAPGKEREFVA	VISWTGGMTYYADSVKG	RFTISRDKAKNTVSLQMNSLKPEDTAVYYCAV	DRRRTYSRWRYYTGVNEYEY	WGQGTQVTVSS
VEGFBII 41B06/100	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	AYDMG	WFRQAPGKEREFVA	VISWTGDMTYYADSVKG	RFTISRDKAKNTVSLQMNSLKPEDTAVYYCAA	DRRRTYSRWRYYTGVNEYEY	WGQGTQVTVSS
VEGFBII 41C05/101	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	VYTMG	WFRQAPGKEREFVA	TISRTGDRTSYANSVKG	RFTISRENAKNTVYLQMNSLKPEDTAVYSCAA	GPIAPSPRPREYYY	WGQGTQVTVSS
VEGFBII 41D11/102	EVQLMESGGGLVQAGGSLRLSCAASGRTFS	AYDMG	WFRQAPGKEREFVA	VISWTGGMTYYADSVKG	RFTISRDKAKNTVSLQMNSLKPEDTAVYYCAV	DRRRTYSRWRYYTGVNEYEY	WGQGTQVTVSS

VEGFBII 42F10/ **103**	EVQLVESGGG LVQAGGSLRL SCAASGRTFS	AYDMG	WFRQAPG KEREFVA	VISWSGGMT DYADSVKG	RFTISRENAKNTQ FLQMNSLKPEDTA VYYCAV	GRRRAYSRWRYYT GVNEYDY	WGQGTQ VTVSS
VEGFBII 86C11/ **104**	EVQLVESGGG LVQAGDSLRL SCTASGRTFN	SYAMG	WFRQAPG KERESVA	HINRSGSST YYADSVKG	RFTISRDNAKNTV YLQLNSLKPEDTA VYYCAA	GRYYSSDGVPSAS FNY	WGQGTQ VTVSS
VEGFBII 86F11/ **105**	EVQLVESGGG LVQAGDSLRL SCFTSARTFD	TWAMA	WFRQAPG KEREFIS	AISWSGSMT YYTDSVKG	RFIISRDNAQNTL FLQMNNTAPEDTA VYYCAA	KTVDYCSAYECYA RLEYDY	WGRGAQ VTVSS
VEGFBII 86G08/ **106**	EVQLVESGGG LMQTGDSLRL SCAASGLRFT	STNMG	WFRQGPG KEREFVA	AITLSGTTY YAEAVKG	RFTISRDNDKNTV ALQMNSLKPEDTA VYYCGA	DPSYYSTSRYTKA TEYDY	WGQGTQ VTVSS
VEGFBII 86G10/ **107**	EVQLVESGGG LVQAGGSLRL SCAASGRTFN	TYTMG	WFRQTPG TEREFVA	AIRWTVNIT YYADSVKG	RFTISRDIVKNTV YLQMNSLKPEDTA VYYCAA	QTSAPRSLIRMSN EYPY	WGQGTQ VTVSS
VEGFBII 86G11/ **108**	EVQLVESGGG LVQAGGSLRL SCAASGLTFS	LYTVG	WFRQAPG KEREFVA	YISRSGSNR YYVDSVKG	RFTLSRDNAKNTV DLQMNSLKTEDTA VYYCAA	TSRGLSSLAGEYN Y	WGRGTQ VTVSS
VEGFBII 86H09/ **109**	EVQLVESGGG LVQAGGSLRL SCTASGSAFK	SYRMG	WFRRTPG KEDEFVA	SISWTYGST FYADSVKG	RFTMSRDKAKNAG YLQMNSLKPEDTA LYYCAA	GAQSDRYNIRSYD Y	WGQGTQ VTVSS

VEGFBII 87B07/ **110**	EVQLVESGGG LVQPGGSLKL SCTASGFTFS	TSWMH	WVRQAPG KGLEWVS	SIPPVGHFA NYAPSVKG	RFTISRDNAKNTL FLQMNSLKSEDTA VYYCAK	DSAGRT	KGQGTQ VTVSS
VEGFBII 88A01/ **111**	KVQLVESGGG LVQAGGSLRL SCAASERTFS	NYAMD	WFRQAPG KEREFVA	AITRSGGGT YYADSVKG	RFTISRDNAKNTV YLQMNSLKPEDTA VYYCAA	TRSSTIVVGVGGM EY	WGKGTL VTVSS
VEGFBII 88A02/ **112**	EVQLVESGGG LVQAGGSLRL SCAASGFTFG	DYDIG	WFRQAPG NEREGVS	CITTDVGTT YYADSVKG	RFTISSDNAKNTV YLQINDLKPEDTA IYYCAV	DTQDLGLDIFCRG NGPFDG	WGQGTQ VTVSS
VEGFBII 88B02/ **113**	EVQLVESGGG LVQPGGSLRL SCTASGLNLD	DYAIG	WFRQAPG KEREGVS	CISSYDSVT YYADHVKG	RFTISRDSAKNTL YLQMNSLSIEDTG VYYCAA	EREQLRRRESPHD ELLRLCFYGMRY	SGKGTL VTVSS
VEGFBII 88E02/ **114**	EVQLVESGGG LVQPGGSLRL SCVASGFRLD	DYAIG	WFRQAPG KEREAVS	CISSSDTSI DYTNSVKG	RFTFSRDNAKNTV YLQMNSLKPEDTA VYYCAA	AFRCSGYELRGFP T	WGQGTQ VTVSS
VEGFBII 88G03/ **115**	EVQLVESGGG LVQAGGSLRL SCAASGGTFS	SLAVG	WFRQAPG KEREFVA	RITWSGATT YYADAVKD	RFTISRDNAKNTM YLQMNSLKPEDTA VYYCAA	DRSPNIINVVTAY EYDY	WGQGTQ VTVSS
VEGFBII 88G05/ **116**	EVQLVESGGG LVQPGASLRL SCAASGDGFT	LYNMG	WFRQAPG KEREFVA	AITSSPMST YYADSVKG	RFSISINNDKTTG FLQMNVLKPEDTG VYFCAA	PEGSFRRQYADRA MYDY	WGQGTQ VTVSS
VEGFBII 88G11/ **117**	EVQLVESGGG LAQAGGSLRL SCAASGRTFS	GSDMG	WFRQSPG KEREIVA	AIRLSGSIT YYPDSVKG	RFTISRDNAKNTV YLQMNSLKPEDTA VYYCAA	RSTYSYYLALADR GGYDY	WGQGTQ VTVSS

VEGFBII 88H01/ **118**	EVQLVESGGG LVQAGGSLRL SCVASGFTLG	TYAIG	WFRQAPG KEREAVS	CMSAGDSIP WYTASVKG	RFTTSTDNARNTV YLQMNSLKPEDTA HYYCAA	ARYHGDYCYYEGY YPF	WGQGTQ VTVSS
VEGFBII 89B04/ **119**	EVQLVESGGG LVQAGGSLRL SCAASTSISS	TNFMG	WYRQAPG KQRELVA	TITSSSITN YVDSVKG	RFTISRDNAKNTV YLQMTSLKPEDTA VYYCHA	RWRWSDVEY	WGKGTL VTVSS
VEGFBII 89B08/ **120**	EVQLVESGGG LVQPGGSLRL SCAASGTTSS	IFAMR	WYRQAPG KQRELVA	SITRSSITT YADSVKG	RFTPSRDNAKNTV SLQMNSLKPEDTA VYYCNA	AIRPELYSVVNDY	WGQGTQ VTVSS
VEGFBII 89D04/ **121**	EVQLVESGGG LVQPGGSLRL SCATSGLTFS	DYNLG	WFRQAPG KERQFVA	VISWRDSFA YYAEPVKG	RFTISRDNAKNTV YLQMNSLKPEDTA VYYCAA	DRVSSRLVLPNTS PDFGS	WGQGTQ VTVSS
VEGFBII 89F09/ **122**	EVQLVESGGG LVQAGDSLRL SCAASGRTFN	NAIMG	WFRQAPG QEREFVA	AMNWRGGPT YYADSVKG	RFTISGDNTKNTV FLQMNFLKPEDTA VYYCAA	DEDLYHYSSYHYS RVDLYHY	WGQGTQ VTVSS
VEGFBII 89G09/ **123**	EVQLVESGGG LVQPGGSLRL SCAASGTTSS	IFAMR	WYRQAPG KQRELVA	SITRSSITT YADSVKG	RFTLSRDNAKNTV SLQMNSLKPEDTA VYYCNA	AIRPELYSVVNDY	WGQGTQ VTVSS
VEGFBII 89H08/ **124**	EVQLVESGGG LVQAGGSLRL SCAASGGSFS	SYAPG	WFRQAPG KEREFVA	AFTRSSNIP YYKDSVKG	RFTISRDNAHTVY LQMNSLKPEDTAI YYCAV	NLGSTWSRDQRTY DY	WGQGTQ VTVSS

Table 3: Sequence IDs and AA sequences of monovalent receptor-blocking anti-VEGF VHHs (FR, framework; CDR, complementary determining region) SEQ ID NO: 9 - 46

VHH ID/ **SEQ ID NO:**	**FR1**	**CDR1**	**FR2**	**CDR2**	**FR3**	**CDR3**	**FR4**
VEGFBII 22A10/**9**	EVQLVESGG GLVQPGDSL KLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTKNT VYLQTPSLKPED TADYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 22A11/**10**	EVQLVESGG GLVQPGDSL KLSCAFSGR TFS	SYSM A	WFRQAQGKE REFVV	AISSGGFIY DAVSLEG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 22B06/**11**	EVQLVESGG GLVQPGDSL KLSCAASGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 22B07/**12**	EVQLVESGG GLVQAGDSL RLSCAASGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGNYK YDSVSLEG	RFTISRDNTKNT VYLQINSLKPED TAVYYCAA	SRAYGSSRLRL GDTYDY	WGQGTQV TVSS
VEGFBII 22E04/**13**	EVQLVESGG GLVQPGDSL KLSCVASGR TSS	SYSM G	WFRQAQGKE REFVV	AISSGGSIY DSVSLQG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYASSRLRL ADTYDY	WGQGTQV TVSS

VEGFBII 23A03/14	EVQLVESGGGLVQPGDSLKLSCVASGRTFS	SYSMG	WFRQAQGKEREFVV	AISSGGYIYDSVSLQG	RFTISRDNTKNTVYLQTPSLKPEDTAVYYCAA	SRAYGSSRLRLADTYDY	WGQGTQVTVSS
VEGFBII 23A06/15	EVQLVESGGGLVQPGDSLKLSCAFSGRTFS	SYSMG	WFRQAQGKEREFVV	AISSGGFIYDAVSLEG	RFTISRDNTKNTVYLQTPSLKPEDTAVYYCAA	SRAYGSSRLRLADTYDY	WGQGTQVTVSS
VEGFBII 23A08/16	EVQLVESGGGLVQTGDSLRLSCVASGRTFS	SYSMG	WFRQAQGKEREFVV	AISNGGYKYDSVSLEG	RFTISRDNTKNTVYLQINSLKPEDTAVYYCAA	SRAYGSSRLRLADTYDY	WGQGTQVTVSS
VEGFBII 23A09/17	EVQLVESGGGLVQPGDSLKLSCAFSGRTFG	SYSMG	WFRQAQGKEREFVV	AISSSGGYIYDSVSLEG	RFTISRDNSKNTVYLQTPSLKPEDTAVYYCAA	SRAYGSSRLRLPDTYDY	WGQGTQVTVSS
VEGFBII 23B04/18	EVQLVESGGGLVQTGDSLRLSCEVSGRTFS	SYSMG	WFRQAQGKEREFVV	AISKGGYKYDSVSLEG	RFTISKDNAKNTVYLQINSLKPEDTAVYYCAS	SRAYGSSRLRLADTYEY	WGQGTQVTVSS
VEGFBII 23D11/19	EVQLVESGGGLVQPGDSLRLSCAFSGRTFS	SYSMA	WFRQAQGKEREFVV	AISSGGFIYDAVSLEG	RFTISRDNTKNTVYLQTPSLKPEDTAVYYCAA	SRAYGSSRLRLADTYDY	WGQGTQVTVSS

VEGFBII 23E05/20	EVQLVESEG GLVQPGDSL KLSCVASGR TSS	SYSM G	WFRQAQGKE REFVV	AISSGGYIY DSVSLQG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 23F02/21	EMQLVESGG GLVQPGDSL KLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTKNT VYLQTPSLKPED TADYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 23F05/22	EVQLVESGG GLVQAGDSL RLSCAASGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGNYK YDSVSLEG	RFTISRDNTKNT VYLQINSLKPKD TAVYYCAA	SRAYGSSRLRL GDTYDY	WGQGTQV TVSS
VEGFBII 23F11/23	EVQLVESGG GLVQPGDSL KLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AISSGGGYI YDSVSLEG	RFTISRDNTKNT VYLQTPSLKPED TADYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 23G03/24	EVQLVESGG GLVQPGDSL KLSCAFSGR TFG	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNSKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL PGTYDY	WGQGTQV TVSS
VEGFBII 24C04/25	EVQLVESGG GLVQPGDSL KLSCVASGR TSS	SYSM G	WFRQAQGKE REFVV	AISSGGYIY DSVSLQG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS

VEGFBII 27D08/26	EVQLVESGG GLVQTGDSL RLSCAASGR TFS	SYSM G	WFRQAQGKE REFVV	AISSGGYKY DSVSLEG	RFTISRDNTQNT VYLQINSLKPED TAVYYCAA	SRAYGSGRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 27G07/27	EVQLVESGG GLVQPGDSL KLSCVASGR TSS	SYSM G	WFRQAQGQE REFVV	AISSGGYIY DSVSLQG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 30C09/28	EVQLVESGG GLVQPGDSL KLSCIASGR TSS	SYSM G	WFRQAQGQE REFVV	AISSGGYIY DSVSLQG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 30E07/29	EVQLVESGG GLVQAGDSL RLSCAASGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGNYK YDSVSLEG	RFTISRDNTKNT VYLQINSLKPED TAVYYCAA	SRAYGSSRLRL GDTYDY	WGQGTRV TVSS
VEGFBII 31C07/30	EVQLVESGG GLVQTGDSL RLSCAASGG TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTKNT VYLQTPSLKPED TADYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 39E02/31	EVQLVESGG GLVQPGDPL KLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTKNT VYLQTPSLKPED TADYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS

VEGFBII 39G04/32	EVPLVESGGGLVQAGDSLRLSCAASGRTFS	SYSMG	WFRQAQGKEREFVV	AISSSGNYKYDSASLEG	RFTISRDNTKNTVYLQINSLKPEDTAVYYCAA	SRAYGSSRLRLGDTYDY	WGQGTQVTVSS
VEGFBII 40F02/33	EVQLVESGGGLVQPGDSLKLSCAFSGRTFS	SYSMA	WFRQAQGKEREFVV	AISSGGFIYDAVSLEG	RFTISRDNTKNTVYLQTPSLKPEGTAVYYCAA	SRAYGSSRLRLADTYDY	WGQGTQVTVSS
VEGFBII 40G07/34	EVQLVESGGGLVQPGDSLKLSCAFSGRTFS	SYSMG	WFRQAQGKEREFVV	AISSSGGYIYDSVSLEG	RFTISRDNTKNAVYLQTPSLKPEDTADYYCAA	SRAYGSSRLRLADTYDY	WGQGTQVTVSS
VEGFBII 40H10/35	EVQLMESGGGLVQPGDSLKLSCAFSGRTFS	SYSMG	WFRQAQGKEREFVV	AISSSGGYIYDSVSLEG	RFTISRDNTKNTVYLQTPSLKPEDTADYYCAA	SRAYGSSRLRLADTYDY	WGQGTQVTVSS
VEGFBII 41B05/36	EVQLVESGGGLVQPGGSLRLSCAFSGRTFS	SYSMG	WFRQAQGKEREFVV	AISSGGFIYDAVSLEG	RFTISRDNTKNTVYLQTPSLKPEDTAVYYCAA	SRAYGSSRLRLADTYDY	WGQGTQVTVSS
VEGFBII 41G03/37	EVQLVESGGGLVQPGDSLKLSCAFSGRTFS	SYSMA	WFRQAQGKEREFVV	AISSGGFIYDAVSLEG	RFTISRENTKNTVYLQTPSLKPEDTAVYYCAA	SRAYGSSRLRLADTYDY	WGQGTQVTVSS

VEGFBII 42A05/38	EVQLVESGG GLVQPGDSL KLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTKNT VYLQMPSLKPED TADYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 42D05/39	EVQLVESGG GLVQPGDSL KLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 42F11/40	EVQLVESGG GLVQPGDSL KLSCVASGR TSS	SYSV G	WFRQAQGKE REFVV	AISSGGYIY DSVSLQG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 56E11/41	EVQLVESGG GLVQPGDSL KLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTKNT VYLQTPSLKPED AADYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 60A09/42	EVQLVESGG GLVQPGDSL KLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTRNT VYLQTPSLKPED TADYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 61A01/43	EVQLVESGG GLVQAGGSL RLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AISSGGYKY DAVSLEG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYASSRLRL ADTYDY	WGQGTQV TVSS

VEGFBII 62A09/**44**	EVQLVESGG DLVQPGDSL KLSCAASGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGGYI YDSVSLEG	RFTISRDNTKNT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS
VEGFBII 62D10/**45**	EVQLVESEG GLVQAGDSL RLSCAASGR TFS	SYSM G	WFRQAQGKE REFVV	AISSSGNYK YDSVSLEG	RFTISRDNTKNT VYLQINSLKPED TAVYYCAA	SRAYGSSRLRL GDTYDY	WGQGTQV TVSS
VEGFBII 62F02/**46**	EVQLVESGG GLVQPGDSL KLSCAFSGR TFS	SYSM G	WFRQAQGKE REFVV	AIASGGYIY DAVSLEG	RFTISRDNTKDT VYLQTPSLKPED TAVYYCAA	SRAYGSSRLRL ADTYDY	WGQGTQV TVSS

Dissociation rates of inhibitory VHHs are analyzed on Biacore (Biacore T100 instrument, GE Healthcare). HBS-EP+ buffer is used as running buffer and experiments are performed at 25◦C. Recombinant human VEGF165 is irreversibly captured on a CM5 sensor chip via amine coupling (using EDC and NHS) up to a target level of +/- 1500RU.
5 After immobilization, surfaces are deactivated with 10 min injection of 1M ethanolamine pH8.5. A reference surface is activated and deactivated with respectively EDC/NHS and ethanolamine. Periplasmic extracts of VHHs are injected at a 10-fold dilution in running buffer for 2 min at 45μl/min and allowed to dissociate for 10 or 15 min. Between different samples, the surfaces are regenerated with regeneration buffer. Data are double
10 referenced by subtraction of the curves on the reference channel and of a blank running buffer injection. The of the processed curves is evaluated by fitting a two phase decay model in the Biacore T100 Evaluation software v2.0.1. Values for k_d-fast, k_d-slow and % fast are listed in Table 4.

Table 4: Off-rate determination of receptor-blocking VHHs with Biacore

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B-cell lineage	Unique sequence variant	Representative VHH ID	k_d(fast)	k_d(slow)	% fast	Binding level (RU)
1	1	VEGFBII22B07	1.50E-02	7.80E-05	31	328
1	2	VEGFBII23A08	1.30E-02	5.00E-05	19	502
1	3	VEGFBII23B04	8.80E-03	4.00E-05	12	768
1	4	VEGFBII27D08	2.40E-02	8.10E-05	13	225
1	5	VEGFBII24C04	1.30E-02	3.40E-05	17	456
1	6	VEGFBII27G07	1.30E-02	3.80E-05	18	471
1	7	VEGFBII22E04	1.80E-02	1.10E-04	14	520
1	8	VEGFBII23A03	1.50E-02	3.20E-05	15	487
1	9	VEGFBII22B06	3.80E-02	9.00E-05	23	168
1	10	VEGFBII23A09	2.70E-02	4.60E-05	20	247
1	11	VEGFBII23G03	2.80E-02	8.60E-05	28	141
1	12	VEGFBII22A11	2.20E-02	4.70E-05	12	461
1	13	VEGFBII23A06	1.70E-02	3.70E-05	13	547
1	14	VEGFBII23F11	2.70E-02	1.30E-04	22	134
1	15	VEGFBII22A10	3.70E-02	4.00E-05	19	229
1	16	VEGFBII23F05	1.60E-02	1.30E-04	29	198

1	17	VEGFBII23D11	1.90E-02	5.80E-05	13	510
1	18	VEGFBII23F02	n/d	n/d	n/d	n/d
1	19	VEGFBII23E05	1.50E-02	6.90E-05	18	275
1	20	VEGFBII31C07	3.70E-02	1.50E-04	25	77
1	21	VEGFBII30C09	1.50E-02	7.60E-05	19	264
1	22	VEGFBII30E07	1.70E-02	1.30E-04	29	226
1	23	VEGFBII39G04	1.40E-02	7.40E-04	40	210
1	24	VEGFBII41G03	1.20E-02	2.70E-04	20	332
1	25	VEGFBII41B05	1.90E-02	1.20E-04	16	324
1	26	VEGFBII40F02	1.20E-02	9.80E-05	20	258
1	27	VEGFBII39E02	1.90E-02	2.40E-04	13	181
1	28	VEGFBII42D05	3.30E-02	1.50E-04	26	77
1	29	VEGFBII40G07	1.80E-02	3.20E-04	19	139
1	30	VEGFBII42A05	1.60E-02	3.40E-04	25	118
1	31	VEGFBII42F11	9.10E-03	5.00E-04	46	100
1	32	VEGFBII40H10	1.40E-02	2.90E-04	17	200
1	33	VEGFBII62A09	4.10E-02	1.10E-04	23	84
1	34	VEGFBII60A09	3.70E-02	9.30E-05	20	106
1	35	VEGFBII62F02	1.40E-02	8.50E-05	21	205
1	36	VEGFBII62D10	1.90E-02	1.60E-04	40	94
1	37	VEGFBII61A01	7.40E-03	1.70E-04	21	275
1	38	VEGFBII56E11	3.30E-02	1.40E-04	24	76

n/d, not determined

Example 5

Characterization of purified VHHs

Three inhibitory anti-VEGF VHHs are selected for further characterization as purified protein: VEGFBII23B04, VEGFBII24C4 and VEGFBII23A6. These VHHs are expressed in E. *coli* TG1 as c-myc, His6-tagged proteins. Expression is induced by addition of 1 mM IPTG and allowed to continue for 4 hours at 37°C. After spinning the cell cultures, periplasmic extracts are prepared by freeze-thawing the pellets. These extracts are used as starting material for VHH purification via IMAC and size exclusion chromatography (SEC). Final VHH preparations show 95% purity as assessed via SDS-PAGE.

5.1 Evaluation of human VEGF165/VEGFR2 blocking VHHs in human VEGF165/human VEGFR2-Fc blocking ELISA

The blocking capacity of the VHHs is evaluated in a human VEGF165/human VEGFR2-Fc blocking ELISA. In brief, 1 µg/mL of VEGFR2-Fc chimera (R&D Systems,
5 Minneapolis, MN, USA) is coated in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Dilution series (concentration range 1 mM - 64pM) of the purified VHHs in PBS buffer containing 0.1% casein and 0.05% Tween 20 (Sigma) are incubated in the presence of 4 nM biotinlyated VEGF165. Residual binding of bio-VEGF165 to VEGFR2 is detected using horseradish peroxidase (HRP) conjugated extravidin (Sigma, St Louis,
10 MO, USA) and TMB as substrate. As controls Bevacizumab (Avastin®) and Ranibizumab (Lucentis®) are taken along. Dose inhibition curves are shown in Figure 1; the corresponding IC_{50} values and % inhibition are summarized in Table 5.

Table 5: IC_{50} (nM) values and % inhibition for monovalent VHHs in hVEGF165/hVEGFR2-Fc competition ELISA
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VHH ID	IC_{50} (nM)	% inhibition
VEGFBII23B04	2.1	100
VEGFBII23A06	3.0	100
VEGFBII24C04	2.5	100
Ranibizumab	1.6	100
Bevacizumab	1.7	100

5.2 Evaluation of human VEGF165/VEGFR2 blocking VHHs in human VEGF165/human VEGFR1-Fc blocking ELISA

VHHs are also evaluated in a human VEGF165/human VEGFR1-Fc blocking ELISA. In
20 brief, 2 µg/mL of VEGFR1-Fc chimera (R&D Systems, Minneapolis, MN, USA) is coated in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Dilution series (concentration range 1 mM - 64pM) of the purified VHHs in PBS buffer containing 0.1% casein and 0.05% Tween 20 (Sigma) are incubated in the presence of 0.5nM biotinlyated

VEGF165. Residual binding of bio-VEGF165 to VEGFR1 is detected using horseradish peroxidase (HRP) conjugated extravidin (Sigma, St Louis, MO, USA) and TMB as substrate. As controls Bevacizumab, Ranibizumab and an irrelevant VHH (2E6) are taken along. Dose inhibition curves are shown in Figure 2; the corresponding IC_{50} values
5 and % inhibition are summarized in Table 6.

Table 6: IC_{50} (nM) values and % inhibition of monovalent VHHs in hVEGF165/hVEGFR1-Fc competition ELISA

VHH ID	IC_{50} (nM)	% inhibition
VEGFBII23B04	0.5	64
VEGFBII23A06	0.9	55
VEGFBII24C04	0.8	71
Ranibizumab	1.2	91
Bevacizumab	1.5	96

10 *5.3 Evaluation of the anti-VEGF165 VHHs in the human VEGF165/human VEGFR2-Fc blocking AlphaScreen*

The blocking capacity of the VHHs is also evaluated in a human VEGF165/human VEGFR2-Fc blocking AlphaScreen. Briefly, serial dilutions of purified VHHs (concentration range: 200 nM – 0.7 pM) in PBS buffer containing 0.03 % Tween 20
15 (Sigma) are added to 4pM bio-VEGF165 and incubated for 15 min. Subsequently VEGFR2-Fc (0.4 nM) and anti-Fc VHH-coated acceptor beads (20 µg/ml) are added and this mixture is incubated for 1 hour in the dark. Finally, streptavidin donor beads (20 µg/ml) are added and after 1 hour of incubation in the dark, fluorescence is measured on the Envision microplate reader. Dose-response curves are shown in the
20 Figure 3. The IC_{50} values for VHHs blocking the human VEGF165 – human VEGFR2-Fc interaction are summarized in Table 7.

Table 7: IC_{50} (pM) values and % inhibition for VHHs in hVEGF165/hVEGFR2-Fc competition AlphaScreen

VHH ID	IC_{50} (pM)	% inhibition
VEGFBII23B04	160	100
VEGFBII23A06	250	100
VEGFBII24C04	250	100
Ranibizumab	860	100

5 *5.4 Evaluation of the anti-VEGF165 VHHs in the human VEGF165/human VEGFR1-Fc blocking AlphaScreen*

The blocking capacity of the VHHs is also evaluated in a human VEGF165/human VEGFR1-Fc blocking AlphaScreen. Briefly, serial dilutions of purified VHHs (concentration range: 500 nM – 1.8 pM)) in PBS buffer containing 0.03 % Tween 20
10 (Sigma) are added to 0.4 nM bio-VEGF165 and incubated for 15 min. Subsequently VEGFR1-Fc (1 nM) and anti-Fc VHH-coated acceptor beads (20 µg/ml) are added and this mixture is incubated for 1 hour in the dark. Finally, streptavidin donor beads (20 µg/ml) are added and after 1 hour of incubation in the dark, fluorescence is measured on the Envision microplate reader. Dose-response curves are shown in the
15 Figure 4. The IC_{50} values and % inhibition for VHHs blocking the human VEGF165 – human VEGFR1-Fc interaction are summarized in Table 8.

Table 8: IC_{50} (nM) values for VHHs in hVEGF165/hVEGFR1-Fc competition AlphaScreen

VHH ID	IC_{50} (nM)	% inhibition
VEGFBII23B04	0.9	41
VEGFBII23A06	0.4	46
VEGFBII24C04	0.2	53
Ranibizumab	3.3	79

20 *5.5 Determination of the affinity of the human VEGF165 –VHH interaction*

Binding kinetics of VHH VEGFBII23B04 with hVEGF165 is analyzed by SPR on a Biacore T100 instrument. Recombinant human VEGF165 is immobilized directly on a CM5 chip via amine coupling (using EDC and NHS). VHHs are analyzed at different concentrations between 10 and 360nM. Samples are injected for 2 min and allowed to dissociate up to 20 min at a flow rate of 45 µl/min. In between sample injections, the chip surface is regenerated with 100 mM HCl. HBS-EP+ (Hepes buffer pH7.4 + EDTA) is used as running buffer. Binding curves are fitted using a Two State Reaction model by Biacore T100 Evaluation Software v2.0.1. The calculated affinities of the anti-VEGF VHHs are listed in Table 9.

Table 9: Affinity K_D (nM) of purified VHHs for recombinant human VEGF165

	VEGF165						
VHH ID	**k_a ($M^{-1}.s^{-1}$)**	**k_{a1} ($M^{-1}.s^{-1}$)**	**k_{a2} ($M^{-1}.s^{-1}$)**	**k_d (s^{-1})**	**k_{d1} (s^{-1})**	**k_{d2} (s^{-1})**	**K_D (nM)**
VEGFBII23B04[(a)]	-	2.1E+05	1.4E-02	-	8.6E-03	2.4E-04	0.7
VEGFBII23A06[(a)]	-	4.2E+05	2.0E-02	-	5.7E-02	1.0E-04	0.7
VEGFBII24C04[(a)]	-	3.2E+05	1.8E-02	-	2.6E-02	9.6E-05	0.4

[(a)] Heterogeneous binding curve resulting in no 1:1 fit, curves are fitted using a Two State Reaction model by Biacore T100 Evaluation Software v2.0.1

5.6 Binding to mouse VEGF164

Cross-reactivity to mouse VEGF164 is determined using a binding ELISA. In brief, recombinant mouse VEGF164 (R&D Systems, Minneapolis, MS, USA) is coated overnight at 4°C at 1 µg/mL in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Wells are blocked with a casein solution (1% in PBS). VHHs are applied as dilution

series (concentration range: 500nM – 32pM) in PBS buffer containing 0.1% casein and 0.05% Tween 20 (Sigma) and binding is detected using a mouse anti-myc (Roche) and an anti-mouse-HRP conjugate (DAKO) and a subsequent enzymatic reaction in the presence of the substrate TMB (3,3',5,5'-tetramentylbenzidine) (Pierce, Rockford, IL, USA) (Figures 5-1 and 5-2). A mouse VEGF164 reactive mAb is included as positive control. As reference, binding to human VEGF165 is also measured. EC_{50} values are summarized in Table 10.

Table 10: EC_{50} (pM) values for VHHs in a recombinant human VEGF165 and mouse VEGF164 binding ELISA

VHH ID	rhVEGF165 EC_{50} (pM)	rmVEGF164 EC_{50} (pM)
VEGFBII23B04	297	NB
VEGFBII24C04	453	NB
VEGFBII23A06	531	NB

NB, no binding

5.7 Binding to VEGF121

Binding to recombinant human VEGF121 is assessed via a solid phase binding ELISA. Briefly, recombinant human VEGF121 (R&D Systems, Minneapolis, MS, USA) is coated overnight at 4°C at 1 µg/mL in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Wells are blocked with a casein solution (1% in PBS). VHHs are applied as dilution series (concentration range: 500nM – 32pM) in PBS buffer containing 0.1% casein and 0.05% Tween 20 (Sigma) and binding is detected using a mouse anti-myc (Roche) and an anti-mouse-HRP conjugate (DAKO) and a subsequent enzymatic reaction in the presence of the substrate TMB (3,3',5,5'-tetramentylbenzidine) (Pierce, Rockford, IL, USA) (Figure 6). As positive control serial dilutions of the VEGFR2 is taken along. EC_{50} values are summarized in Table 11.

Table 11: EC_{50} (pM) values for monovalent VHHs in a recombinant human VEGF121 binding ELISA

VHH ID	EC_{50} (pM)
VEGFBII23B04	510
VEGFBII24C04	792
VEGFBII23A06	928

5 *5.8 Binding to VEGF family members VEGFB, VEGFC, VEGFD and PlGF*

Binding to VEGFB, VEGFC, VEGFD and PlGF is assessed via a solid phase binding ELISA. In brief, VEGFB, VEGFC, VEGFD and PlGF (R&D Systems, Minneapolis, MS, USA) are coated overnight at 4°C at 1 µg/mL in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Wells are blocked with a casein solution (1% in PBS). VHHs are
10 applied as dilution series (concentration range: 500nM – 32pM) and binding is detected using a mouse anti-myc (Roche) and an anti-mouse-AP conjugate (Sigma, St Louis, MO, USA). As positive controls serial dilutions of the appropriate receptors are taken along and detected with horseradish peroxidase (HRP)-conjugated goat anti-human IgG, Fc specific antibody (Jackson Immuno Research Laboratories Inc., West Grove,
15 PA, USA) and a subsequent enzymatic reaction in the presence of the substrate TMB (3,3',5,5'-tetramentylbenzidine) (Pierce, Rockford, IL, USA). Dose-response curves of VHHs and controls are shown in Figures 7-1 through 7-4. The results show that there was no detectable binding of the selected VHHs to VEGFB, VEGFC, VEGFD or PlGF.

5.9 Epitope binning

20 Biacore-based epitope binning experiments are performed to investigate which VEGF binders bind to a similar or overlapping epitope as VEGFBII23B04. To this end, VEGFBII23B04 is immobilized on a CM5 sensor chip. For each sample, human VEGF165 is passed over the chip surface and reversibly captured by VEGFBII23B4. Purified VHHs (100 nM) or periplasmic extracts (1/10 diluted) are then injected with a

surface contact time of 240 seconds and a flow rate of 10 uL/minute. Between different samples, the surface is regenerated with regeneration buffer (100 mM HCl). Processed curves are evaluated with Biacore T100 Evaluation software. VHHs could be divided within two groups: group one which gave additional binding to VEGFBII23B04 captured
5 VEGF165 and a second group which is not able to simultaneously bind to VEGFBII23B04 captured VEGF165. Table 12-A summarizes the binding epitopes of the tested VHHs.

The same assay set-up is used to assess whether VEGFR1, VEGFR2, Ranibizumab and Bevacizumab are able to bind to human VEGF-165 simultaneously with
10 VEGFBII23B04. Table 12-B presents the additional binding responses to VEGFBII23B04-captured VEGF165. Only VEGFR2 is not able to bind to VEGFBII23B04-captured VEGF165, underscoring the blocking capacity of VEGFBII23B04 for the VEGF-VEGFR2 interaction. In addition, these data show that the VEGFBII23B04 epitope is different from the Bevacizumab and Ranibizumab epitope.

15 **Table 12-A:** Epitope binning of anti-VEGF VHHs – simultaneous binding with VEGFBII23B04

No or low additional binding to 23B04-captured VEGF165*	1C02	1E07	4B08	8E07	8F07	12A07	12B01	86C11	86F11	86G08
	86G10	86G11	87B07	88A01	88A02	88B02	88E02	88G03	88G05	88G11
	88H01	89B04	89D04	89F09	89G09	89H08	24C04	23A6	27G07	23B04
Additional binding to 23B04-captured VEGF165	3D12	5B02	5B03	5B05	6G02	7D08	8D09	8F06	10C07	10E07
	10G04	10G05	11C08	11D09	11E04	11E05	11F12	86H09	41C05	

* indicating same or overlapping epitopes

Table 12-B: Epitope binning of VEGFBII23B04 – binding of benchmark inhibitors or cognate receptors on VEGFBII23B04 captured VEGF165

Injection step	Binding	[sample]	Binding level (RU)
1	VEGF165	100 nM	1727
2	VEGFBII23B04	100 nM	-
3	Ranibizumab	100 nM	763
4	Bevacizumab	100 nM	1349
5	VEGFR1	100 nM	1011
6	VEGFR2	100 nM	-

5 *5.10 Characterization of the anti-VEGF VHHs in the HUVEC proliferation assay*

The potency of the selected VHHs is evaluated in a proliferation assay. In brief, primary HUVEC cells (Technoclone) are supplement-starved over night and then 4000 cells/well are seeded in quadruplicate in 96-well tissue culture plates. Cells are stimulated in the absence or presence of VHHs with 33ng/mL VEGF. The proliferation rates are
10 measured by [^{3}H] Thymidine incorporation on day 4. The results of the HUVEC proliferation assay are shown in Table.

Table 13: IC_{50} (nM) values and % inhibition of monovalent VEGFBII23B04, VEGFBII23A06 and VEGFBII24C04 in VEGF HUVEC proliferation assay

VHH ID	IC_{50} (nM)	% inhibition
VEGFBII23B04	0.36	91
Bevacizumab	0.21	92

15

VHH ID	IC_{50} (nM)	% inhibition
VEGFBII23A06	4.29	73
VEGFBII24C04	3.8	79
Bevacizumab	0.78	78

5.11 Characterization of the anti-VEGF VHHs in the HUVEC Erk phosphorylation assay

The potency of the selected VHHs is assessed in the HUVEC Erk phosphorylation
5 assay. In brief, primary HUVE cells are serum-starved over night and then stimulated in the absence or presence of VHHs with 10ng/mL VEGF for 5 min. Cells are fixed with 4% Formaldehyde in PBS and ERK phosphorylation levels are measured by ELISA using phosphoERK-specific antibodies (anti-phosphoMAP Kinase pERK1&2, M8159, Sigma) and polyclonal Rabbit Anti-Mouse-Immunoglobulin-HRP conjugate (PO161,
10 Dako). As shown in Table 14, VEGFBII23B04 and Bevacizumab inhibit the VEGF induced Erk phosphoryaltion by at least 90%, with IC_{50}s <1nM.

Table 14: IC_{50} (nM) values and % inhibition of monovalent VEGFBII23B04 in VEGF HUVEC Erk phosphorylation assay

VHH ID	IC_{50} (nM)	% inhibition
VEGFBII23B04	0.37	90
Bevacizumab	0.63	98

15

Example 6

Generation of multivalent anti-VEGF blocking VHHs

VHH VEGFBII23B04 is genetically fused to either VEGFBII23B04 resulting in a homodimeric VHH (AA sequence see Table 15) or different VEGF binding VHHs
5 resulting in heterodimeric VHHs. To generate the heterodimeric VHHs, a panel of 10 unique VEGF binding VHHs are linked via a 9 or 40 Gly-Ser flexible linker in two different orientations to VEGFBII23B04 (AA sequences see Table 15). Homodimeric VEGFBII23B04 (VEGFBII010) and the 40 heterodimeric bivalent' VHHs are expressed in E. *coli* TG1 as c-myc, His6-tagged proteins. Expression is induced by addition of
10 1 mM IPTG and allowed to continue for 4 hours at 37°C. After spinning the cell cultures, periplasmic extracts are prepared by freeze-thawing the pellets. These extracts are used as starting material and VHHs are purified via IMAC and desalting resulting in 90% purity as assessed via SDS-PAGE.

Table 15: Sequence ID, VHH ID and AA sequence of bivalent anti-VEGF VHHs (each of the used linkers is highlighted in one relevant sequence)

Sequence ID/ SEQ ID NO:	VHH ID	AA sequence
VEGFBII23B04-35GS-23B04/**128**	VEGFBII010	EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII23B04-9GS-4B08/**129**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGSAVGDITVAWYRQAPGIQRQLVATITPSGYTYYWDFVKGRFTISRDNSKNIVYLQMNSLKPEDTAAYYCNTQFYWGQGTQVTVSS
VEGFBII23B04-9GS-5B03/**130**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLAQAGDSLRLSCAASGRSFSHYNMGWFRQAPGKEREFVASIRGGGGSTTYANSVKDRFTISRENAKNTVYLQMNSLKPEDTAVYYCAATAFYRGPYDYDYWGQGTQVTVSS
VEGFBII23B04-9GS-5B05/**131**	VEGFBII022	EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCVASGIRFMSMAWYRQAPGKHRELVARISSGGTTAYVDSVKGRFTISRDNSKNTVYLQMNSLKAEDTAVYYCNTFSSRPNPWGAGTQVTVSS

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VEGFBII23B04-9GS-6G02/**132**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYK YDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWG QGTQVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGNIFSNNAMAWYR QAPGKQRELVARISSGGGFTYYLDSVKGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYC NAAYRTYNYWGQGTQVTVSS
VEGFBII23B04-9GS-10E07/**133**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYK YDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWG QGTQVTVSSGGGGSGGGSEVQLVESGGGLVQAGGSLRLSCAASGRTFSNYAMGWF RQAPGKERVLVADISSSGINTYVADAVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYC AASAWWYSQMARDNYRYWGQGTQVTVSS
VEGFBII23B04-9GS-12B01/**134**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYK YDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWG QGTQVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLACAASGFTLSSSWMYWV RQAPGKGLEWVSRISPGGLFTYYVDSVKGRFSVSTDNANNTLYLQMNSLKPEDTALYS CAKGGAPNYTPRGRGTQVTVSS
VEGFBII23B04-9GS-86C11/**135**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYK YDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWG QGTQVTVSSGGGGSGGGSEVQLVESGGGLVQAGDSLRLSCTASGRTFNSYAMGWF RQAPGKERESVAHINRSGSSTYYADSVKGRFTISRDNAKNTVYLQLNSLKPEDTAVYY CAAGRYYSSDGVPSASFNYWGQGTQVTVSS
VEGFBII23B04-9GS-86H09/**136**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYK YDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWG QGTQVTVSSGGGGSGGGSEVQLVESGGGLVQAGGSLRLSCTASGSAFKSYRMGWF RRTPGKEDEFVASISWTYGSTFYADSVKGRFTMSRDKAKNAGYLQMNSLKPEDTALYY CAAGAQSDRYNIRSYDYWGQGTQVTVSS

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VEGFBII23B04-9GS-87B07/**137**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLKLSCTASGFTFSTSWMHWVRQAPGKGLEWVSSIPPVGHFANYAPSVKGRFTISRDNAKNTLFLQMNSLKSEDTAVYYCAKDSAGRTKGQGTQVTVSS
VEGFBII23B04-9GS-88A01/**138**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQAGGSLRLSCAASERTFSNYAMDWFRQAPGKEREFVAAITRSGGGTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAATRSSTIVVGVGGMEYWGKGTQVTVSS
VEGFBII23B04-40GS-4B08/**139**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGSAVGDITVAWYRQAPGIQRQLVATITPSGYTYYWDFVKGRFTISRDNSKNIVYLQMNSLKPEDTAAYYCNTQFYWGQGTQVTVSS
VEGFBII23B04-40GS-5B03/**140**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLAQAGDSLRLSCAASGRSFSHYNMGWFRQAPGKEREFVASIRGGGGSTTYANSVKDRFTISRENAKNTVYLQMNSLKPEDTAVYYCAATAFYRGPYDYDYWGQGTQVTVSS
VEGFBII23B04-40GS-5B05/**141**	VEGFBII021	EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGIRFMSMAWYRQAPGKHRELVARISSGGTTAYVDSVKGRFTISRDNSKNTVYLQMNSLKAEDTAVYYCNTFSSRPNPWGAGTQVTVSS

VEGFBII23B04-40GS-6G02/**142**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGNIFSNNAMAWYRQAPGKQRELVARISSGGGFTYYLDSVKGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYCNAAYRTYNYWGQGTQVTVSS
VEGFBII23B04-40GS-10E07/**143**	VEGFBII023	EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCAASGRTFSNYAMGWFRQAPGKERVLVADISSSGINTYVADAVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAASAWWYSQMARDNYRYWGQGTQVTVSS
VEGFBII23B04-40GS-12B01/**144**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLACAASGFTLSSSWMYWVRQAPGKGLEWVSRISPGGLFTYYVDSVKGRFSVSTDNANNTLYLQMNSLKPEDTALYSCAKGGAPNYTPRGRGTQVTVSS
VEGFBII23B04-40GS-86C11/**145**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGDSLRLSCTASGRTFNSYAMGWFRQAPGKERESVAHINRSGSSTYYADSVKGRFTISRDNAKNTVYLQLNSLKPEDTAVYYCAAGRYYSSDGVPSASFNYWGQGTQVTVSS
VEGFBII23B04-40GS-86H09/**146**	VEGFBII024	EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCTASGSAFKSYRMGWFRRTPGKEDEFVASISWTYGSTFYADSVKGRFTMSRDKAKNAGYLQMNSLKPEDTALYYCAAGAQSDRYNIRSYDYWGQGTQVTVSS

VEGFBII23B04-40GS-87B07/**147**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLKLSCTASGFTFSTSWMHWVRQAPGKGLEWVSSIPPVGHFANYAPSVKGRFTISRDNAKNTLFLQMNSLKSEDTAVYYCAKDSAGRTKGQGTQVTVSS
VEGFBII23B04-40GS-88A01/**148**		EVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGSLRLSCAASERTFSNYAMDWFRQAPGKEREFVAAITRSGGGTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAATRSSTIVVGVGGMEYWGKGTQVTVSS
VEGFBII4B08-9GS-23B04/**149**		EVQLVESGGGLVQPGGSLRLSCAASGSAVGDITVAWYRQAPGIQRQLVATITPSGYTYYWDFVKGRFTISRDNSKNIVYLQMNSLKPEDTAAYYCNTQFYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII5B03-9GS-23B04/**150**		EVQLVESGGGLAQAGDSLRLSCAASGRSFSHYNMGWFRQAPGKEREFVASIRGGGGSTTYANSVKDRFTISRENAKNTVYLQMNSLKPEDTAVYYCAATAFYRGPYDYDYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII5B05-9GS-23B04/**151**		EVQLVESGGGLVQPGGSLRLSCVASGIRFMSMAWYRQAPGKHRELVARISSGGTTAYVDSVKGRFTISRDNSKNTVYLQMNSLKAEDTAVYYCNTFSSRPNPWGAGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS

VEGFBII6G02-9GS-23B04/**152**		EVQLVESGGGLVQPGGSLRLSCAASGNIFSNNAMAWYRQAPGKQRELVARISSGGGFTYYLDSVKGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYCNAAYRTYNYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII10E07-9GS-23B04/**153**		EVQLVESGGGLVQAGGSLRLSCAASGRTFSNYAMGWFRQAPGKERVLVADISSSGINTYVADAVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAASAWWYSQMARDNYRYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII12B01-9GS-23B04/**154**		EVQLVESGGGLVQPGGSLRLACAASGFTLSSSWMYWVRQAPGKGLEWVSRISPGGLFTYYVDSVKGRFSVSTDNANNTLYLQMNSLKPEDTALYSCAKGGAPNYTPRGRGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII86C11-9GS-23B04/**155**		EVQLVESGGGLVQAGDSLRLSCTASGRTFNSYAMGWFRQAPGKERESVAHINRSGSSTYYADSVKGRFTISRDNAKNTVYLQLNSLKPEDTAVYYCAAGRYYSSDGVPSASFNYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII86H09-9GS-23B04/**156**		EVQLVESGGGLVQAGGSLRLSCTASGSAFKSYRMGWFRRTPGKEDEFVASISWTYGSTFYADSVKGRFTMSRDKAKNAGYLQMNSLKPEDTALYYCAAGAQSDRYNIRSYDYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS

VEGFBII87B07-9GS-23B04/**157**		EVQLVESGGGLVQPGGSLKLSCTASGFTFSTSWMHWVRQAPGKGLEWVSSIPPVGHFANYAPSVKGRFTISRDNAKNTLFLQMNSLKSEDTAVYYCAKDSAGRTKGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII88A01-9GS-23B04/**158**		EVQLVESGGGLVQAGGSLRLSCAASERTFSNYAMDWFRQAPGKEREFVAAITRSGGGTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAATRSSTIVVGVGGMEYWGKGTQVTVSSGGGGSGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII4B08-40GS-23B04/**159**		EVQLVESGGGLVQPGGSLRLSCAASGSAVGDITVAWYRQAPGIQRQLVATITPSGYTYYWDFVKGRFTISRDNSKNIVYLQMNSLKPEDTAAYYCNTQFYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII5B03-40GS-23B04/**160**		EVQLVESGGGLAQAGDSLRLSCAASGRSFSHYNMGWFRQAPGKEREFVASIRGGGGSTTYANSVKDRFTISRENAKNTVYLQMNSLKPEDTAVYYCAATAFYRGPYDYDYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII5B05-40GS-23B04/**161**		EVQLVESGGGLVQPGGSLRLSCVASGIRFMSMAWYRQAPGKHRELVARISSGGTTAYVDSVKGRFTISRDNSKNTVYLQMNSLKAEDTAVYYCNTFSSRPNPWGAGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS

VEGFBII6G02-40GS-23B04/**162**		EVQLVESGGGLVQPGGSLRLSCAASGNIFSNNAMAWYRQAPGKQRELVARISSGGGFTYYLDSVKGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYCNAAYRTYNYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII10E07-40GS-23B04/**163**	VEGFBII025	EVQLVESGGGLVQAGGSLRLSCAASGRTFSNYAMGWFRQAPGKERVLVADISSSGINTYVADAVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAASAWWYSQMARDNYRYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII12B01-40GS-23B04/**164**		EVQLVESGGGLVQPGGSLRLACAASGFTLSSSWMYWVRQAPGKGLEWVSRISPGGLFTYYVDSVKGRFSVSTDNANNTLYLQMNSLKPEDTALYSCAKGGAPNYTPRGRGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII86C11-40GS-23B04/**165**		EVQLVESGGGLVQAGDSLRLSCTASGRTFNSYAMGWFRQAPGKERESVAHINRSGSSTYYADSVKGRFTISRDNAKNTVYLQLNSLKPEDTAVYYCAAGRYYSSDGVPSASFNYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII86H09-40GS-23B04/**166**		EVQLVESGGGLVQAGGSLRLSCTASGSAFKSYRMGWFRRTPGKEDEFVASISWTYGSTFYADSVKGRFTMSRDKAKNAGYLQMNSLKPEDTALYYCAAGAQSDRYNIRSYDYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS

VEGFBII87B07-40GS-23B04/**167**		EVQLVESGGGLVQPGGSLKLSCTASGFTFSTSWMHWVRQAPGKGLEWVSSIPPVGHFANYAPSVKGRFTISRDNAKNTLFLQMNSLKSEDTAVYYCAKDSAGRTKGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS
VEGFBII88A01-40GS-23B04/**168**		EVQLVESGGGLVQAGGSLRLSCAASERTFSNYAMDWFRQAPGKEREFVAAITRSGGGTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAATRSSTIVVGVGGMEYWGKGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQTGDSLRLSCEVSGRTFSSYSMGWFRQAQGKEREFVVAISKGGYKYDSVSLEGRFTISKDNAKNTVYLQINSLKPEDTAVYYCASSRAYGSSRLRLADTYEYWGQGTQVTVSS

The panel of 40 bivalent VHHs is tested in the VEGFR2 and VEGFR1 blocking AlphaScreen assay, as described in Example 5.3 and 5.4, respectively. Based on potency and maximum level of inhibition, the 5 best bivalent VHHs (VEGFBII021, VEGFBII022, VEGFBI023, VEGFBI024 and VEGFBII025) are chosen for further
5 characterization. An overview of the screening results for the 5 selected bivalent VHHs in the competitive VEGFR2 and VEGFR1 AlphaScreen is shown in Table 16.

Table 16: Potency and efficacy of 5 best bivalent VHHs in the VEGF/VEGFR1 and VEGF/VEGFR2 competition AlphaScreen assay

VHH ID	VEGFR2	VEGFR1	
	IC_{50} (pM)	IC_{50} (pM)	% inhibition
VEGFBII021	9	16	100
VEGFBII022	7	8	100
VEGFBII023	38	44	91
VEGFBII024	12	46	100
VEGFBII025	51	39	82

10

Example 7

Characterization of formatted VHHs

VHHs VEGFBII010, VEGFBII021, VEGFBII022, VEGFBII023, VEGFBII024 and VEGFBII025 are compared side-by-side in the VEGFR2 and VEGFR1 blocking ELISA
15 (Figures 8-1 and 8-2 and 9, Table 17 and Table 18 respectively) and AlphaScreen assay (Figure 10 and 11, Table 19 and 20) as described in Examples 5.1, 5.2, 5.3 and 5.4, respectively.

Table 17: IC_{50} (pM) values and % inhibition for formatted VHHs in hVEGF165/hVEGFR2-Fc competition ELISA

VHH ID	IC_{50} (pM)	% inhibition
VEGFBII010	49	100
VEGFBII021	204	100
VEGFBII022	164	100
VEGFBII023	213	100
VEGFBII024	292	100
VEGFBII025	577	100
Bevacizumab	315	100
Ranibizumab	349	100

5

Table 18: IC_{50} (pM) values and % inhibition of formatted VHHs in VEGF165/hVEGFR1-Fc competition ELISA

VHH ID	IC_{50} (pM)	% inhibition
VEGFBII010	73.5	67
VEGFBII021	254	97
VEGFBII022	225	89
VEGFBII023	279	91
VEGFBII024	326	92
VEGFBII025	735	91
Bevacizumab	484	91
Ranibizumab	594	96

10

Table 19: IC_{50} (pM) values and % inhibition for formatted VHHs in hVEGF165/hVEGFR2-Fc competition AlphaScreen

VHH ID	IC_{50} (pM)	% inhibition
VEGFBII010	16	100
VEGFBII021	7	100
VEGFBII022	7	100
VEGFBII023	46	100
VEGFBII024	50	100
VEGFBII025	51	100
Ranibizumab	600	100

5 **Table 20**: IC_{50} (pM) values and % inhibition of formatted VHHs in VEGF165/hVEGFR1-Fc competition AlphaScreen

VHH ID	IC_{50} (pM)	% inhibition
VEGFBII010	21	70
VEGFBII021	12	100
VEGFBII022	9	98
VEGFBII023	48	87
VEGFBII024	69	98
VEGFBII025	71	82
Ranibizumab	1300	87

In addition, formatted VHHs are also tested for their capacity to block the
10 mVEGF164/mVEGFR2-huFc interaction. In brief, serial dilutions of purified VHHs (concentration range: 4μM – 14.5 pM) in PBS buffer containing 0.03 % Tween 20 (Sigma) are added to 0.1 nM biotinylated mVEGF164 and incubated for 15 min.

Subsequently mouse VEGFR2-huFc (0.1 nM) and anti-huFc VHH-coated acceptor beads (20 µg/ml) are added and this mixture is incubated for 1 hour. Finally, streptavidin donor beads (20 µg/ml) are added and after 1 hour of incubation fluorescence is measured on the Envision microplate reader. Dose-response curves are shown in
5 Figure 12. The IC_{50} values for VHHs blocking the mouse VEGF164/VEGFR2-hFC interaction are summarized in Table 21.

Table 21: IC_{50} (pM) values and % inhibition for formatted VHHs in mVEGF164/mVEGFR2-hFc competition AlphaScreen

VHH ID	IC_{50} (nM)	% inhibition
VEGFBII022	108	100
VEGFBII024	-	-
mVEGF164	0.05	100
Ranibizumab	-	-

10

The formatted VHHs are also tested in ELISA for their ability to bind mVEGF164 and human VEGF165 (Example 5.6; Figures 13-1 and 13-2; Table 22); VEGF121 (Example 5.7; Figure 15; Table 23) and the VEGF family members VEGFB, VEGFC, VEGFD and PlGF (Example 5.8; Figures 14-1 through 14-8). Binding kinetics for human VEGF165
15 are analyzed as described in Example 5.5. The K_D values are listed in Table 24.

Table 22 EC_{50} (pM) values for formatted VHHs in a recombinant human VEGF165 and mouse VEGF164 binding ELISA

VHH ID	rhVEGF165 EC_{50} (pM)	rmVEGF164 EC_{50} (pM)
VEGFBII010	428	-
VEGFBII021	334	502
VEGFBII022	224	464
VEGFBII023	221	-
VEGFBII024	320	-
VEGFBII025	668	-

Table 23: EC_{50} (pM) values for formatted VHHs in a recombinant human VEGF121 binding ELISA

VHH ID	rhVEGF121 EC_{50} (pM)
VEGFBII010	920
VEGFBII022	540
VEGFBII024	325
VEGFBII025	475

5 **Table 24:** Affinity K_D (nM) of purified formatted VHHs for recombinant human VEGF165

VHH ID	k_{a1} (1/Ms)	k_{d1} (1/s)	k_{a2} (1/s)	k_{d2} (1/s)	K_D (nM)[(a)]
VEGFBII010 [(b)]	4.5E+05	1.7E-02	2.9E-02	1.3E-04	0.16
VEGFBII021[(b)]	1.2E+06	1.1E-02	2.3E-02	1.9E-04	0.07
VEGFBII022[(b)]	1.2E+06	9.1E-03	1.4E-02	2.6E-04	0.14
VEGFBII023[(b)]	3.0E+05	1.8E-02	2.4E-02	2.7E-04	0.69
VEGFBII024[(b)]	3.0E+05	1.3E-02	2.6E-02	2.8E-04	0.47
VEGFBII025[(b)]	3.3E+05	1.7E-02	1.8E-02	3.7E-04	1.1

[(a)] $K_D = k_{d1}/k_{a1}*(k_{d2}/(k_{d2}+k_{a2}))$

[(b)] Curves are fitted using a Two State Reaction model by Biacore T100 Evaluation Software v2.0.1

10 VHHs VEGFBII010, VEGFBII022, VEGFBII024 and VEGFBII025 are also tested in the VEGF-mediated HUVEC proliferation and Erk phosphorylation assay.

The potency of the selected formatted VHHs is evaluated in a proliferation assay. In brief, primary HUVEC cells (Technoclone) are supplement-starved over night and then 4000 cells/well are seeded in quadruplicate in 96-well tissue culture plates. Cells are 15 stimulated in the absence or presence of VHHs with 33ng/mL VEGF. The proliferation rates are measured by [^{3}H] Thymidine incorporation on day 4. The results shown in Table 25 demonstrate that the formatted VHHs and Bevacizumab inhibit the VEGF-induced HUVEC proliferation by more than 90%, with IC_{50}s <1nM.

Table 25: IC_{50} (nM) values and % inhibition of formatted VHHs in VEGF HUVEC proliferation assay

VHH ID	IC_{50} (nM)	% inhibition
VEGFBII010	0.22	95
VEGFBII021	0.40	98
VEGFBII022	0.34	100
VEGFBII023	0.52	98
VEGFBII024	0.38	96
VEGFBII025	0.41	104
Bevacizumab	0.21	92

The potency of the selected formatted VHHs is assessed in the HUVEC Erk phosphorylation assay. In brief, primary HUVE cells are serum-starved over night and then stimulated in the absence or presence of VHHs with 10ng/mL VEGF for 5 min. Cells are fixed with 4% Formaldehyde in PBS and ERK phosphorylation levels are measured by ELISA using phosphoERK-specific antibodies (anti-phosphoMAP Kinase pERK1&2, M8159, Sigma) and polyclonal Rabbit Anti-Mouse-Immunoglobulin-HRP conjugate (PO161, Dako). As shown in Table 26, the formatted VHHs and Bevacizumab inhibit the VEGF induced Erk phosphoryaltion by more than 90%, with IC_{50}s <1nM.

Table 26: IC_{50} (nM) values and % inhibition of formatted VHHs in VEGF HUVEC Erk phosphorylation assay

VHH ID	IC_{50} (nM)	% inhibition
VEGFBII010	0.19	92
VEGFBII021	0.21	103
VEGFBII022	0.18	94
VEGFBII023	0.25	100
VEGFBII024	0.23	94
VEGFBII025	0.23	99
Bevacizumab	0.63	98

Example 8

Sequence optimization

8.1 Sequence optimization of VEGFBII23B04

The amino acid sequence of VEGFBII23B04 is aligned to the human germline sequence VH3-23/JH5, see Figure 16 (SEQ ID NO: 179)

The alignment shows that VEGFBII23B04 contains 19 framework mutations relative to the reference germline sequence. Non-human residues at positions 14, 16, 23, 24, 41, 71, 82, 83 and 108 are selected for substitution with their human germline counterparts. A set of 8 VEGFBII23B04 variants is generated carrying different combinations of human residues at these positions (AA sequences are listed in Table 27). One additional variant is constructed in which the potential isomerization site at position D59S60 (CDR2 region, see Figure 16, indicated as bold italic residues) is removed by introduction of a S60A mutation.

Table 27: AA sequence of sequence-optimized variants of VHH VEGFBII23B04 (FR, framework; CDR, complementary determining region)

VHH ID/ SEQ ID NO:	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VEGFBII 111D05/**47**	EVQLVESGG GLVQTGGSL RLSCEASGR TFS	SYSM G	WFRQAPGKER EFVV	AISKGGY KYDSVS LEG	RFTISRDNAKNTVYL QINSLRPEDTAVYYC AS	SRAYGS SRLRLA DTYEY	WGQGTLVT VSS
VEGFBII 111G06/**48**	EVQLVESGG GLVQPGGSL RLSCAASGR TFS	SYSM G	WFRQAPGKER EFVV	AISKGGY KYDSVS LEG	RFTISRDNAKNTVYL QMNSLRPEDTAVYYC AS	SRAYGS SRLRLA DTYEY	WGQGTLVT VSS
VEGFBII 112D11/**49**	EVQLVESGG GLVQPGGSL RLSCEASGR TFS	SYSM G	WFRQAPGKER EFVV	AISKGGY KYDSVS LEG	RFTISRDNAKNTVYL QINSLRPEDTAVYYC AS	SRAYGS SRLRLA DTYEY	WGQGTLVT VSS
VEGFBII 113A08/**50**	EVQLVESGG GLVQTGGSL RLSCEVSGR TFS	SYSM G	WFRQAPGKER EFVV	AISKGGY KYDSVS LEG	RFTISKDNAKNTVYLQ INSLRPEDTAVYYCAS	SRAYGS SRLRLA DTYEY	WGQGTLVT VSS

12-0319-pct

VEGFBII 113E03/**51**	EVQLVESGG GLVQTGDSL RLSCEVSGR TFS	SYSM G	WFRQAQGKER EFVV	AISKGGY KYDSVS LEG	RFTISKDNAKNTVYLQ MNSLRPEDTAVYYCA S	SRAYGS SRLRLA DTYEY	WGQGTLVT VSS
VEGFBII 114C09/**52**	EVQLVESGG GLVQPGDSL RLSCEVSGR TFS	SYSM G	WFRQAPGKER EFVV	AISKGGY KYDSVS LEG	RFTISKDNAKNTVYLQ INSLRPEDTAVYYCAS	SRAYGS SRLRLA DTYEY	WGQGTLVT VSS
VEGFBII 114D02/**53**	EVQLVESGG GLVQTGGSL RLSCEVSGR TFS	SYSM G	WFRQAPGKER EFVV	AISKGGY KYDSVS LEG	RFTISRDNAKNTVYL QINSLRPEDTAVYYC AS	SRAYGS SRLRLA DTYEY	WGQGTLVT VSS
VEGFBII 114D03/**54**	EVQLVESGG GLVQTGDSL RLSCAVSGR TFS	SYSM G	WFRQAQGKER EFVV	AISKGGY KYDSVS LEG	RFTISKDNAKNTVYLQ INSLRPEDTAVYYCAS	SRAYGS SRLRLA DTYEY	WGQGTLVT VSS
VEGFBII 118E10/**55**	EVQLVESGG GLVQTGDSL RLSCEVSGR TFS	SYSM G	WFRQAQGKER EFVV	AISKGGY KYDAVS LEG	RFTISKDNAKNTVYLQ INSLKPEDTAVYYCAS	SRAYGS SRLRLA DTYEY	WGQGTQVT VSS

These variants are characterized as purified proteins in the VEGF165/VEGFR2 AlphaScreen (Example 5.3, Figure 17). The melting temperature (T_m) of each clone is determined in a thermal shift assay, which is based on the increase in fluorescence signal upon incorporation of Sypro Orange (Invitrogen) (Ericsson et al, Anal. Biochem.
5 357 (2006), pp289-298). All variants displayed comparable IC_{50} when compared to VEGFBII23B04 and T_m values which are similar or higher when compared to the parental VEGFBII23B04. Table 28 summarizes the IC_{50} values and T_m values at pH 7 for the 9 clones tested.

Table 28: IC_{50} (pM) values, % inhibition and melting temperature (@pH 7) of sequence-
10 optimized variants of VEGFBII23B04

VHH ID	IC_{50} (pM)	% inhibition	T_m @ pH 7 (°C)
VEGFBII23B04 (wt)	*169*	*100*	*63*
VEGFBII111D05	209	100	68
VEGFBII111G06	366	100	71
VEGFBII112D11	221	100	70
VEGFBII113A08	253	100	69
VEGFBII113E03	290	100	68
VEGFBII114C09	215	100	71
VEGFBII114D02	199	100	74
VEGFBII114D03	227	100	64
VEGFBII118E10	189	100	62

In a second cycle, tolerated mutations from the humanization effort (VEGFBII111G06) and mutations to avoid potential posttranslational modification at selected sites (the
15 D16G, the S60A substitution and an E1D mutation) are combined resulting in a sequence-optimized clone derived from VEGFBII23B04: VEGFBII0037. One extra sequence-optimized variant (VEGFBII038) is anticipated which contains the same substitutions as VEGFBII0037, with the exception of the I82M mutation, as this mutation

may be associated with a minor drop in potency. The sequences from both sequence-optimized clones are listed in Table 29. VEGFBII0037 and VEGFBII0038 are characterized in the VEGF165/VEGFR2 blocking AlphaScreen (Example 5.3, Figure 18), the melting temperature is determined in the thermal shift assay as described above and the affinity for binding on VEGF165 is determined in Biacore (Example 5.5). An overview of the characteristics of the 2 sequence-optimized VHHs is presented in Table 30.

Table 29: AA sequences of sequence-optimized variants of VHH VEGFBII23B04

VHH ID/ SEQ ID NO:	**FR 1**	**CDR 1**	**FR2**	**CDR 2**	**FR3**	**CDR 3**	**FR 4**
VEGFBII037 **56**	DVQLVESGGGLVQPGGSLRLSCAASGRTFS	SYSMG	WFRQAPGKEREFVV	AISKGGYKYDAVSLEG	RFTISRDNAKNTVYLQMNSLRPEDTAVYYCAS	SRAYGSSRLRLADTYEY	WGQGTLVTVSS
VEGFBII038 **57**	DVQLVESGGGLVQPGGSLRLSCAASGRTFS	SYSMG	WFRQAPGKEREFVV	AISKGGYKYDAVSLEG	RFTISRDNAKNTVYLQINSLRPEDTAVYYCAS	SRAYGSSRLRLADTYEY	WGQGTLVTVSS

Table 30: IC_{50} (pM) values, % inhibition, melting temperature (@pH 7) and affinity (pM) of sequence-optimized clones VEGFBII037 and VEGFBII038

VHH ID	IC_{50} (pM)	% inhibition	T_m (°C) @ pH 7	K_D (pM)
VEGFBII23B04	*152*	*100*	*63*	*560*
VEGFBII037	300	100	72	270
VEGFBII038	143	100	71	360

5 *8.2 Sequence optimization of VEGFBII5B05*

The amino acid sequence of VEGFBII5B05 is aligned to the human germline sequence VH3-23/JH5, see Figure 19 (SEQ ID:NO: 179 The alignment shows that VEGFBII5B05 contains 15 framework mutations relative to the reference germline sequence. Non-human residues at positions 23, 60, 83, 105, 108 are selected for substitution with their
10 human germline counterparts while the histidine at position 44 is selected for substitution by glutamine. One humanization variant is constructed carrying the 6 described mutations (AA sequence is listed in Table 31).

Table 31: AA sequences of sequence-optimized variants of VHH VEGFBII5B05 (FR, framework; CDR, complementary determining region)
15

VHH ID/ SEQ ID NO:	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VEGFBII119G11/ **125**	EVQLVESGGGLVQPGGSLRLSCAASGIRFM	SMA	WYRQAPGKQRELVA	RISSGGTTAYADSVKG	RFTISRDNSKNTVYLQMNSLRAEDTAVYYCNT	FSSRPNP	WGQGTLVTVSS
VEGFBII120E10/ **126**	EVQLVESGGGLVQPGGSLRLSCVASGIRFI	SMA	WYRQAPGKHRELVA	RISSGGTTAYVDSVKG	RFTISRDNSKNTVYLQMNSLKAEDTAVYYCNT	FSSRPNP	WGAGTQVTVSS

One additional variant is constructed in which the potential oxidation site at position M30 (CDR1 region, see Figure 19 indicated as bold italic residue) is removed by introduction of a M30I mutation. Both variants are tested for their ability to bind hVEGF165 using the ProteOn. In brief, a GLC ProteOn Sensor chip is coated with human VEGF165.
5 Periplasmic extracts of the variants are diluted 1/10 and injected across the chip coated with human VEGF165. Off-rates are calculated and compared to the off-rates of the parental VEGFBII5B05. Off-rates from the 2 variants are in the same range as the off-rates from the parental VEGFBII5B05 indicating that all mutations are tolerated (Table 32).

10 **Table 32**: Off-rates sequence-optimized variants VEGFBII5B05

VHH ID	**binding level (RU)**	**k_d (1/s)**
VEGFBII5B05	*242*	*6.15E-02*
VEGFBII119G11	234	7.75E-02
VEGFBII120E10	257	4.68E-02

In a second cycle, mutations from the humanization effort and the M30I substitution are combined resulting in a sequence-optimized clone of VEGFBII5B05, designated
15 VEGFBII032. The sequence is listed in Table 33. Affinity of VEGFBII032 is determined by Biacore (see Example 5.5) and the melting temperature is determined in the thermal shift assay as described above. An overview of the characteristics of the sequence-optimized VHH VEGFBII032 is presented in Table 34.

Table 33: AA sequence of sequence-optimized clone VEGFBII032 (FR, framework;
20 CDR, complementary determining region)

VHH ID/ SEQ ID NO:	**FR1**	**CDR1**	**FR2**	**CDR2**	**FR3**	**CDR3**	**FR4**
VEGFBII032/ **127**	EVQLVE SGGGLV QPGGSL RLSCAA SGIRFI	SMA	WYRQA PGKQR ELVA	RISSG GTTA YADS VKG	RFTISRDNSK NTVYLQMNS LRAEDTAVY YCNT	FSSR PNP	WGQGTL VTVSS

Table 34: Melting temperature (@pH 7) and affinity (nM) of sequence-optimized clone VEGFBII032

VHH ID	T_m (°C) @ pH 7	K_D (nM)
VEGFBII5B05(wt)	*69*	*32*
VEGFBII0032	71	44

5 The potency of the sequence-optimized clones VEGFBII037 and VEGFBII038 is evaluated in a proliferation assay. In brief, primary HUVEC cells (Technoclone) are supplement-starved over night and then 4000 cells/well are seeded in quadruplicate in 96-well tissue culture plates. Cells are stimulated in the absence or presence of VHHs with 33ng/mL VEGF. The proliferation rates are measured by [^{3}H] Thymidine
10 incorporation on day 4. The results shown in Table 35, demonstrate that the activity (potency and degree of inhibition) of the parental VHH VEGFBII23B04 is conserved in the sequence optimized clone VEGFBII038.

Table 35: IC_{50} (nM) values and % inhibition of the sequence optimized clones VEGFBII037 and VEGFBII038 in VEGF HUVEC proliferation assay

15

VHH ID	IC_{50} (nM)	% inhibition
VEGFBII23B04	0.68	92
VEGFBII037	1.54	78
VEGFBII038	0.60	92
Bevacizumab	0.29	94

Claims

5 1. VEGF-binding molecule comprising at least a variable domain with four framework regions and three complementarity determining regions CDR1, CDR2 and CDR3, respectively, wherein said CDR3 has the amino acid sequence Ser Arg Ala Tyr Xaa Ser Xaa Arg Leu Arg Leu Xaa Xaa Thr Tyr Xaa Tyr as shown in SEQ ID NO: 1, wherein

10 Xaa at position 5 is Gly or Ala;
Xaa at position 7 is Ser or Gly;
Xaa at position 12 is Gly, Ala or Pro;
Xaa at position 13 is Asp or Gly;
Xaa at position 16 is Asp or Glu; and
15 wherein said VEGF-binding molecule is capable of blocking the interaction of human recombinant VEGF165 with the human recombinant VEGFR-2 with an inhibition rate of ≥60%.

2. A VEGF-binding molecule of claim 1, wherein said CDR3 has a sequence selected from

20 SEQ ID NO: 2 SRAYGSSRLRLGDTYDY,
SEQ ID NO: 3 SRAYGSSRLRLADTYDY;
SEQ ID NO: 4 SRAYGSSRLRLADTYEY;
SEQ ID NO: 5 SRAYGSGRLRLADTYDY;
SEQ ID NO: 6 SRAYASSRLRLADTYDY;
25 SEQ ID NO: 7 SRAYGSSRLRLPDTYDY;
SEQ ID NO: 8 SRAYGSSRLRLPGTYDY.

3. A VEGF-binding molecule of claim 2, which comprises one or more immunoglobulin single variable domains each containing

   a) a CDR3 with an amino acid sequence selected from a first group of sequences shown in SEQ ID NO: 2 to 8**;**

5 b) a CDR1 and a CDR2 with an amino acid sequences that is contained, as indicated in Table 3, in a sequence selected from a second group of sequences shown in SEQ ID NOs: 9 to 46, wherein said second sequence contains the respective CDR3 in said selected sequence according to a).

4. A VEGF-binding molecule of claim 3, wherein said one or more
10 immunoglobulin single variable domains are VHHs.

5. A VEGF-binding molecule of claim 4, wherein said one or more VHHs have amino acid sequences selected from the amino acid sequences shown in SEQ ID NOs: 9 - 46.

6. A VEGF-binding molecule of claim 5, which comprises one or more VHHs
15 having amino acid sequences selected from SEQ ID NO: 15, SEQ ID NO: 18 and SEQ ID NO: 25.

7. A VEGF-binding molecule which has been obtained by affinity maturation and/or sequence optimization of a VHH defined in claim 6.

8. A VEGF-binding molecule of claim 7 which has been obtained by sequence
20 optimization of a VHH having an amino acid sequence shown in SEQ ID NO: 18.

9. A VEGF-binding molecule of claim 8 having an amino acid sequence selected from sequences shown in SEQ ID NOs: 47 – 57.

10. A VEGF-binding molecule of claim 4, comprising two or more VHHs, which are

a) identical VHHs that are capable of blocking the interaction between recombinant human VEGF and the recombinant human VEGFR-2 with an inhibition rate of ≥ 60% or

b) different VHHs that bind to non-overlapping epitopes of VEGF, wherein at least one VHH is capable of blocking the interaction between recombinant human VEGF and the recombinant human VEGFR-2 with an inhibition rate of ≥ 60% and wherein at least one VHH is capable of blocking said interaction with an inhibition rate of ≤ 60 %.

11. A VEGF-binding molecule of claim 10, wherein said identical VHHs a) are selected from VHHs having amino acid sequences shown in SEQ ID NOs: 9 – 46 or VHHs that have been obtained by affinity maturation and/or sequence optimization of such VHH.

12. A VEGF-binding molecule of claim 11, wherein said VHH is selected from VHHs having the amino acid shown in SEQ ID NO: 18 or SEQ ID NO: 47 – 57.

13. The VEGF-binding molecule of claim 12 comprising two VHHs each having the amino acid sequence shown in SEQ ID NO: 57.

14. A VEGF-binding molecule of claim 13, wherein

a) said one or more VHHs with an inhibition rate of ≥ 60% are selected from

i. VHHs having an amino acid sequence selected from amino acid sequences shown in SEQ ID NOs: 9 – 46 or

ii. VHHs that have been obtained by affinity maturation and/or sequence optimization of such VHHs, and wherein

b) said one or more VHHs with an inhibition rate of ≤ 60 % are selected from

i. SEQ ID NOs: 58 – 124 or

ii. VHHs that have been obtained by affinity maturation and/or sequence optimization of such VHH.

5 15. A VEGF-binding molecule of claim 14, wherein two VHHs are contained in polypeptides with amino acid sequences shown in SEQ ID NOs: 128 – 168, separated by linker sequences as indicated in Table 13.

16. A VEGF-binding molecule of claim 15, wherein said VHH a) i. has an amino acid sequence shown in SEQ ID NO: 18 and said VHH b) i. has an amino acid
10 sequence shown in SEQ ID NO: 64.

17. A VEGF-binding molecule of claim 16, wherein said VHHs according to a) ii) are selected from VHHs having an amino acid sequence shown in SEQ ID NOs: 47 – 57 and wherein said VHHs according to b) ii) are selected from VHHs having an amino acid sequence shown in SEQ ID NOs: 125 – 127.

15 18. A VEGF-binding molecule of claim 17, comprising two VHHs, one of them having the amino acid shown in SEQ ID NO: 57 and one of them having the amino acid shown in SEQ ID NO: 127.

19. A nucleic acid molecule encoding a VEGF-binding molecule of any one of claims 1 to 18 or a vector containing same.

20 20. A host cell containing a nucleic acid molecule of claim 19.

21. A pharmaceutical composition containing at least one VEGF-binding molecule of any one of claims 1 to 18 as the active ingredient.

22. The pharmaceutical composition of claim 21 for the treatment of a disease that is associated with VEGF-mediated effects on angiogenesis.

23. The pharmaceutical composition of claim 22 for the treatment of cancer and cancerous diseases.

24. The pharmaceutical composition of claim 22 for the treatment of eye diseases.

5

Fig. 1

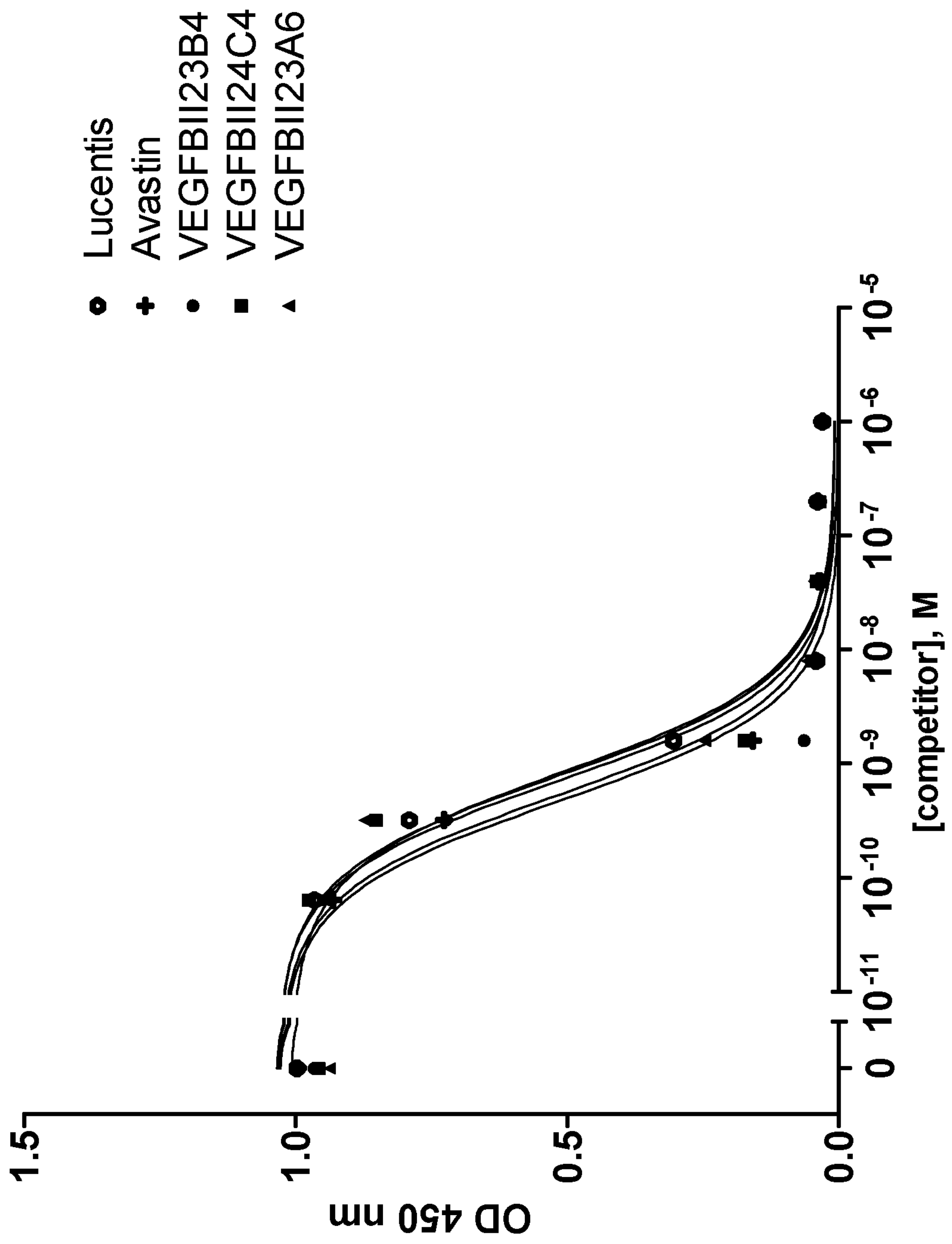

Fig. 2

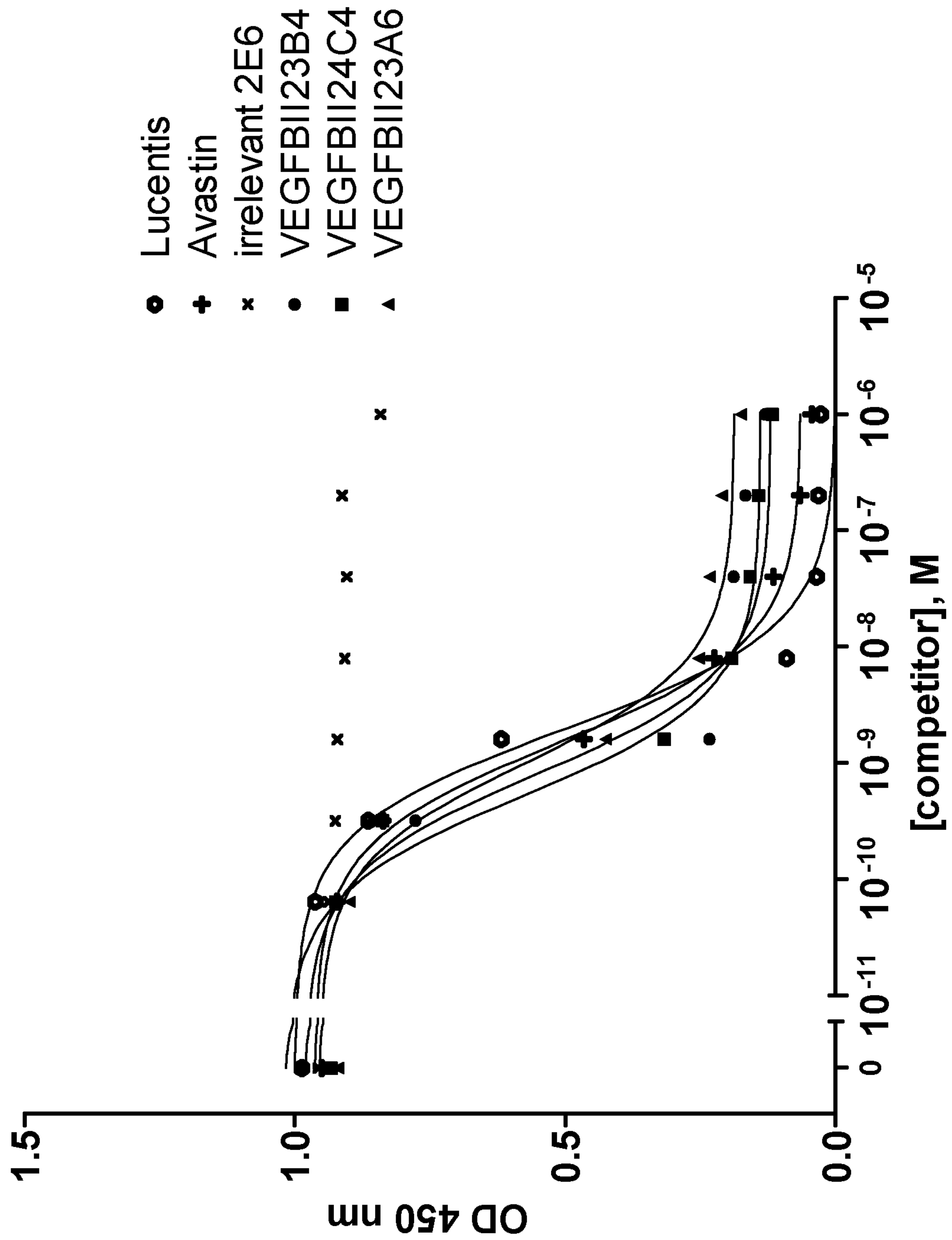

Fig. 3

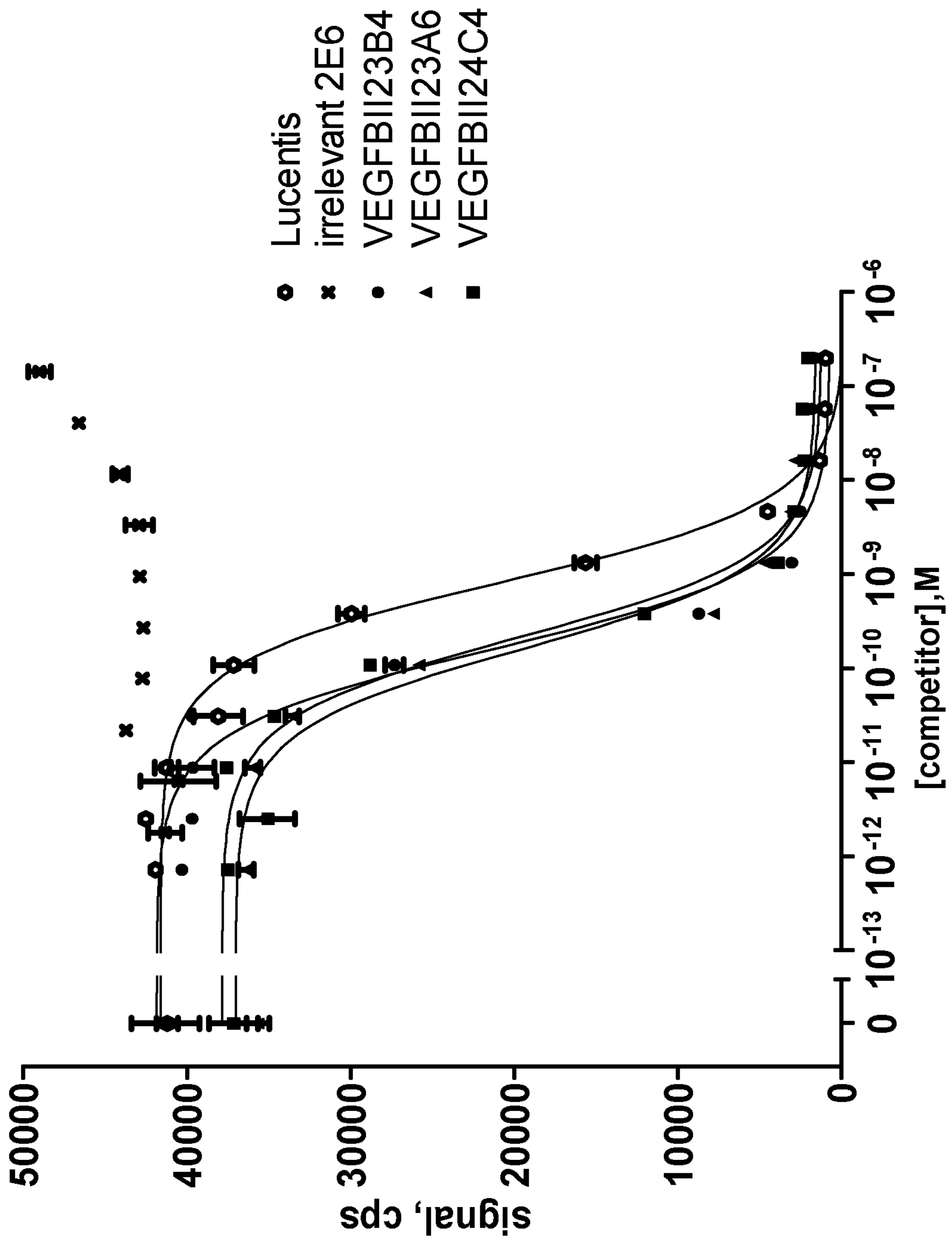

Fig. 4

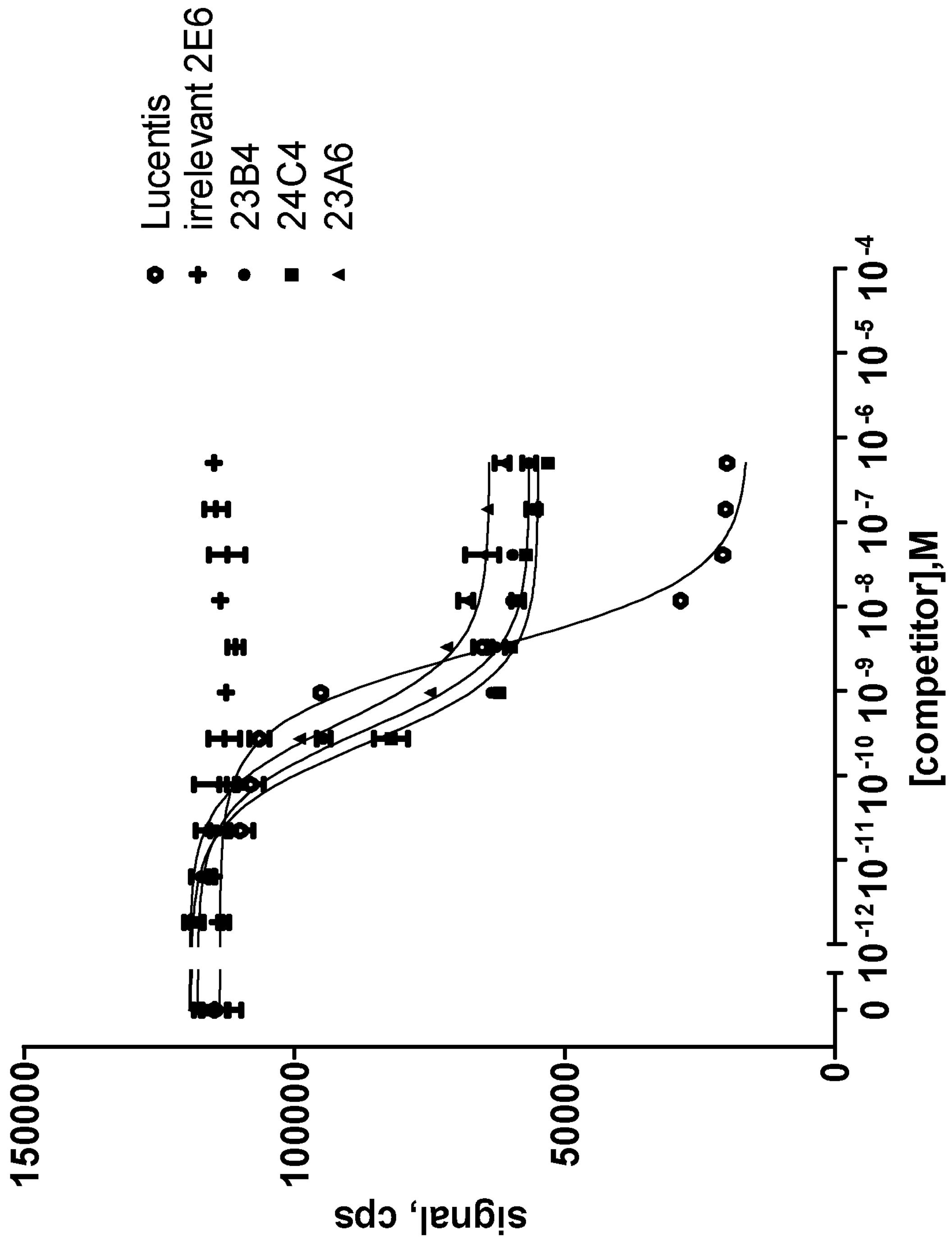

Fig. 5-1

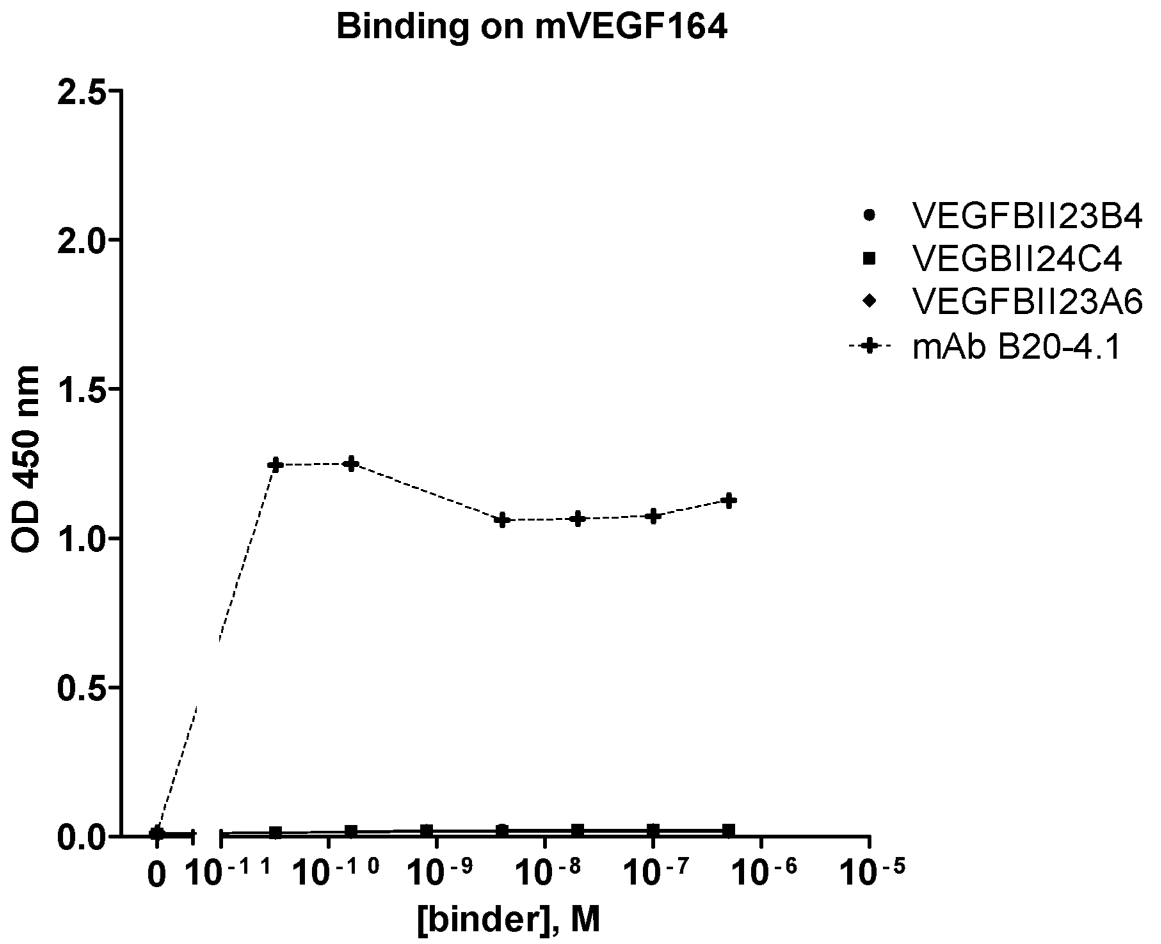

Fig. 5-2

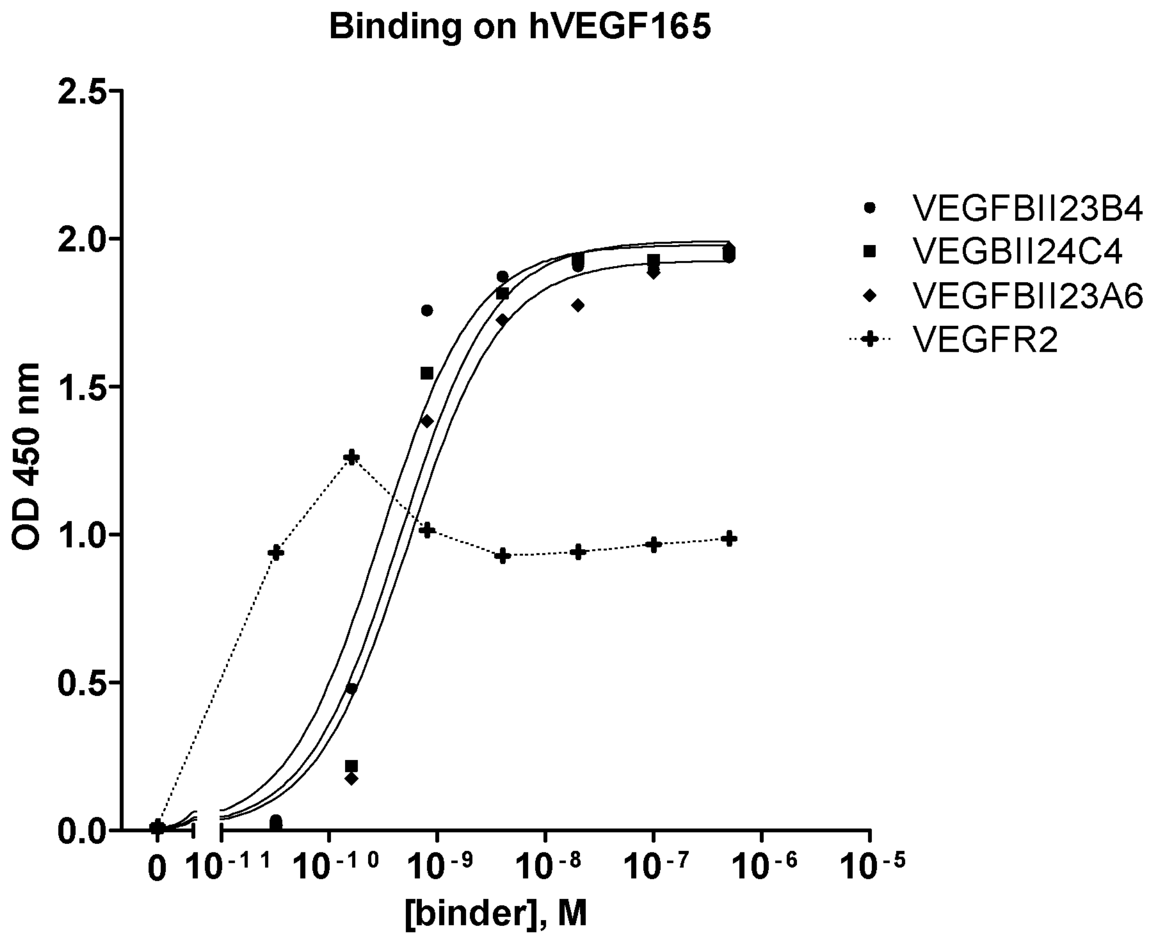

Fig. 6

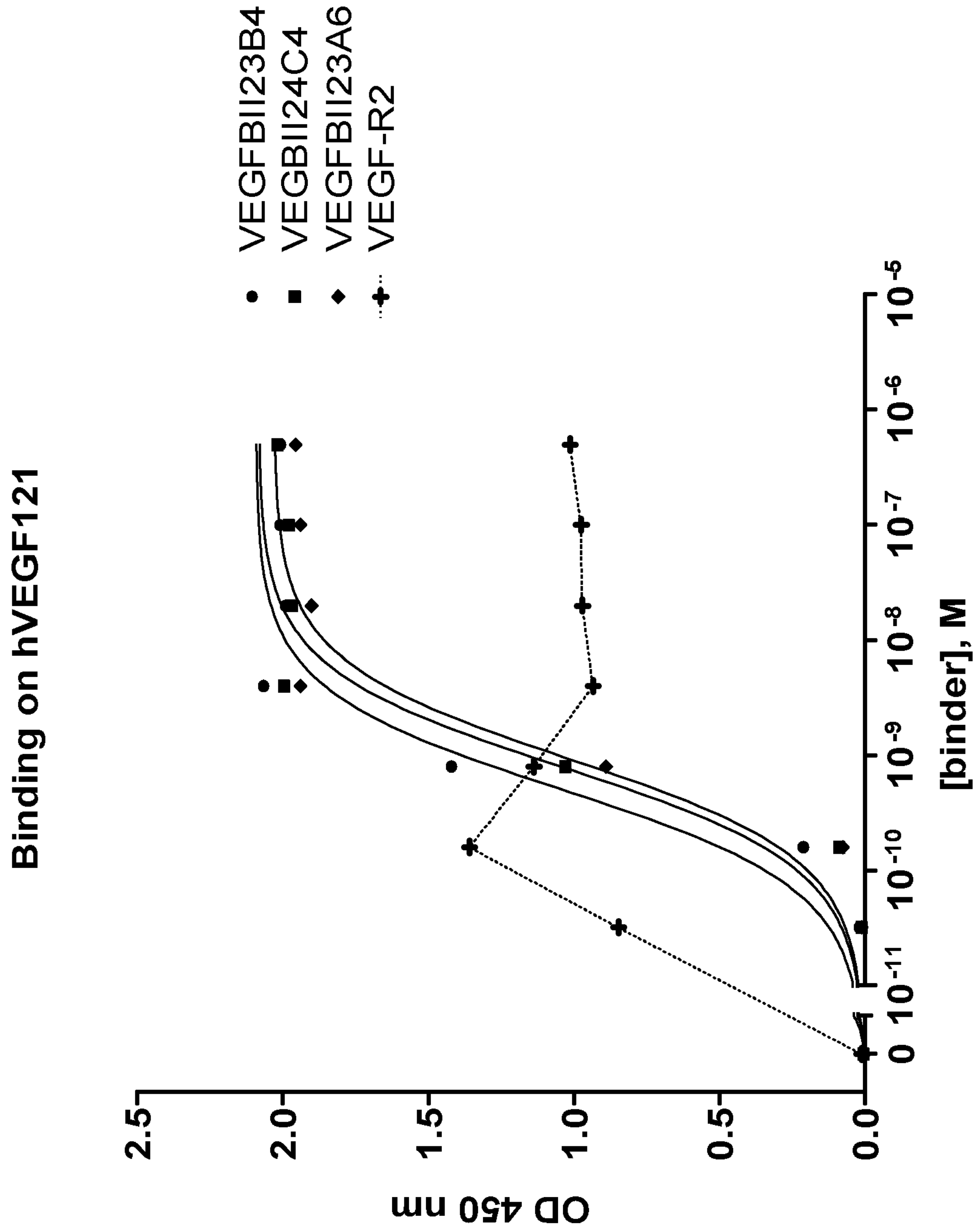

Fig. 7-1

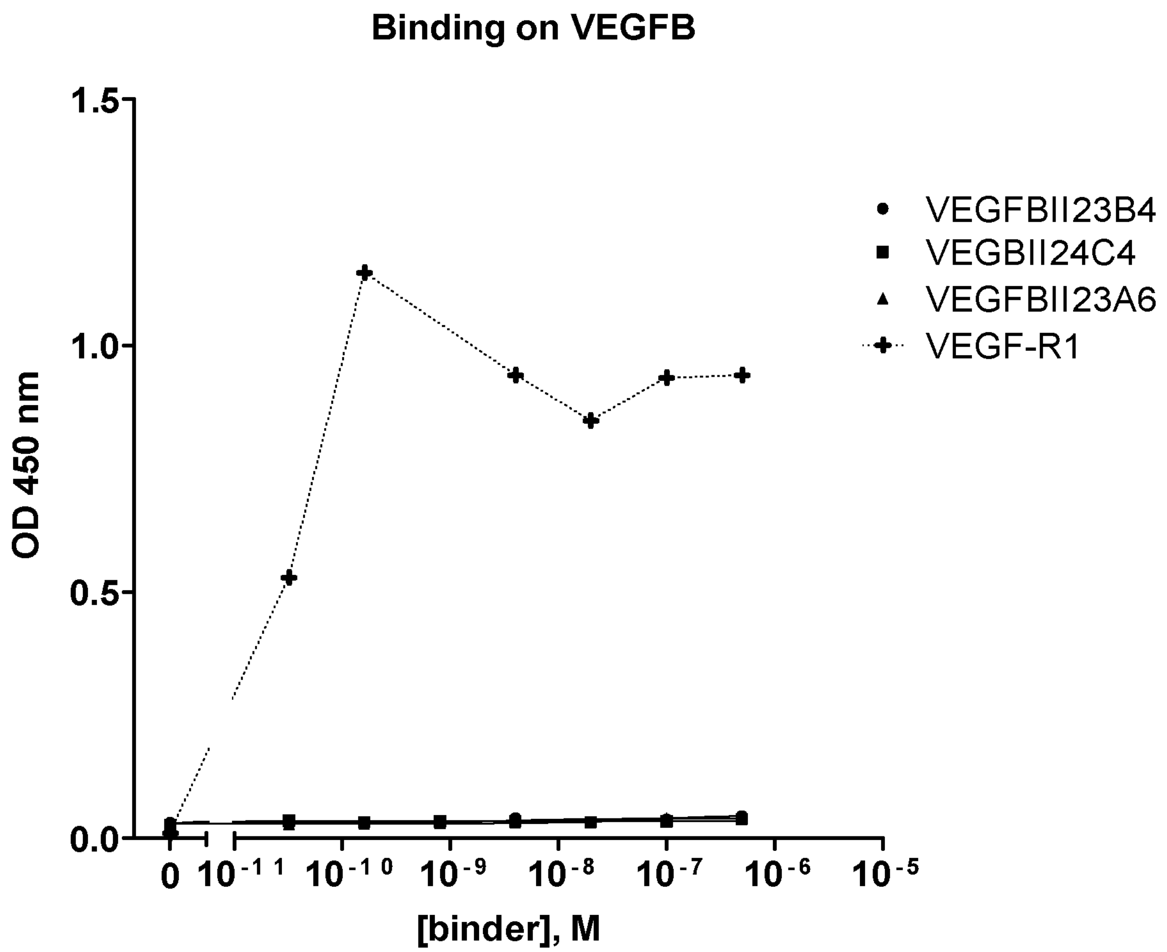

Fig. 7-2

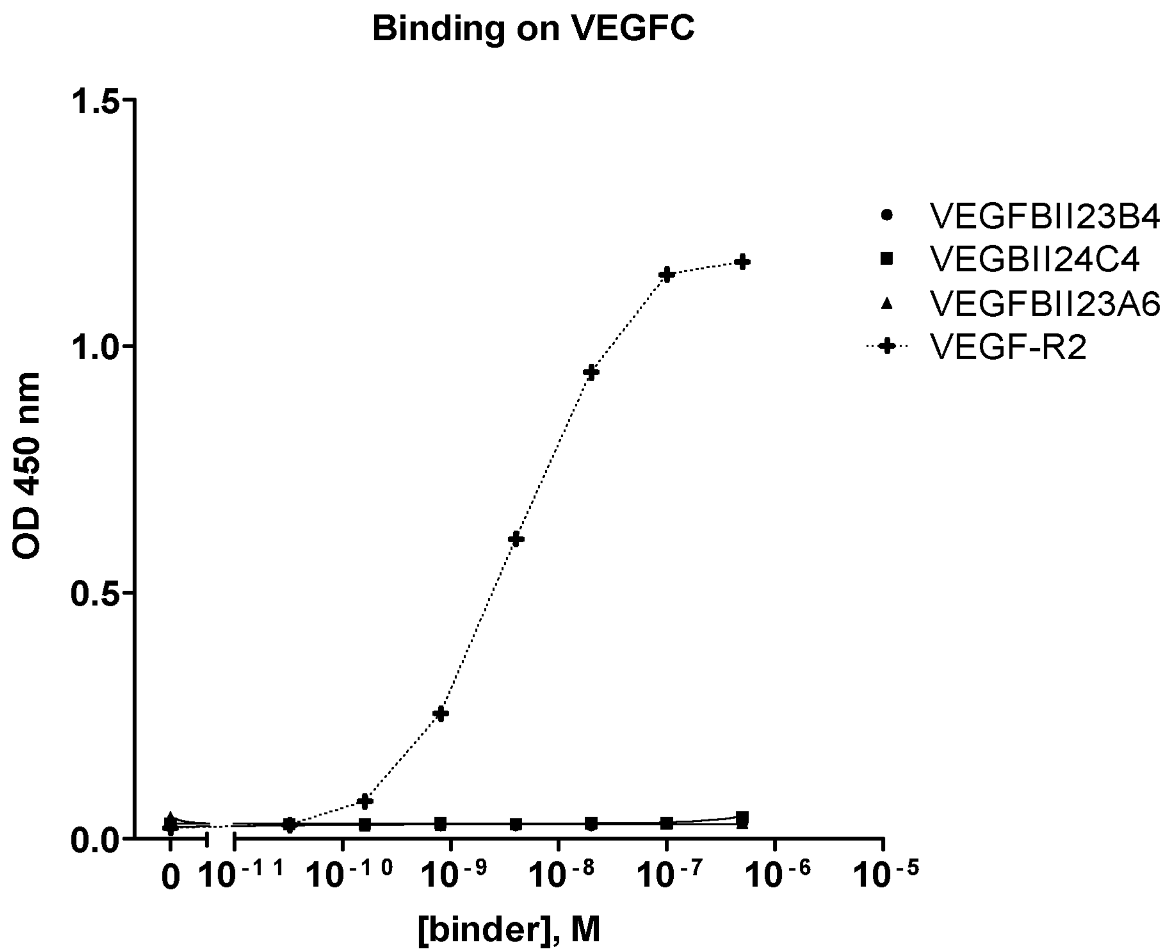

Fig. 7-3

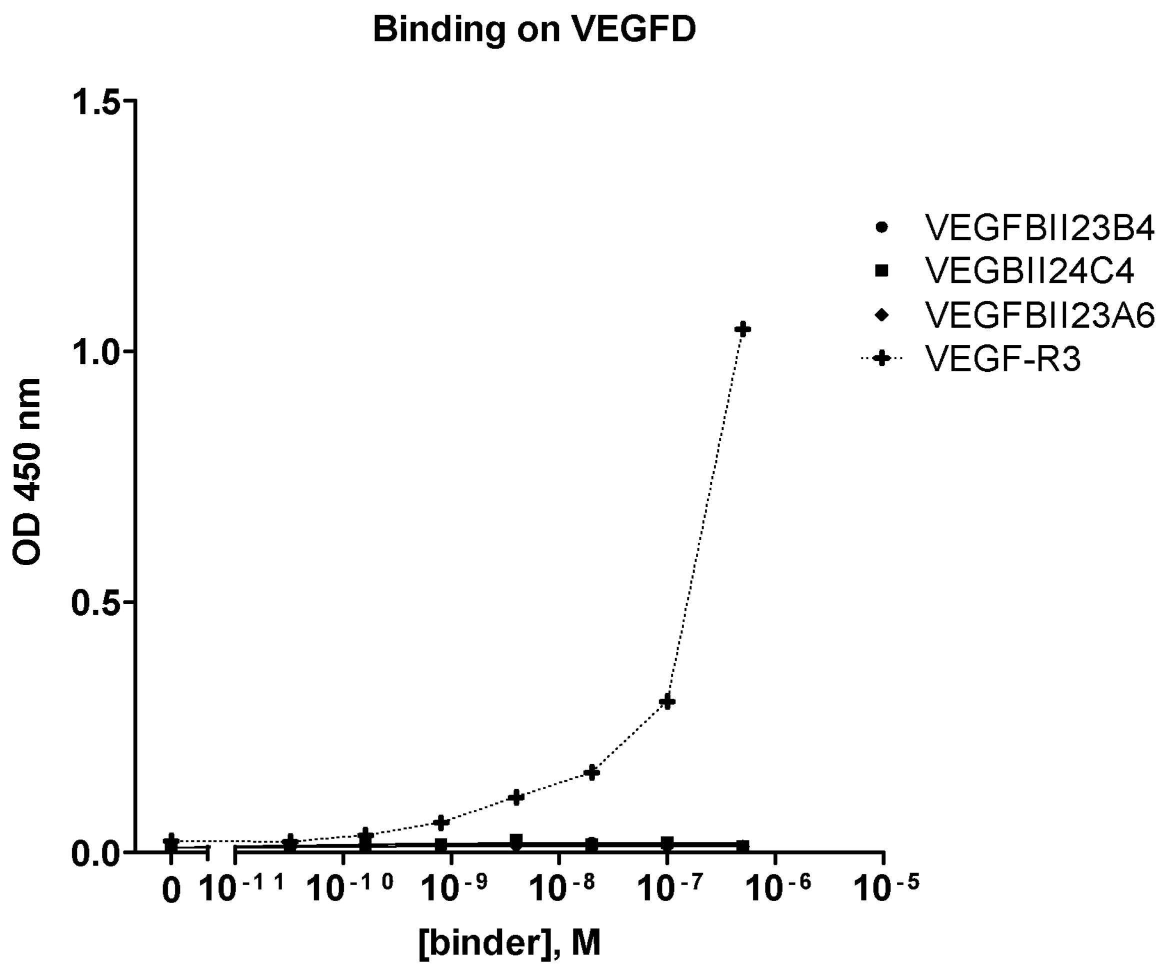

Fig. 7-4

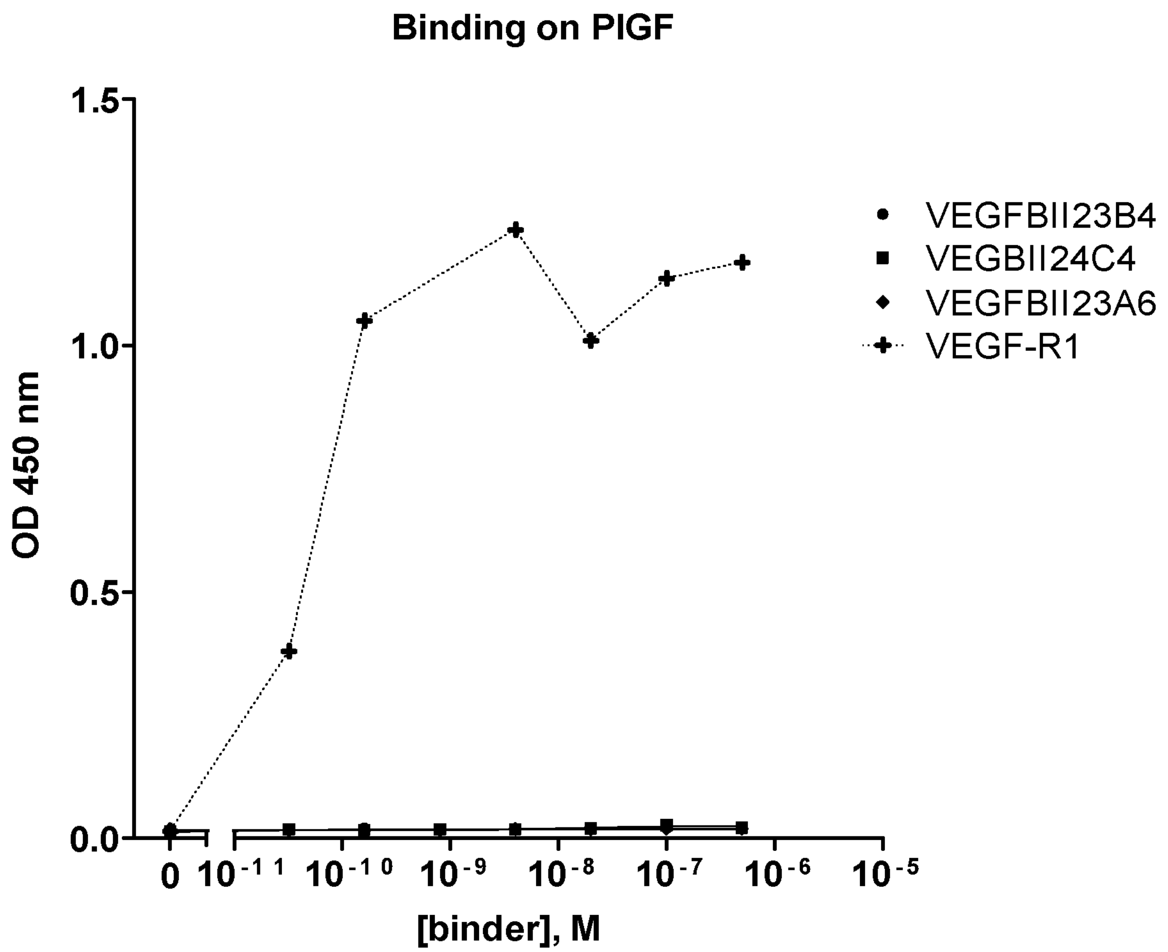

Fig. 8-1

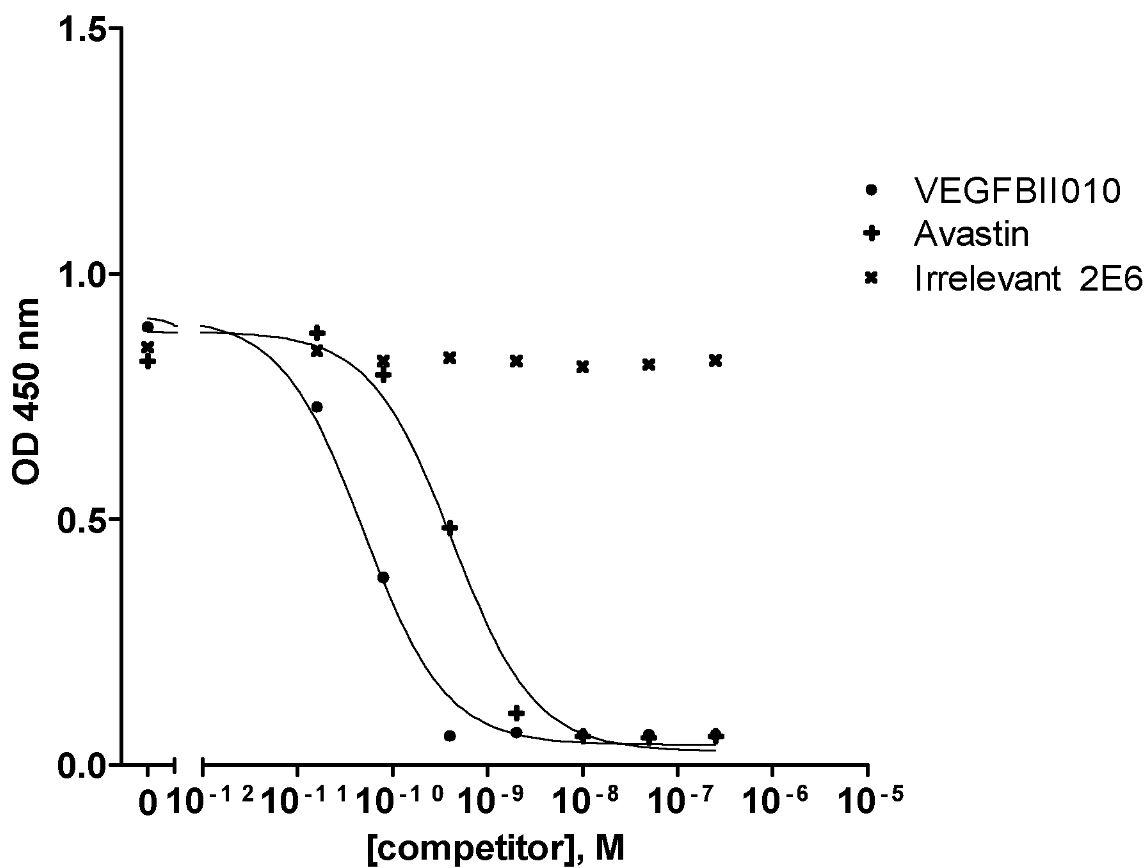

Fig. 8-2

OD 450 nm

1.5
1.0
0.5
0.0

0 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}

[inhibitor], M

Lucentis
Avastin
Irrelevant 2E6
VEGFBII021
VEGFBII022
VEGFBII023
VEGFBII024
VEGFBII025

Fig. 9-1

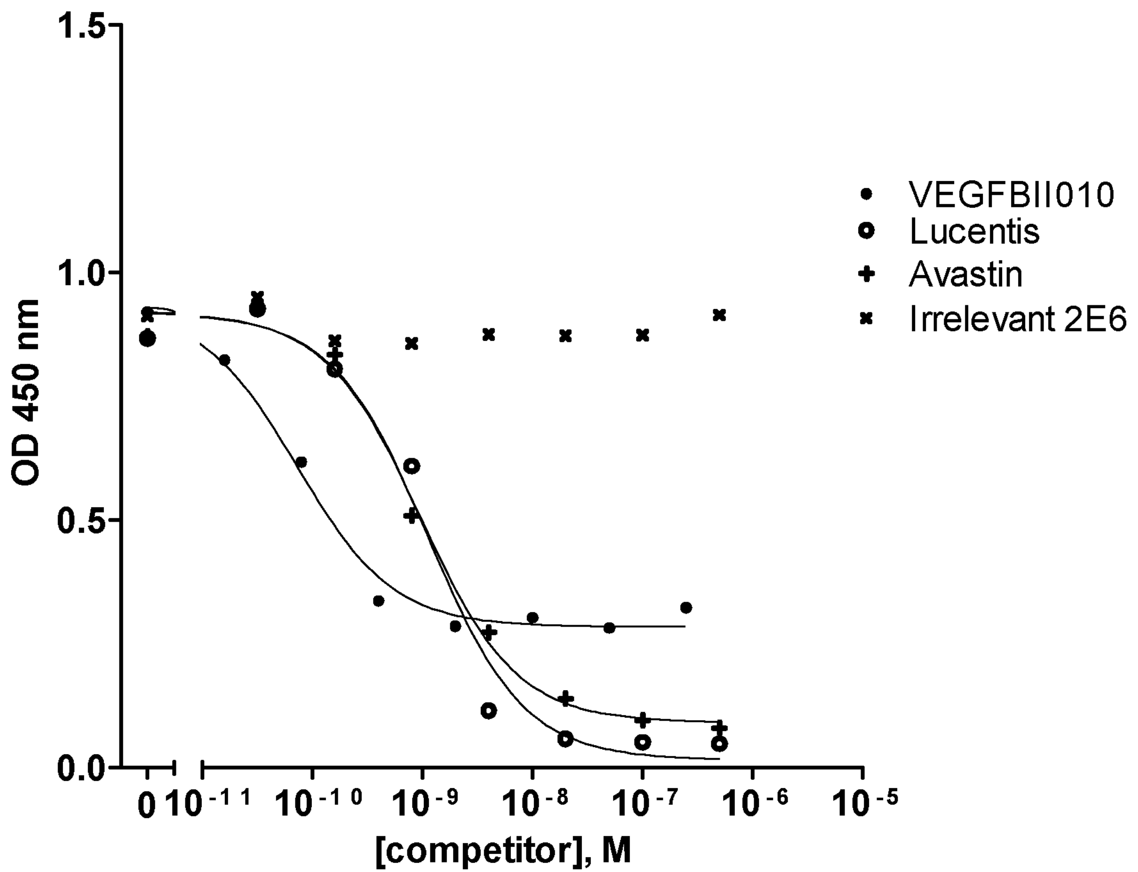

Fig. 9-2

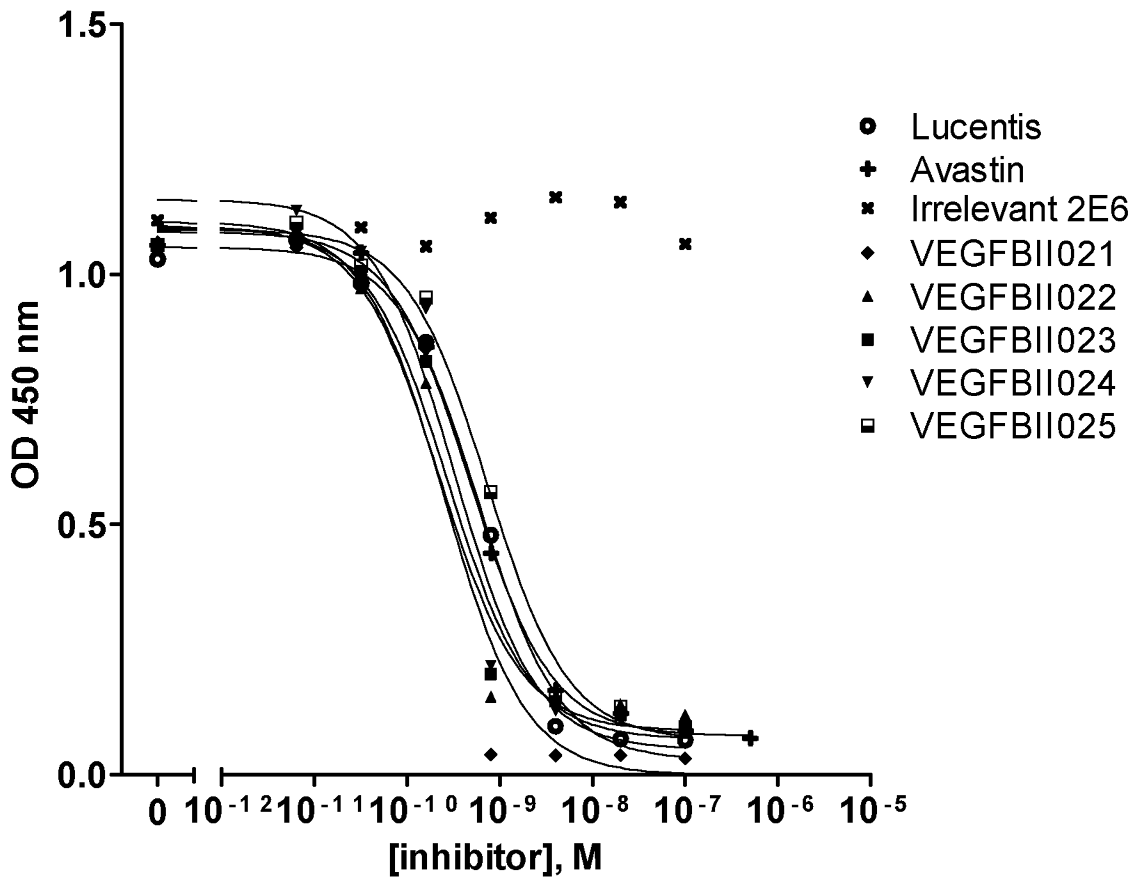

Fig. 10

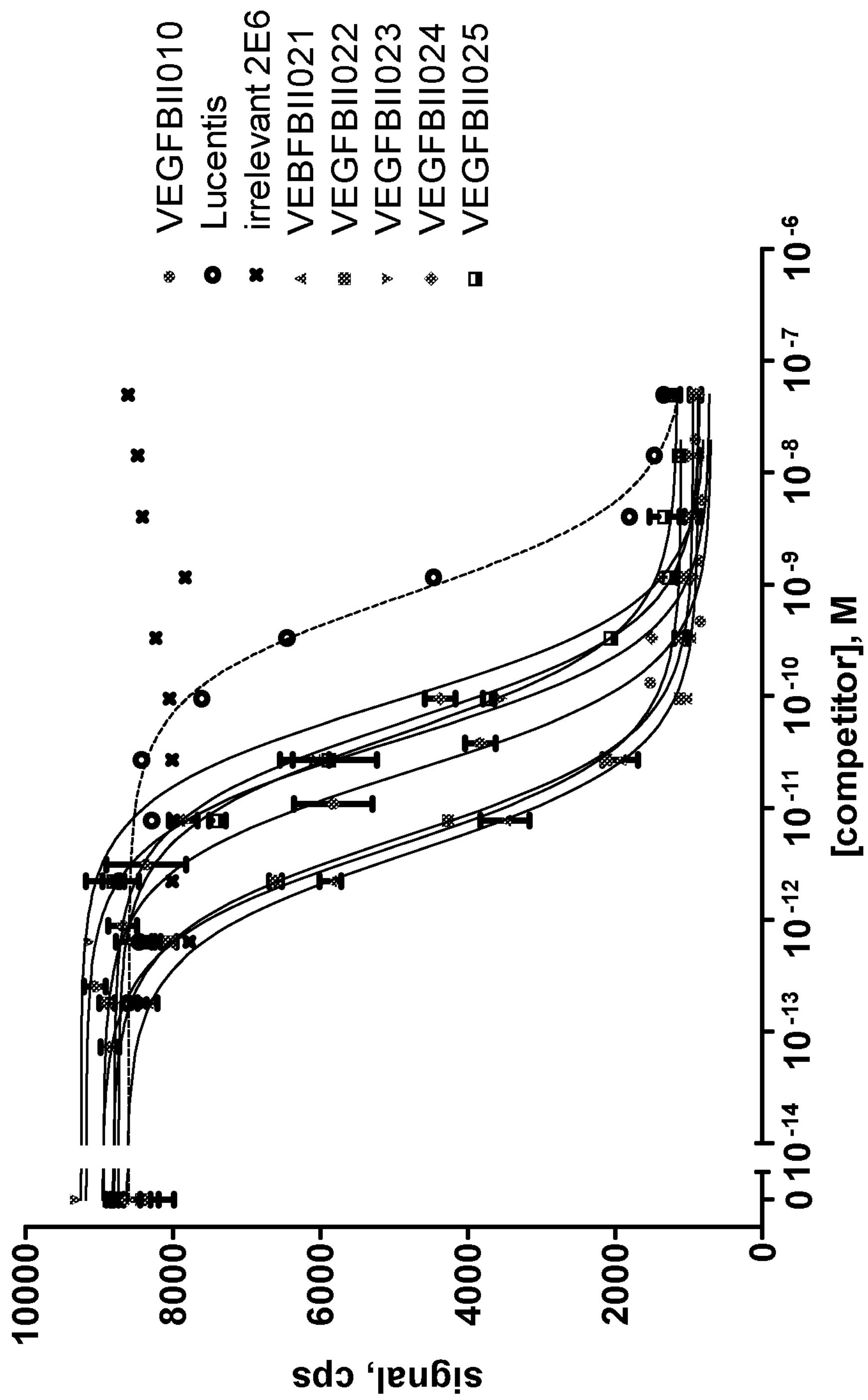

Fig. 11

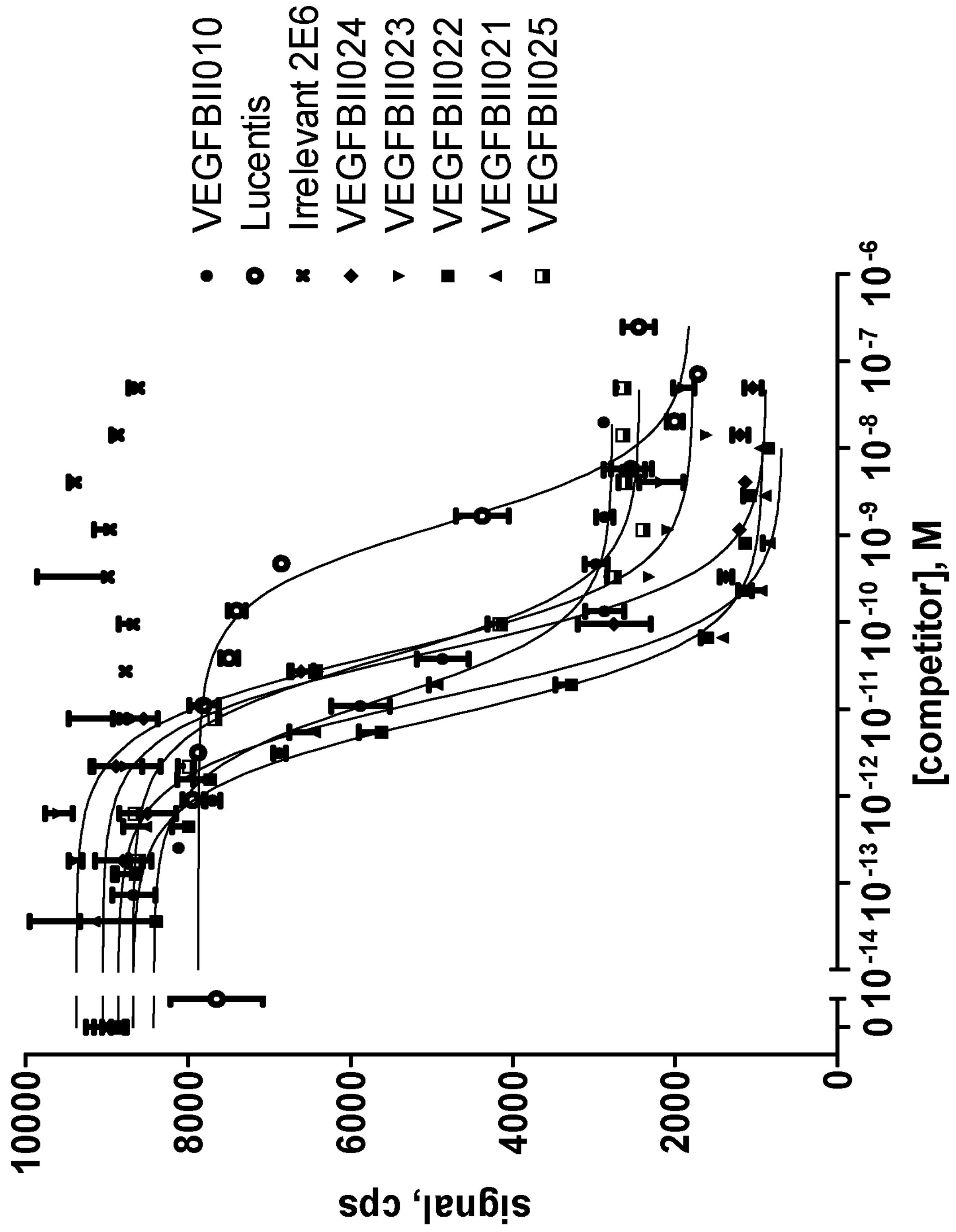

Fig. 12

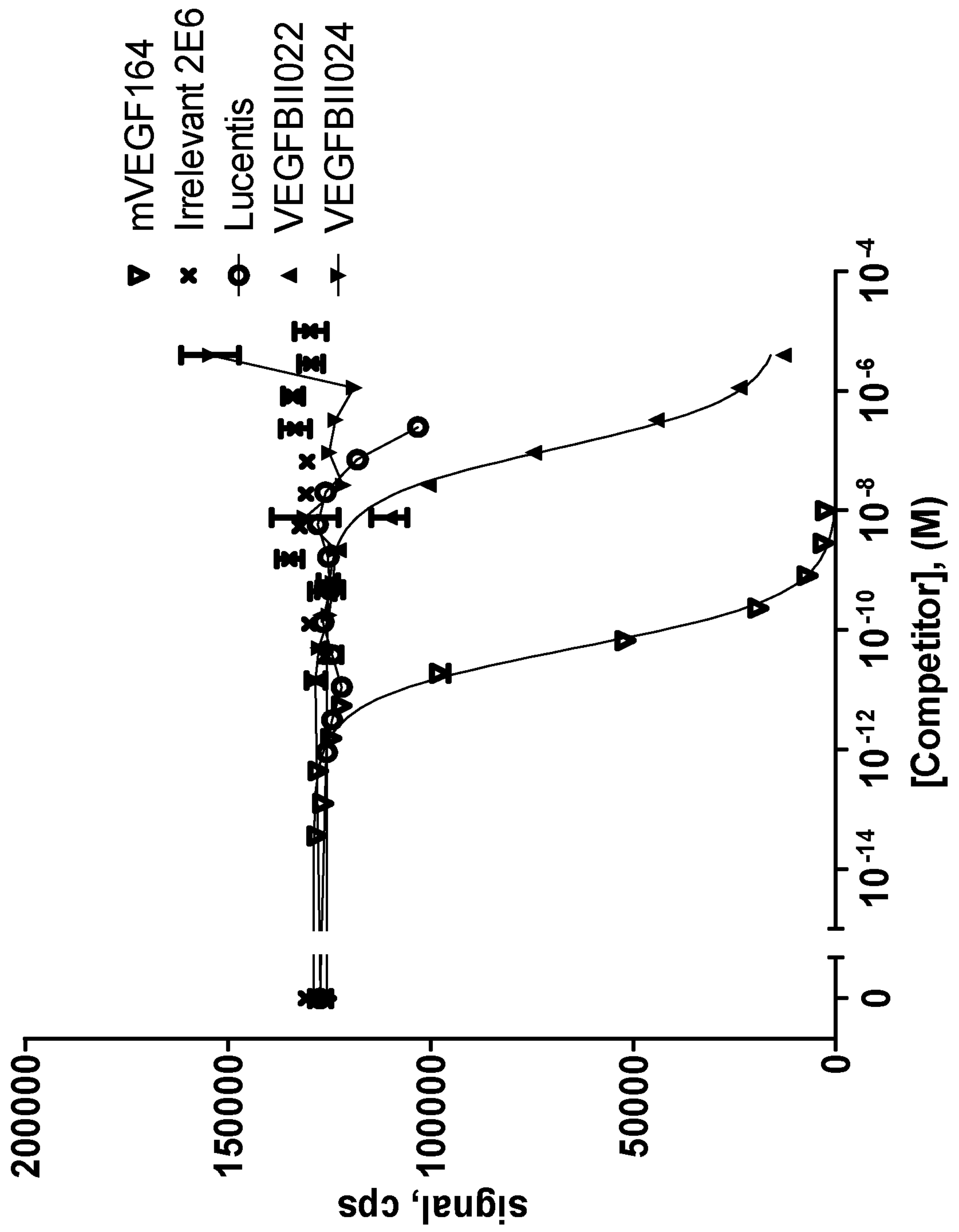

Fig. 13-1

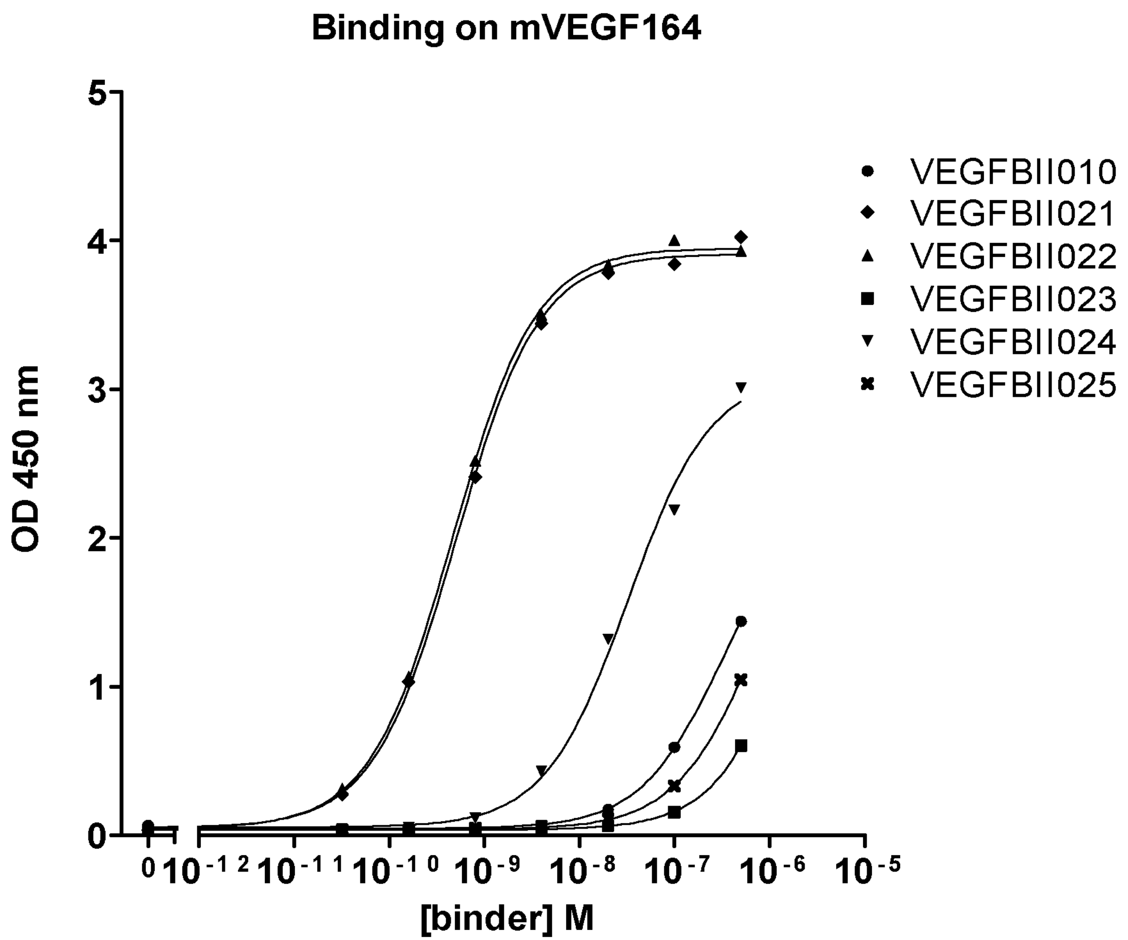

Fig. 13-2

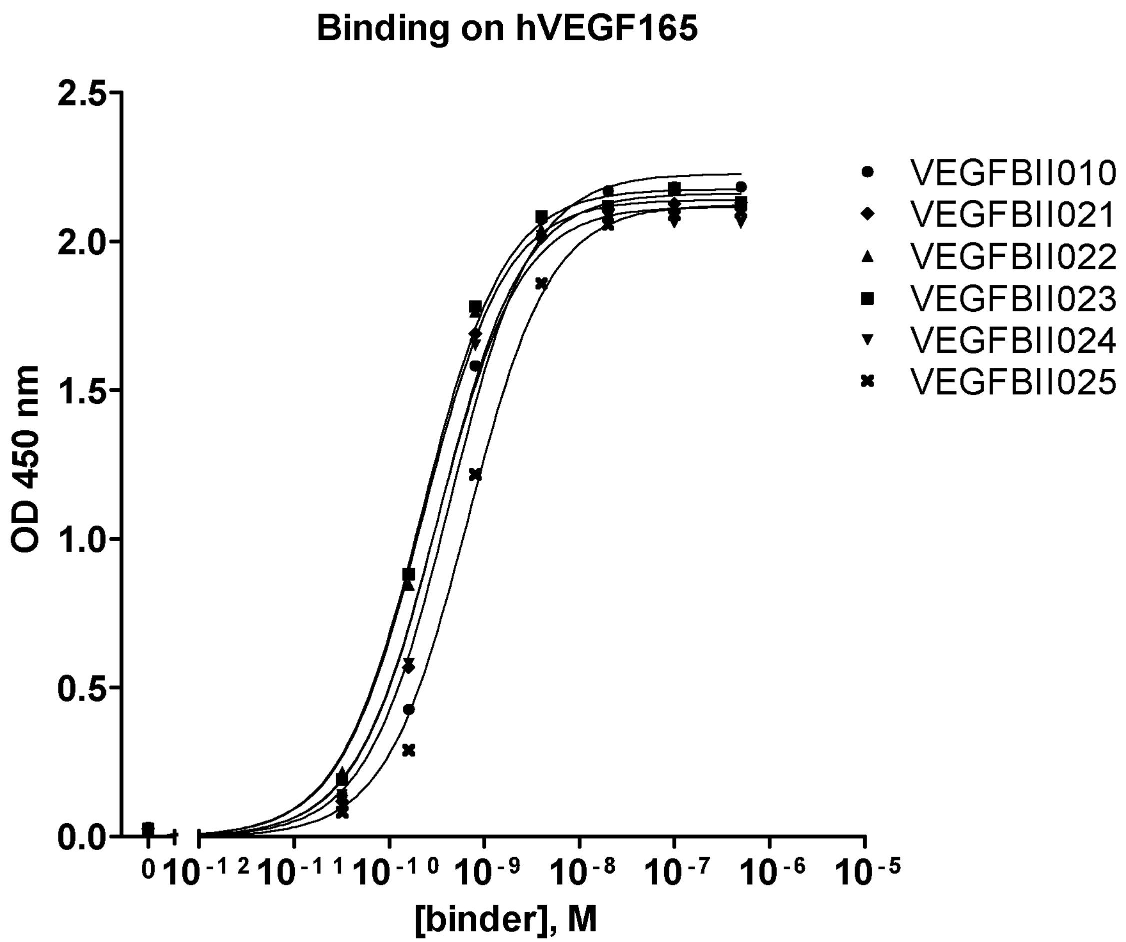

Fig. 14-1

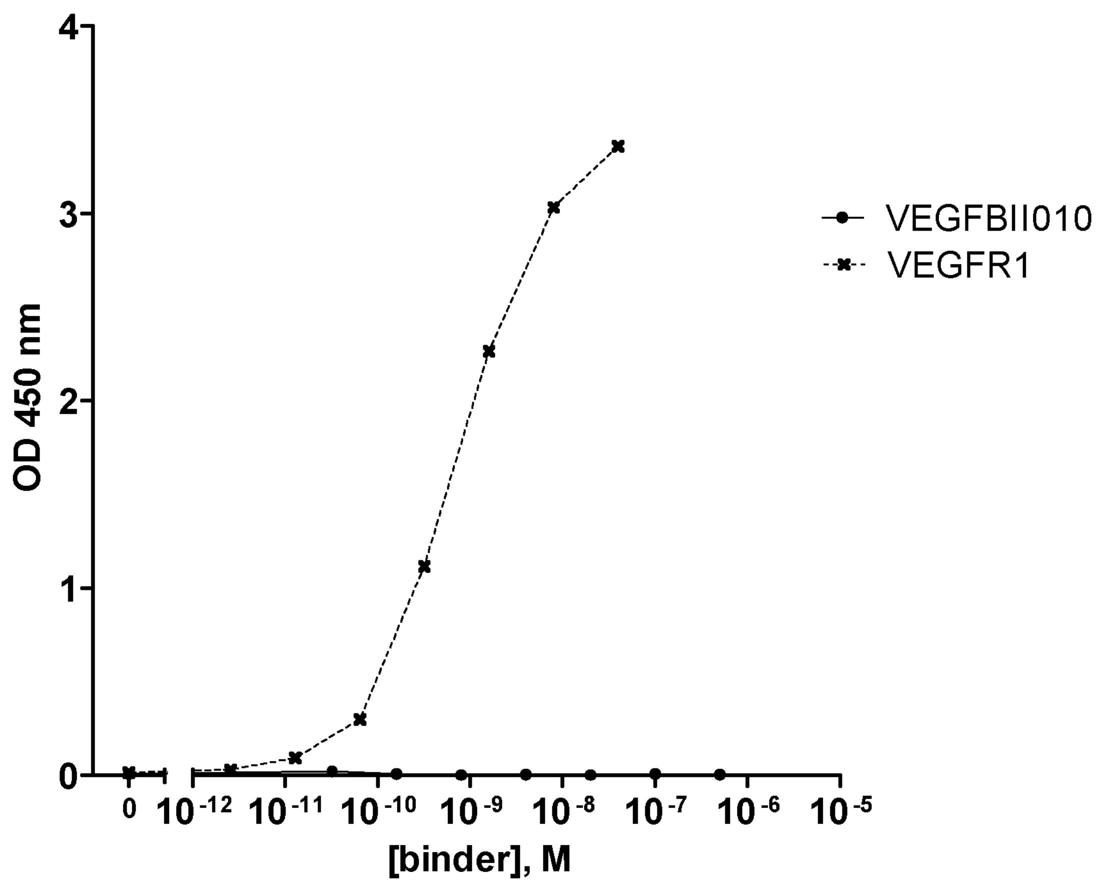

Fig. 14-2

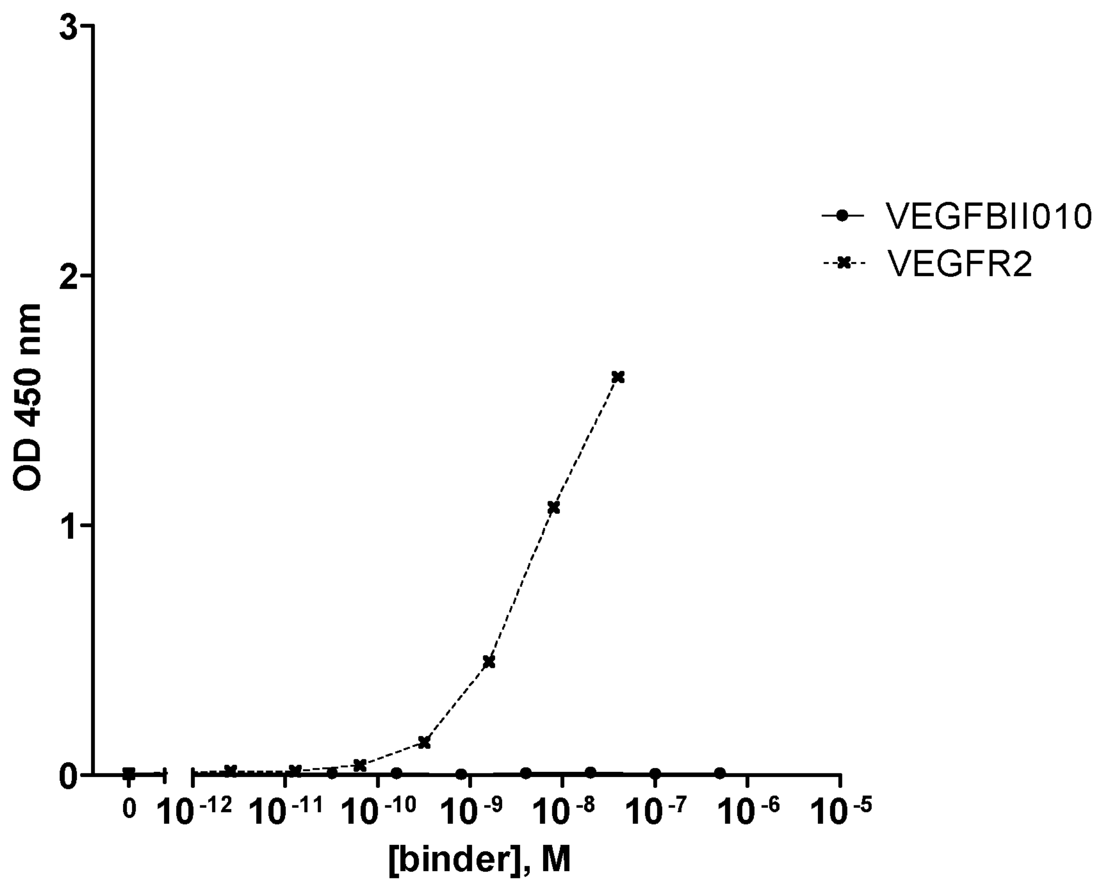

Fig. 14-3

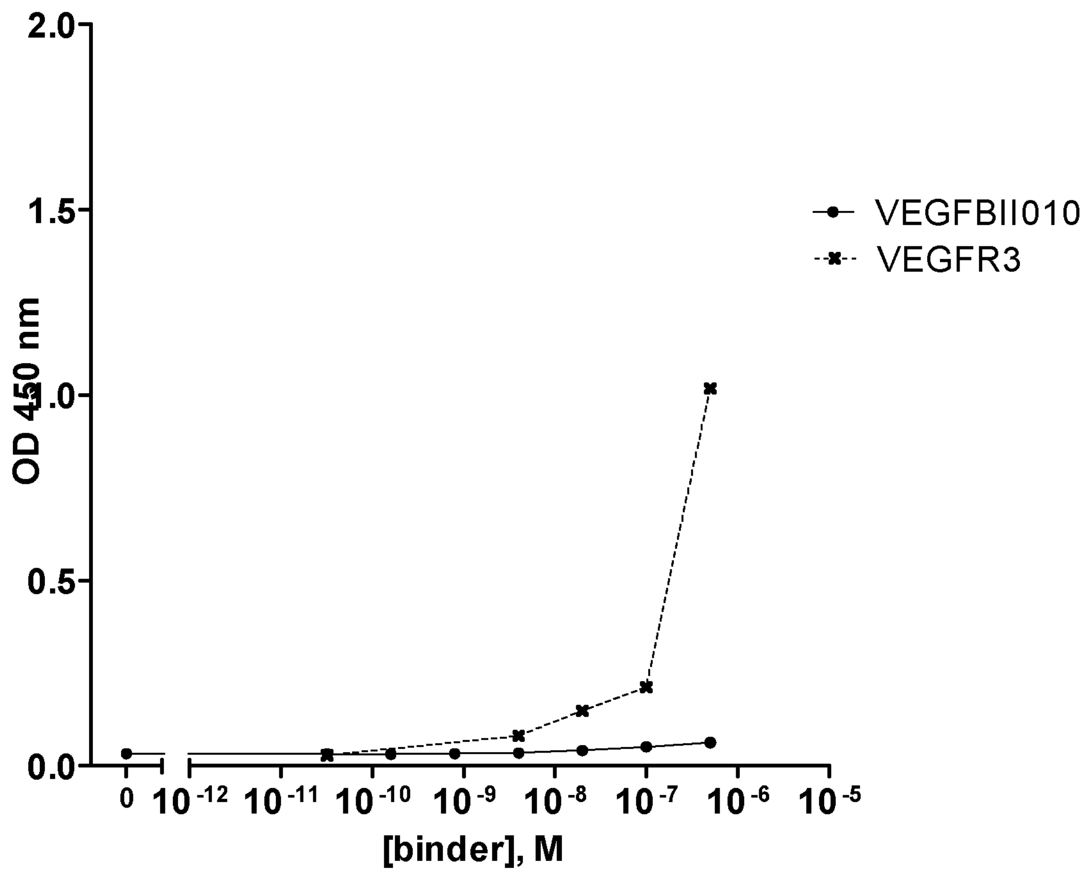

Fig. 14-4

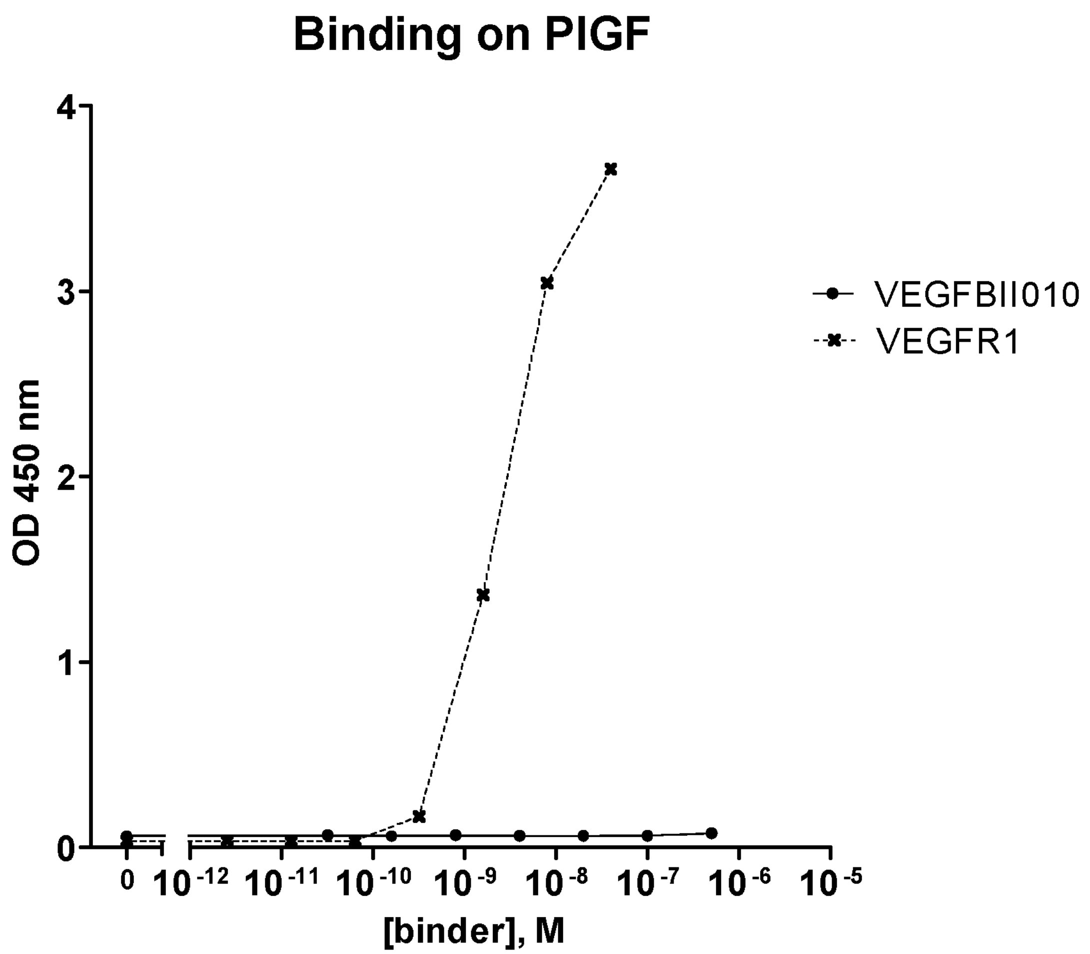

Fig. 14-5

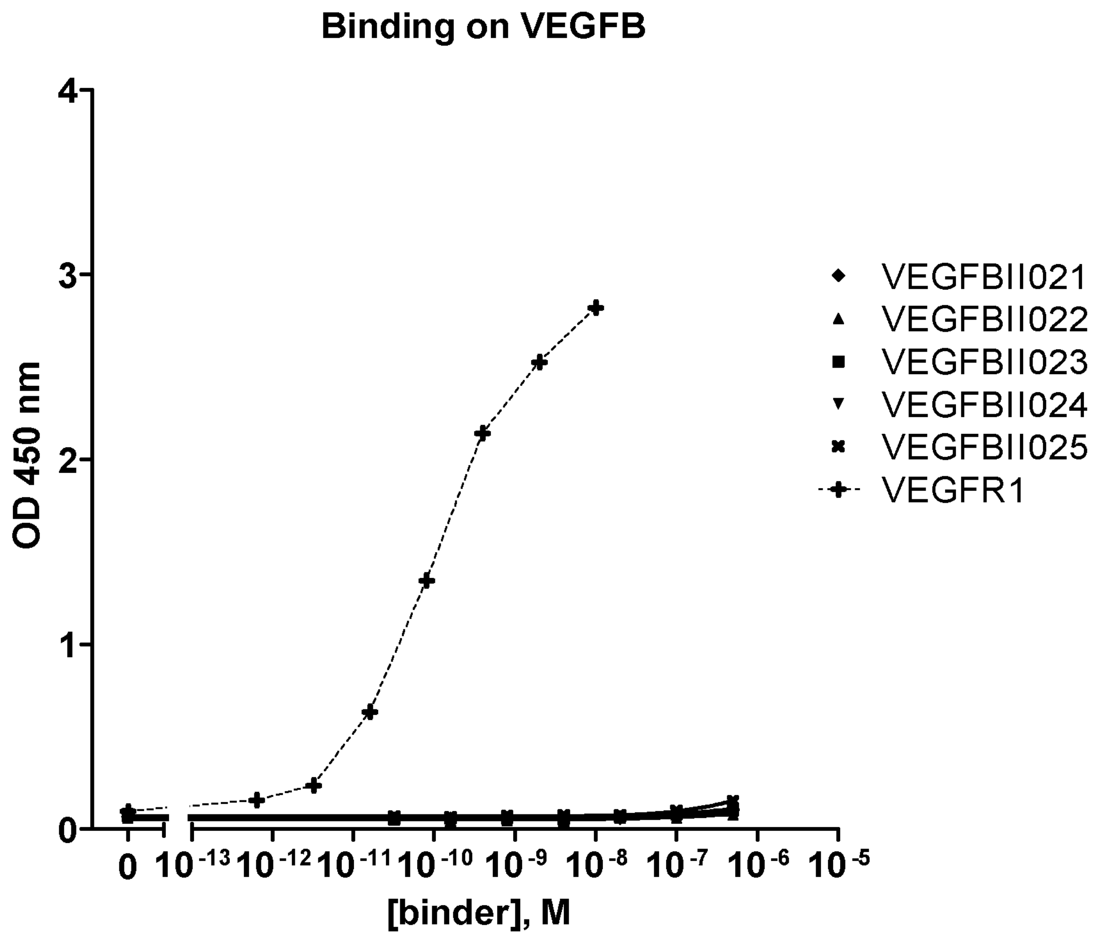

Fig. 14-6

Binding on VEGFC

OD 450 nm

3
2
1
0

0 10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}

[binder], M

- VEGFBII021
- VEGFBII022
- VEGFBII023
- VEGFBII024
- VEGFBII025
- VEGFR2

Fig. 14-7

Binding on VEGF D

OD 450 nm: 0, 1, 2

[binder], M: 0, 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}

VEGFBII021
VEGFBII022
VEGFBII023
VEGFBII024
VEGFBII025
VEGFR3

Fig. 14-8

Binding on PlGF

Fig. 15

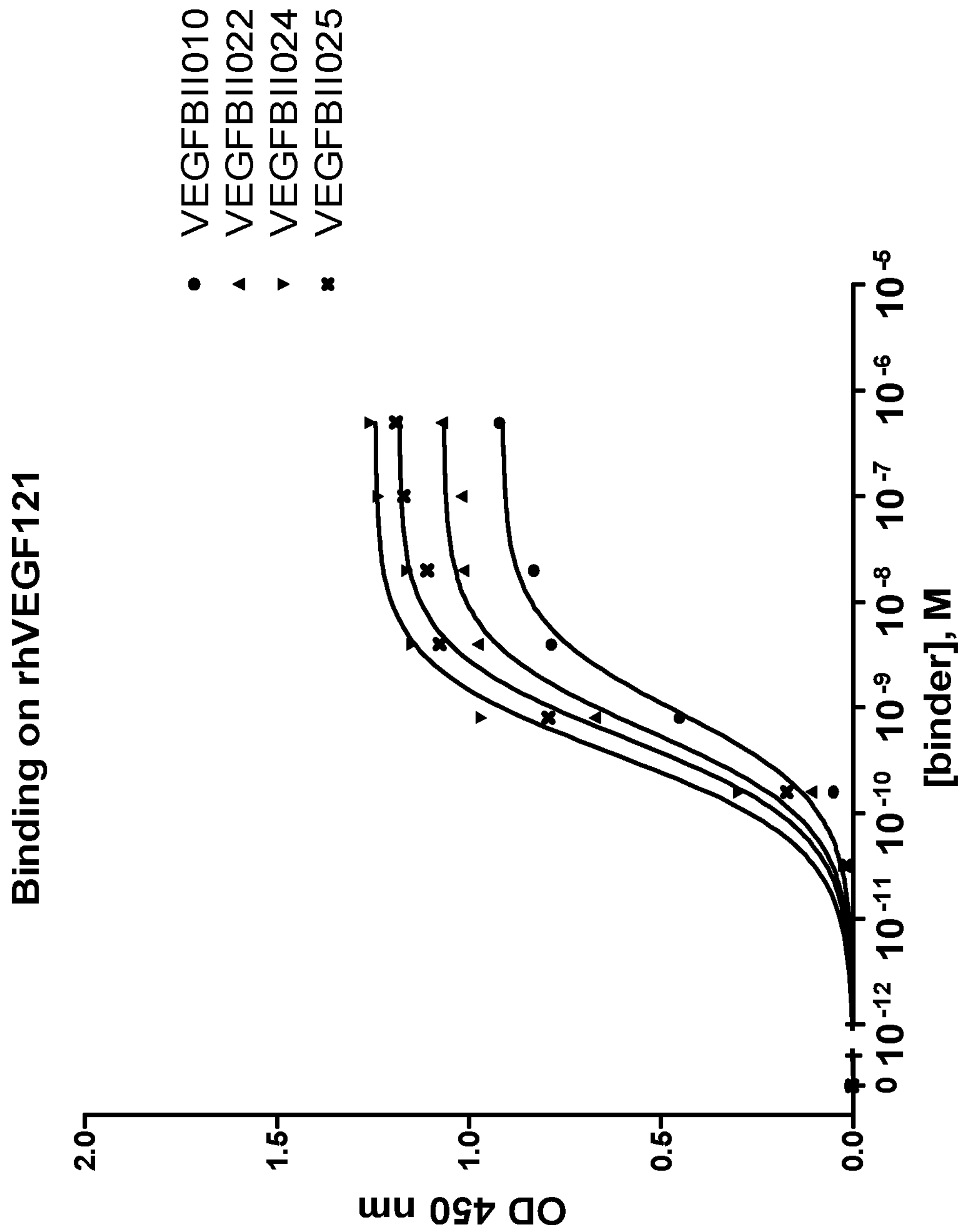

Fig. 16

```
                         10        20        30        40
Kabat#        :  .........|.........|.........|.........|
VH3-23/JH5    :  EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA
VEGFBII23B04  :  .............T.D......EV..R.....S.G.F...

                         50        60        70        80
Kabat#        :  .........|..a.......|.........|.........|
VH3-23/JH5    :  PGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYL
VEGFBII23B04  :  Q..ER.F.V...-K..YK.DSV.LE......K..A...V..

                            90       100                110
Kabat#        :  ..abc.......|.........|abcdefghi.........|...
VH3-23/JH5    :  QMNSLRAEDTAVYYCAK-----------------WGQGTLVTVSS
VEGFBII23B04  :  .I...KP.........SSRAYGSSRLRLADTYEY.....Q.....
```

Fig. 17

80000
60000
40000
20000
0

signal, cps

0 10^{-14} 10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}

[Competitor], (M)

23B4
Neg Control
VEGFBII111D05
VEGFBII111G06
VEGFBII112D11
VEGFBII113A08
VEGFBII113E03
VEGFBII114C09
VEGFBII114D02
VEGFBII114D03
VEGFBII118E10

Fig. 18

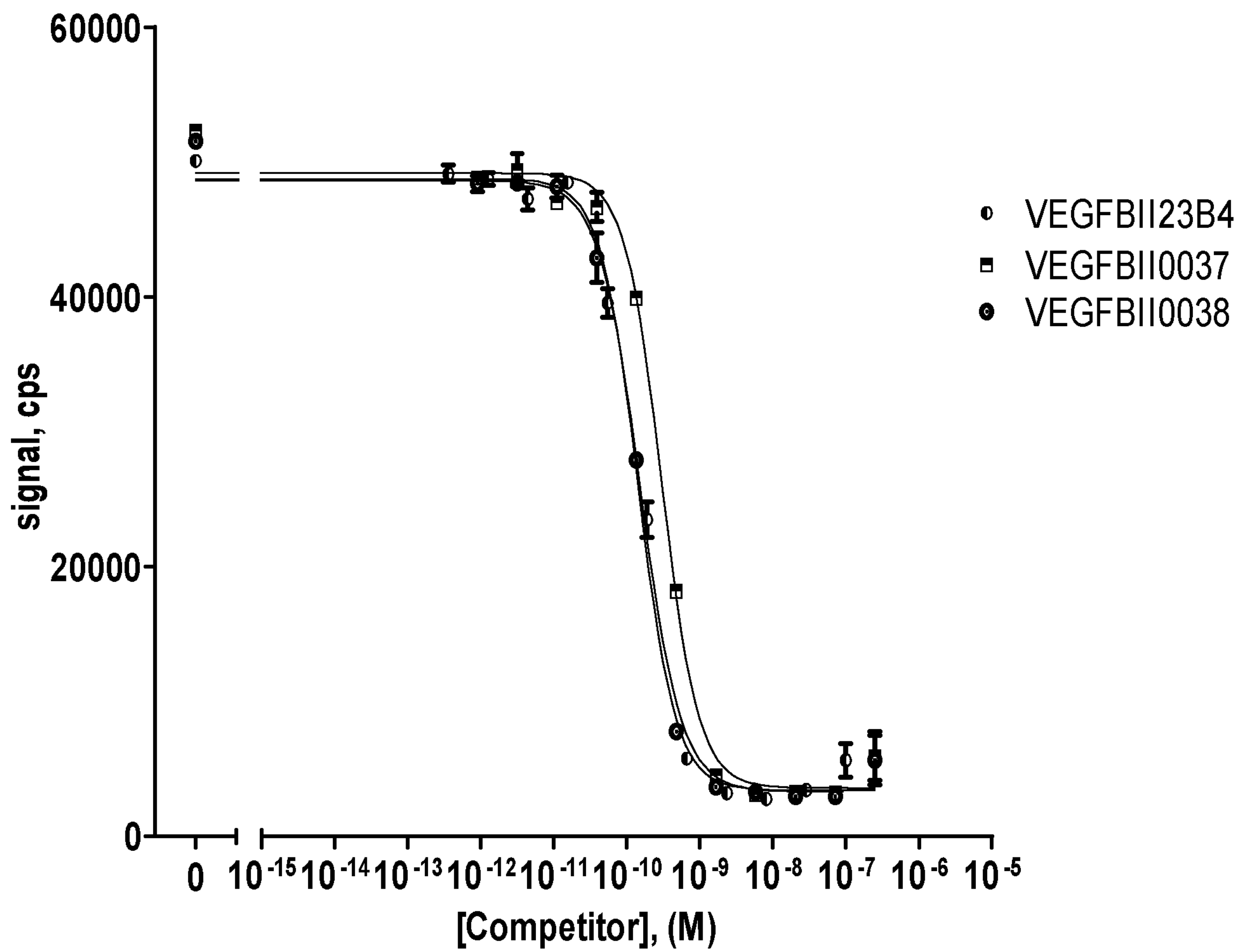

Fig. 19

```
                          10        20        30        40
Kabat#          : .........|.........|.........|.........|
VH3-23/JH5      : EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA
PVEGFBIIPMP5B5  : ......................V...IR.MSM.--.Y...

                          50         60        70        80
Kabat#          : .........|..a.......|.........|.........|
VH3-23/JH5      : PGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYL
PVEGFBIIPMP5B5  : ...HR.L.AR..-...T.A.V.................V..

                             90        101       110
Kabat#          : ..abc......|.........|........|...
VH3-23/JH5      : QMNSLRAEDTAVYYCAK-------WGQGTLVTVSS
PVEGFBIIPMP5B5  : .....KA........NTFSSRPNP..A..Q.....
```

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/065199

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/22
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/101985 A2 (ABLYNX NV [BE]; MERCHIERS PASCAL GERARD [BE]; VERHEESEN PETER [BE]; HO) 28 August 2008 (2008-08-28) cited in the application The whole document, in particular Example 8	1-24
X	US 2007/027102 A1 (GUYER DAVID R [US] ET AL) 1 February 2007 (2007-02-01) the whole document	21-24
X	US 6 884 879 B1 (BACA MANUEL [US] ET AL) 26 April 2005 (2005-04-26) the whole document	21-24

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 October 2011	22/11/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Chapman, Rob

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/133706 A2 (SCHERING CORP [US]; XOMA TECHNOLOGY LTD; RAMACHANDRA SUMANT [US]; BISH) 6 November 2008 (2008-11-06) the whole document -----	1-24
X	WO 2009/055343 A2 (SCHERING CORP [US]; XOMA TECHNOLOGY LTD; RAMACHANDRA SUMANT [US]; BISH) 30 April 2009 (2009-04-30) the whole document -----	1-24
T	WO 2010/124009 A2 (SCHERING CORP [US]; XOMA TECHNOLOGY LTD; RAMACHANDRA SUMANT [US]; HUAN) 28 October 2010 (2010-10-28) the whole document -----	1-24
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1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2011/065199

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-24(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-24(partially)

   A VEGF-binding molecule comprising at least one VHH as embodied by SEQ ID NO: 9

2-38. claims: 1-24(partially)

   A VEGF-binding molecule comprising at least one VHH as embodied by SEQ ID NO: 10-46

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/065199

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