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(54) Title: MODIFIED MULTIMERIC UBIQUITIN PROTEINS BINDING VEGF-A

(57) Abstract: The present invention refers to novel multimeric proteins obtained from modified ubiquitin capable of binding VEGF-A and VEGF-A isoforms with high affinity. The invention is further directed to the use of said proteins in medical diagnosis or treatment methods such as the treatment of cancer and eye diseases such as age-related macular degeneration.

MODIFIED MULTIMERIC UBIQUITIN PROTEINS BINDING VEGF-A

FIELD OF THE INVENTION

The present invention relates to novel and specific ubiquitin-based multimeric proteins capable of binding VEGF-A with high affinity. Furthermore, the invention refers to fusion proteins comprising said multimeric binding protein fused to a pharmaceutically or diagnostically active component. The invention is further directed to methods for the production and identifying of said multimeric binding protein or fusion protein and pharmaceutical or diagnostic compositions containing said multimeric VEGF-A binding proteins and fusion proteins or conjugates thereof.

In further embodiments, the invention is directed to polynucleotides coding for said multimeric binding protein or fusion protein or conjugate, vectors comprising said polynucleotide and host cells comprising said protein, fusion protein or conjugate, and/or polynucleotide. In a preferred embodiment, said multimeric binding protein or fusion protein or conjugate is included in a medicament or a diagnostic composition. Additionally, methods for using of said proteins in medical treatment methods such as treatment of cancer and eye diseases or in diagnostic methods are described.

BACKGROUND OF THE INVENTION

There is a growing demand for binding molecules consisting of amino acids which are not immunoglobulins. While until now antibodies represent the best-established class of binding molecules there is still a need for new binding molecules in order to target ligands with high affinity and specificity since immunoglobulin molecules suffer from major drawbacks. Although they can be produced quite easily and may be directed to almost any target, they have a quite complex molecular structure. There is an ongoing need to substitute antibodies by smaller molecules which can be handled in an easy way. These alternative binding agents can be beneficially used for instance in the medical fields of diagnosis, prophylaxis and treatment of diseases.

Small proteins having relatively defined 3-dimensional structures, commonly referred to as protein scaffolds, may be used as starting material for the design of said alternative binding

agents. These scaffolds typically contain one or more regions which are amenable to specific or random sequence variation, and such sequence randomisation is often carried out to produce a library of proteins from which the specific binding molecules may be selected. Molecules with a smaller size than antibodies and a comparable or even better affinity towards a target antigen are expected to be superior to antibodies in terms of pharmacokinetic properties and immunogenicity.

Specific targeting proteins based on modified ubiquitin

For example, WO 04/106368 describes the generation of artificial binding proteins on the basis of ubiquitin, also referred to as Affilin® (registered trademark of Scil Proteins GmbH). Ubiquitin is a small, monomeric, and cytosolic protein which is highly conserved among Eukaryota and is present in all known eukaryotic cells from protozoans to vertebrates. Ubiquitin is particularly characterized by beta sheets arranged in an antiparallel manner and subdivided into α and ß segments. A characteristic of ubiquitin protein thus is an antiparallel beta sheet exposed to one surface of the protein onto the back side of which an α helix is packed which lies perpendicularly on top of it. This ubiquitin-like folding motif clearly distinguishes ubiquitin from other proteins.

The polypeptide chain of ubiquitin consists of 76 amino acids (SEQ ID NO:1) folded in an extraordinary compact α/β structure (Vijay-Kumar, 1987 J. Mol. Biol. 194(3) 531-544): almost 87% of the polypeptide chain is involved in the formation of the secondary structural elements by means of hydrogen bonds. Secondary structures are three and a half alpha-helical turns as well as an antiparallel β sheet consisting of four strands. The characteristic arrangement of these elements - an antiparallel β sheet exposed on the protein surface onto the back side of which an alpha helix is packed which lies vertically on top of it - is generally considered as so-called ubiquitin-like folding motif. A further structural feature is a marked hydrophobic region in the protein interior between the alpha helix and the β sheet.

The amino acids of the four beta strands which contribute to the formation of the antiparallel beta sheet are according to the invention and according to the structure 1UBQ in the following amino acid positions of SEQ ID NO: 1: First strand (amino terminal): 2 to 7; second beta sheet strand: 12 to 16; third strand: 41 to 45; fourth strand (carboxy terminal): 65 to 71. The position of the strands if the sheet is viewed from the top (amino terminus at the

bottom, carboxy terminus on top) from left to right is: 2nd, 1st, 4th, 3rd strand wherein the polypeptide chain between the 1st and 4th strand forms the alpha helix.

Compared to antibodies or other alternative scaffolds, artificial binding proteins on the basis of ubiquitin proteins (also referred to as Affilin have many advantages: high target affinity and specificity, small size, high stability, and cost effective manufacturing. However, there is still a need to further develop those proteins in terms of new therapeutic approaches with high affinities to specific targets. While WO 05/05730 generally describes the use of ubiquitin scaffolds in order to obtain artificial binding proteins, no solution is provided on how to modify a ubiquitin protein in order to obtain a specific and high affinity directed against vascular epithelial growth factor-A (VEGF-A).

VEGF-A as specific target

In humans, multiple spliced isoforms of VEGF-A have been identified. The most common isoforms are composed of 121, 165 and 189 amino acids, and the murine homologues lack one amino acid per isoform (see Figure 1). The longer splice isoforms of VEGF-A, including VEGF165, contain a highly basic heparin-binding domain. This domain allows these isoforms to interact with and localize to the heparan sulfate (HS)-rich extracellular matrix, and bind to the co-receptor Nrp-1 (neuropilin-1).

Cellular responses to VEGF-A are primarily mediated by binding to two structurally similar VEGF receptors: VEGFR-1 (also referred to as Flt-1 or Fms-like tyrosine kinase 1) and VEGFR-2 (also referred to as KDR or kinase insert domain receptor/Flk-1 (fetal liver kinase 1).

Studies suggest a pathological role of VEGF-A in eye disorders such as retinal disorders. Several therapeutics are used in medical treatments. A therapeutic compound derived from a modified 2-fluoro pyrimidine acts as RNA inhibitor to VEGF and is used for the treatment of the wet form of age related macular degeneration (AMD) (for example, Pegatanib (Macugen®)). Another compound with antagonistic binding property to VEGF-A is used in the AMD treatment (Lucentis® (Ranibizumab)). In addition to treatments in eye disorders, VEGF-A antagonists are used for the treatment of cancer. The antibody Bevacizumab (Avastin®) which is inhibiting angiogenesis is approved for use against several cancers

including colon, lung, breast, and kidney cancers. For further details, reference is made to Krilleke et al., Biochem. Soc. Trans. (2009) 37, 1201–1206.

Designed repeat domains have been described for neutralizing VEGF. For example, WO2010060748 describes binding proteins wherein the designed proteins comprise a mutated ankyrin repeat domain to inhibit VEGF-A binding to VEGFR-2 (vascular epidermal growth factor receptor 2). Uses for developing protein therapeutics based on ankyrin repeat domains binding to VEGF-A for cancer and eye diseases are described.

WO2008015239 describes muteins derived from human tear lipocalin that bind to a short recombinant fragment of amino acids 8 to 109 of the mature VEGF-A polypeptide chain. Binding proteins based on lipocalin are mutated in specific positions in flexible, naturally occuring loop regions of lipocalin.

The above-discussed prior art documents describe the use of various protein scaffolds including antibodies to generate VEGF-A binding proteins. However, targeting VEGF-A with currently available compounds has certain disadvantages including toxicity, side effects, low affinity and a large size of the binding modules .

Technical problems underlying the present invention and their solution

Since cancer represents one of the leading causes for death worldwide, there is a growing need for improved agents for treating cancer. Current chemotherapeutic agents and radiation treatment suffer from poor selectivity due to an undirected cytotoxic mechanism of action. Most chemotherapeutic agents do not accumulate at the tumor site and thus fail to achieve adequate levels within the tumor. This results in significant side effects. Further, the toxicological profile of many chemotherapeutics limits dosing and thus the beneficial effect. Chemotherapeutic drugs, if given alone, often show poor tissue penetration and poor tumor uptake resulting in the accumulation of chemotherapeutic drugs in healthy tissue.

Targeting VEGF-A with currently available therapeutics is not effective in all patients. Drugs which are available on the market or in clinical development such as Avastin® (e.g. WO96/30046, WO98/45331, WO98/45332) or VEGF-Trap® (e.g. WO00/75319) show inhibition of angiogenesis but with cytotoxic and dose-limiting severe side effects (such as

bleeding, thromboembolic events, hypertension, gastrointestinal perforations and proteinuria) and low efficacy. Needless to say that thereis a strong medical need to effectively treat cancer with improved novel anti-angiogenic agents. There remains a strong need in the art for efficient tumor targeted therapeutics.

For severe eye pathologies such as age-related macular degeneration (AMD) or the common diabetes linked eye condition diabetic macular edema (DME) there is also a strong need to develop new drugs with an excellent tissue penetration, higher efficacies and less side effects.

It is thus an object of the present invention to provide novel targeted therapeutics for human diseases. In particular, it is an object to provide novel proteins which have high affinity to VEGF-A. The invention provides novel proteins which are advantageous as compared to antibodies or other scaffold proteins by their ability of high specific and high affinity binding to VEGF-A or its isoforms. It is a further object of the present invention to provide a method to generate and identify novel binding proteins with high binding specificity to VEGF-A or its isoforms, for example for use in the diagnosis and treatment of cancer or other diseases such as pathologic eye conditions. An advantage of this invention is to provide smaller molecules with high affinity towards the VEGF-A antigen. The proteins of the invention are expected to have significant advantages to antibodies or other antibody mimetics binding proteins.

The above-described objects are solved by the enclosed independent claims. Preferred embodiments of the invention are included in the dependent claims as well as in the following description, examples and figures. The above overview does not necessarily describe all problems solved by the present invention.

SUMMARY OF THE INVENTION

In a first aspect the present invention relates to a multimeric modified ubiquitin protein capable of binding VEGF-A or its isoforms, comprising at least two monomeric ubiquitin units linked together in a head-to-tail arrangement, wherein each monomeric unit of said multimeric protein is differently or identically modified at least by substitutions of at least 5 amino acids corresponding to and selected from positions 6, 8, 62, 63, 64, 65, 66 of SEQ ID NO: 1, and wherein optionally at least one monomeric ubiquitin unit contains an insertion of 2 - 15 amino acids within or in close proximity of 1 - 3 amino acids in direction of the N- or

C- terminus of said amino acid substitutions, and wherein said modified monomeric ubiquitin unit having an amino acid identity to SEQ ID NO: 1 of at least one of the group of at least 75%, at least 80%, at least 85%, and at least 90%, said protein having a specific binding affinity to said VEGF-A or its isoforms of Kd = 10^{-7} - 10^{-12} M.

In a second aspect the present invention relates to a fusion protein or conjugate comprising a protein according to the first aspect fused to or conjugated with a diagnostic component, wherein said diagnostically active component is optionally a fluorescent compound, a photosensitizer, or a radionuclide, or fused to or conjugated with a therapeutically or pharmaceutically active component, wherein said pharmaceuticallyor therapeutically active component is optionally a cytokine, a chemokine, a cytotoxic compound, or an enzyme, or a fusion protein or conjugate wherein said multimeric, preferably dimeric modified ubiquitin unit capable of binding VEGF-A or its isoforms is fused with or conjugated to a second multimeric, preferably dimeric modified ubiquitin with different target specificity.

In a third aspect the present invention relates to polynucleotide coding for a recombinant protein according to the first aspect or fusion protein or a conjugate according to the second aspect.

In a fourth aspect the present invention relates to a vector comprising a polynucleotide according to the third aspect.

In a fifth aspect the present invention relates to a host cell comprising a protein according to according to the first aspect or fusion protein or a conjugate according to the second aspect, a polynucleotide according to the third aspect and/or a vector according to the fourth aspect.

In a sixth aspect the present invention relates to the protein according to the first aspect or fusion protein according to the second aspect for use in medicine.

In a seventh aspect pharmaceutical or diagnostic composition comprising to the protein according to the first aspect or fusion protein according to the second aspect with one or more pharmaceutically acceptable carriers or excipients or with one or more diagnostically acceptable carrier.

In a eigth aspect the invention relates to a method for generating a multimeric modified ubiquitin protein according to the first aspect comprising the following steps:

a) providing a population of modified multimeric ubiquitin proteins originating from monomeric ubiquitin proteins, said population comprising multimeric ubiquitin proteins comprising differently or identically modified ubiquitin monomers linked together in a head-to-tail arrangement wherein each monomer of said multimeric protein is differently or identically modified at least by substitutions of at least 5 amino acids corresponding to and selected from positions 6, 8, 62, 63, 64, 65, and/or 66 of SEQ ID NO: 1 and wherein optionally at least one monomeric ubiquitin unit contains an insertion of 2 - 15 amino acids within or in close proximity of 1 - 3 amino acids in direction of the N- or C-terminus of said amino acid substitutions

b) providing VEGF-A or its isoforms as potential target,

c) contacting said population of modified proteins with VEGF-A;

d) identifying a modified multimeric ubiquitin protein by a screening process, wherein said modified multimeric ubiquitin protein binds to said target with a specific binding affinity of Kd in a range of 10^{-7} - 10^{-12} M, and optionally

e) isolating said modified multimeric ubiquitin protein with said binding affinity.

In a ninth aspect the present invention relates to a method for the preparation of a protein as defined in the first aspect, said method comprising the following steps:

(a) preparing a nucleic acid encoding a protein as defined in the first aspect

(b) introducing said nucleic acid into an expression vector;

(c) introducing said expression vector into a host cell;

(d) cultivating the host cell;

(e) subjecting the host cell to culturing conditions under which a fusion protein is expressed from said vector, thereby producing a fusion protein as defined in the first aspect;

(f) optionally isolating the fusion protein produced in step (e).

In a tenth aspect the present invention relates to a method of generating a fusion protein or a conjugate according to the second aspect wherein a protein according to the first aspect is fused with or conjugated to a diagnostic or pharmaceutical or therapeutical component.

This summary of the invention does not necessarily describe all features of the present invention. Other embodiments will become apparent from a review of the ensuing detailed description.

DETAILED DESCRIPTION OF THE INVENTION

Before the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

All sequences referred to herein are disclosed in the attached sequence listing that, with its whole content and disclosure, is a part of this specification.

More specifically, the invention provides a multimeric modified ubiquitin protein capable of binding VEGF-A or its isoforms, comprising at least two monomeric modified ubiquitin units linked together in a head-to-tail arrangement, wherein each monomeric unit of said multimeric protein is differently or identically modified or at least two monomeric ubiquitin units are differently or identically modified and one ubiquitin unit is unmodified, said modifications comprising at least substitutions of at least 5 amino acids corresponding to and selected from positions 6, 8, 62, 63, 64, 65, 66 of SEQ ID NO: 1, wherein optionally at least one monomeric ubiquitin unit contains an insertion of 2 - 15 amino acids amino acids, or 8 amino acids within or in close proximity of 1 - 3 amino acids in direction of the N- or C-terminus of said amino acid substitutions, said monomeric modified ubiquitin unit having an amino acid identity to SEQ ID NO: 1 of at least 75%, at least 80%, at least 85% or at least 90%, said protein having a specific binding affinity to said VEGF-A or its isoforms of Kd = 10^{-7} - 10^{-12} M.

In order to cover embodiments wherein the modifications are introduced into a ubiquitin protein which is not identical but similar to SEQ ID NO: 1, the term "corresponding to" has been used. In said not identical but similar ubiquitins the positions of amino acids specified herein might be different to SEQ ID NO: 1; nevertheless they can be allocated to those positions which are designated by the positions referring to SEQ ID NO: 1. "Not identical to but similar" describes e.g. ubiquitins which are of non-human origin or which are derived from SEQ ID NO: 1 and differ therefore in their amino acid sequence to. SEQ ID NO: 1.

The multimeric modified ubiquitin protein with binding capability to VEGF-A or its isoforms of the invention is recombinant.

In one embodiment, said multimeric ubiquitin protein is a dimeric or trimeric or tetrameric protein comprising two, three, or four differently or identically modified monomeric ubiquitin units.

In another embodiment, said monomeric units are differently or identically modified and/or wherein 6 - 10 amino acids or 7 - 9 amino acids or 8 amino acids are inserted in at least one monomeric ubiquitin protein, optionally wherein said multimeric protein comprises at least one additional unmodified monomeric ubiquitin unit.

In a preferred embodiment, substitutions in the N-terminal (first) ubiquitin monomer of the multimeric binding protein are L8D and are selected from at least K6Y or K6F or K6W.

In another embodiment of the invention, substitutions in the first monomer are at least K6Y, L8D, K63W, E64M, S65P, preferably K6Y, L8D, Q62S, K63W, E64M, S65P, and T66A wherein said insertion comprises or consists of the amino acid sequence DVAEYLGI (SEQ ID NO: 37).

In a preferred embodiment, the multimeric modified ubiquitin protein capable of binding VEGF-A or its isoforms, contains an insertion in at least one monomeric ubiquitin unit. It is also possible that two or more or all monomeric ubiquitin units contain each one identical or a different insertion. In a still further embodiment, 2 or 3 or 4 insertions are included in one or in several monomeric ubiquitin units. The total number of amino acids of all insertions is however limited by maintaining the structural integrity of the modified ubiquitin and its

binding capability to VEGF-A and its isoforms. At the most insertions of 15 amino acids may be tolerated by a monomeric ubiquitin, preferably 5-9 amino acids, most preferred 8 amino acids. The insertion is preferred within or in close proximity of 1 - 3 amino acids in direction of the N- or C-terminus of said amino acid substitutions. Preferably said insertion of amino acids is in one loop region of said modified monomeric ubiquitin. A loop region in ubiquitin refers to residues 7 - 11, 18 - 21, 37 - 40, 45 - 48, 51 - 54, 57 - 60, 62 - 65 of SEQ ID NO: 1, as further defined below. It is further preferred that the insertion of amino acids is closely adjacent, optionally 0, 1, 2, 3, 4, or 5 amino acids, distant from beta sheet strands, preferably distant from the fourth (C-terminal) or the first (N-terminal) beta-strand, optionally wherein said insertion is located in the N-terminal (first) ubiquitin monomer.

In further embodiments of the invention, 5, 6, or 7 of the amino acids corresponding to and selected from positions 6, 8, 62, 63, 64, 65, 66 of SEQ ID NO: 1 are modified in at least one monomeric ubiquitin unit. It is to be understood that the present invention allows a combination of each of these variations in each monomeric unit, i.e. in the first and the second and any further unit. For instance the first monomeric unit can comprise 5 modifications while the second unit comprises 6 or 7 modifications, the first unit may comprise 7 modifications and the second unit 5 modifications etc. Each of the amino acids listed above can be selected in the first and/or second unit or any further units which are then combined. Preferred substitutions and insertions are described herein below.

In a still further embodiment of the invention, the multimeric, preferably dimeric modified ubiquitin protein is fused with or conjugated to a modified ubiquitin protein capable of binding a target different from VEGF-A. The combination of two binding molecules based on modified ubiquitin molecules with different target specificities can be achieved preferably by fusion or conjugation, e.g. by using linkers. The resulting protein will be able to bind to two different target molecules providing therefore two different specificities. A further embodiment of the invention covers a fusion protein or conjugate comprising a multimeric VEGF-A binding protein claims fused to or conjugated with a diagnostic component, wherein said diagnostically active component is optionally a fluorescent compound, a photosensitizer, or a radionuclide, or fused to or conjugated with a therapeutically active component, wherein said pharmaceutically active component is optionally a cytokine, a chemokine, a cytotoxic compound, or an enzyme, or a fusion protein or conjugate wherein said multimeric, preferably dimeric modified ubiquitin unit capable of binding VEGF-A or its isoforms is fused with or

conjugated to a second multimeric, preferably dimeric modified ubiquitin with different target specificity.

Further embodiments cover a polynucleotide coding for a recombinant VEGF-A binding protein or fusion protein or a conjugate, a vector comprising said polynucleotide, a host cell comprising said VEGF-A binding protein said fusion protein or conjugate, said vector and/or a polynucleotide.

Further embodiments cover a pharmaceutical or diagnostic composition comprising a VEGF-A binding protein or a fusion protein or a conjugate with one or more pharmaceutically acceptable carriers or excipients or with one or more diagnostically acceptable carrier.

Even further embodiments cover a method of generating a fusion protein or a conjugate, wherein a VEGF-A binding protein is fused with or conjugated to a diagnostic component or a pharmaceutical component.

Another embodiment covers a recombinant VEGF-A binding protein or a fusion protein or conjugate for use in a method of medical treatment or diagnosis.

Definitions of important terms used in the application

The term “VEGF-A” or briefly designated as “VEGF” comprises all proteins which show a sequence identity to SEQ ID NO: 2 (FIGURE 1a; accession number P15692) of at least 70%, optionally 75%, further optionally 80%, 85%, 90%, 95%, 96% or 97% or more, or 100% and having the above defined functionality of VEGF. The term “VEGF-A” or briefly designated as “VEGF” also comprises isoforms of VEGF-A; well-known isoforms of VEGF-A are VEGF 121 und VEGF 165.

The terms "protein capable of binding" or "binding protein" according to this invention refer to an ubiquitin protein comprising a binding domain to VEGF-A as further defined below.

Any such binding protein based on ubiquitin may comprise additional protein domains that are not binding domains, such as, for example, multimerization moieties, polypeptide tags, polypeptide linkers and/or non- proteinaceous polymer molecules. Some examples of non-proteinaceous polymer molecules are hydroxyethyl starch, polyethylene glycol, polypropylene glycol, or polyoxyalkylene.

While antibodies and fragments thereof are well known to the person skilled in the art, the binding protein of the invention is **not** an antibody or a fragment thereof, such as Fab or scFv fragments. Further, the binding domain of the invention does not comprise an immunoglobulin fold as present in antibodies**.** The binding proteins of the invention comprise only modified multimeric ubiquitin-based proteins.

In the present specification, the terms "ligand" and "target" and "binding partner" are used synonymously and can be exchanged. A ligand or target is any molecule (here: VEGF-A and its isoforms) capable of binding with an affinity as defined herein to the homo- or hetero-multimeric modified ubiquitin protein.

The term "ubiquitin protein" covers the ubiquitin in accordance with SEQ ID NO: 1 and modifications thereof according to the following definition. Ubiquitin is highly conserved in eukaryotic organisms. For example, in all mammals investigated up to now ubiquitin has the identical amino acid sequence. Particularly preferred are ubiquitin molecules from humans, rodents, pigs, and primates. Additionally, ubiquitin from any other eukaryotic source can be used. For instance ubiquitin of yeast differs only in three amino acids from the wild-type human ubiquitin. Generally, the unmodified monomeric ubiquitin covered by said term "ubiquitin protein" shows an amino acid identity of at least 70%, preferably at least 75% or at least 80%, of at least 85%, of at least 90%, of at least 95%, of at least 96% or of at least 97% %, or of at least a sequence identity of 98% to SEQ ID NO: 1.

The phrase "a modified ubiquitin protein" refers to modifications of the monomeric ubiquitin protein as defined in the previous paragraph by any one of substitutions, insertions or deletions of amino acids or a combination thereof while substitutions are the most preferred modifications which may be supplemented by any one of the modifications described above. The number of modifications is strictly limited as each of said modified monomeric ubiquitin units has an amino acid identity to SEQ ID NO: 1 of at least one of the group of 80%, at least

83%, at least 85%, at least 83% and at least 90%. At the most, the overall number of substitutions in a monomeric unit is, therefore, limited to 16 amino acids corresponding to 80% amino acid identity taking into account only substitutions and deletions. This calculation includes insertions related to the total amino acid identity, said identity to SEQ ID NO: 1 may be between 75% and 90%. The total number of modified amino acids in the multimeric ubiquitin molecule may be up to 32 amino acids corresponding to about 20% amino acid modifications based on the unmodified dimeric ubiquitin protein. The amino acid identity of the dimeric modified ubiquitin protein compared to a dimeric unmodified ubiquitin protein with a basic monomeric sequence of SEQ ID NO: 1 is selected from at least one of the group of at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89% and at least 90%.

The term "loop" or "loop region" refers to regions of non-repetitive conformations connecting regular secondary structure elements such as alpha-helix or beta-strands of ubiquitin. The structure of human ubiquitin reveals 7 reverse turns (loops) which connect secondary structure elements: 7 - 11, 18 - 21, 37 - 40, 45 - 48, 51 - 54, 57 - 60, 62 - 65 (Vijay-Kumar *et al.* 1987 J. Mol. Biol.;194(3):531-44).

The term "insertions" comprises the addition of amino acids to the original amino acid sequence of ubiquitin wherein the ubiquitin remains stable without significant structural change. The invention covers insertions of 2 - 15 amino acids preferably in the VEGF-A binding region of the ubiquitin monomers. Specifically, the number of amino acids to be inserted is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15. One embodiment of the invention shows an insert of 8 amino acids of the N-terminal (first) ubiquitin monomer (e.g. see SEQ ID NO: 37). Most likely, the insertion of the additional 2 - 15 amino acids, preferably 8 amino acids, insertion is located in loop-structures of ubiquitin. The insertion is generally not located in a beta sheet but adjacent to beta sheets, optionally 0, 1, 2, 3, 4, or 5 amino acids, distant from beta sheets. It is preferred that the insertion is 0, 1, 2, 3, 4, or 5 amino acids distant from the fourth (C-terminal) or the first (N-terminal) beta-strand. In one embodiment of the invention, an insertion of additional amino acids is before (position 61 - 62) or within the loop region 62-64 (positions 62 – 63, 63 – 64, 64 – 65) which is adjacent to the C-terminal beta sheet. The insertion is further adjacent to the substituted amino acids. Thus, the insertion of amino acids is preferred between amino acids corresponding to 61 - 62 or 62 - 63 or 63 - 64 or 64 - 65 of human ubiquitin of SEQ ID NO: 1. The insertion is most preferred between amino

acid residues corresponding to amino acids 61 - 62 or 62 - 63 or 63 - 64 or 64 - 65 of SEQ ID NO: 1 or 0, 1, 2, 3, 4, or 5 amino acids distant from beta sheets, in particular adjacent to the C-terminal beta sheet.

Preferably, there is only one insertion in one monomeric ubiquitin unit. Said insertion which is preferably in the N-terminal (first) monomeric ubiquitin unit may participate in the binding of the modified ubiquitin to VEGF-A. A further positive effect of the insertion is an increase of the number of amino acids which may be substituted and may therefore participate in binding to the target VEGF-A and its isoforms. The insert itself may optionally form a loop structure. In spite of the insertion, the modified ubiquitin remains soluble (data not shown).

For determining the extent of sequence identity of a derivative of the ubiquitin to the amino acid sequence of SEQ ID NO: 1, for example, the SIM Local similarity program (Xiaoquin Huang and Webb Miller, " Advances in Applied Mathematics, vol. 12: 337- 357, 1991) or Clustal, W. can be used (Thompson et al., Nucleic Acids Res., 22(22): 4673-4680, 1994.). The extent of the sequence identity of the modified protein to SEQ ID NO: 1 as defined herein is determined relative to the complete sequence of SEQ ID NO: 1.

The “multimeric fusion protein” or “multimeric protein” of the invention is considered as a protein which comprises homo-multimers and hetero-multimers. In one embodiment, at least two differently modified monomeric ubiquitin proteins with two binding domain regions providing together a specific binding property (binding domain) for VEGF-A as the specific binding partner. A hetero-dimer is accomplished by fusing two monomeric ubiquitin molecules wherein both of these molecules are differently modified as described herein. The “homo-multimeric fusion protein” or “homo-multimeric protein” of the invention is considered as a protein which comprises at least two identically modified monomeric ubiquitin proteins with two binding domain regions providing together a binding property (binding domain) for VEGF-A as the specific binding partner. A homo-dimer is accomplished by fusing two monomeric ubiquitin molecules wherein both of these molecules are identically modified as described herein. Preferred are dimeric or tetrameric proteins or multimers thereof. Examples are: A-A (Homo-Dimer), A-B (Hetero-Dimer), A-B-A-B, A-A-A-B, A-B-A-A, A-A-A-A, etc.

An advantage of multimerization, for example dimerization, of differently or identically modified ubiquitin monomers in order to generate multimeric binding proteins (here: hetero- or homo-dimeric proteins) with binding activity lies in the increase of the total number of amino acid residues that can be modified or in multimerization of the binding region to generate a new high affinity binding property to VEGF-A. The main advantage is that while even more amino acids are modified, the protein-chemical integrity is maintained without decreasing the overall stability of the scaffold of said newly created binding protein to VEGF-A. The total number of residues which can be modified in order to generate a novel binding site for VEGF-A is increased as the modified residues can be allocated to two monomeric ubiquitin proteins. The number of modifications can so be both of SEQ ID NO: 1 multiplied and allotted to different monomeric molecules of ubiquitin corresponding to the number of modified monomeric ubiquitin molecules. A modular structure of the ubiquitin-based VEGF-A binding protein allows increasing the overall number of modified amino acids as said modified amino acids are included on e.g. two monomeric ubiquitin molecules.

Thus, the use of hetero-dimers binding to the binding partner VEGF-A opens up the possibility to introduce an increased number of modified residues which do not unduly influence the protein-chemical integrity of the final binding molecule, since the overall amount of those modified residues is distributed over the e.g. two monomeric units which form the dimer. Said hetero-dimeric modified ubiquitin proteins binding to VEGF-A are present in a library of proteins. . In one embodiment of the invention the monomeric proteins are fused to each other. The dimerized molecules can be used for further multimerization. The comments provided for dimerized ubiquitin molecules are *mutatis mutandis* also valid for higher multimerized molecules. Thus, the homo- or hetero-dimers binding to VEGF-A can be used for further multimerization. Thus, the dimeric protein can be further multimerised with the same dimeric protein or with a different dimeric protein. The different dimeric protein can have other specificities than the first dimeric protein. Preferred are dimeric or tetrameric proteins or multimers thereof. Examples are: A-A (Homo-Dimer), A-B (Hetero-Dimer), A-B-A-B, A-A-A-B, A-B-A-A, A-A-A-A, A-B-C-D, A-A-C-D. The constructs A-B-C-D and A-A-C-D can be bi-specific binding proteins with specificity for VEGF-A or isoforms and to a different target.

According to the invention, the at least two differently or identically modified ubiquitin monomers which bind to one ligand are to be linked by head-to-tail fusion to each other using

e.g. genetic methods. A “binding domain region” is defined herein as region on a ubiquitin monomer that has modified amino acids selected from positions corresponding to amino acids 6, 8, 62, 63, 64, 65, 66 of SEQ ID NO: 1 which are involved in binding the target VEGF-A.

A "head-to-tail fusion" is to be understood as fusing two proteins together by connecting them in the direction N-C-N-C- depending on the number of units contained in the dimer. In this head-to-tail fusion, the ubiquitin monomers may be connected directly without any linker. Alternatively, the fusion of ubiquitin monomers can be performed via linkers, for example, a polypeptide linker.

The “polypeptide linker” is an amino acid sequence which is able to link two ubiquitin monomers. As used herein, the term "linker" refers to a molecule that joins at least two other molecules either covalently or noncovalently, e.g., through hydrogen bonds, ionic or van der Waals interactions, e.g., a nucleic acid molecule that hybridizes to one complementary sequence at the 5’ end and to another complementary sequence at the 3’ end, thus joining two non-complementary sequences. A “linker” is to be understood in the context of the present application as a moiety that connects a first polypeptide with at least a further polypeptide. The second polypeptide may be the same as the first polypeptide or it may be different.

Preferred herein are peptide linkers. This means that the peptide linker is an amino acid sequence that connects a first polypeptide with a second polypeptide. The peptide linker is connected to the first polypeptide and to the second polypeptide by a peptide bond. Typically, a peptide linker has a length of between 1 and 20 amino acids; e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids. It is preferred that the amino sequence of the peptide linker is not immunogenic to human beings. An example of such linker is a glycine-serine-linker of variable length, for example, having at least the amino acid sequence GIG or having at least the amino acid sequence SGGGG, for example GIG (SEQ ID NO: 31), SGGGG (SEQ ID NO: 32), SGGGGIG (SEQ ID NO: 33), SGGGGSGGGGIG (SEQ ID NO: 34) or SGGGGSGGGG (SEQ ID NO: 35) or any other peptide linker can be used in the present invention. In generell, linkers of the structure (SGGG)n can be used wherein n can be any number between 1 and 6, preferably 1 or 2.The linkers may have a length between 2 and 16 amino acids. Also other linkers for the genetic fusion of two ubiquitin monomers are known in the art and can be used.

The modified ubiquitin proteins of the invention are engineered, artificial proteins with novel binding affinities to VEGF-A. This means that the binding affinity to a target was created de novo by substituting certain amino acids in wild-type ubiquitin. After substituting 1- 8 amino acids in a ubiquitin monomer and optionally inserting 1- 8 amino acids in at least one monomer and linking two modified ubiquitin monomers, these novel artificial protein – heterodimeric ubiquitin – has binding capabilities that did not exist before. The term "substitution" comprises also the chemical modification of amino acids by e.g. substituting or adding chemical groups or residues to the original amino acid. The substitution of surface-exposed amino acids is crucial.

The substitution of amino acids for the generation of the novel binding domain specific to the VEGF-A can be performed according to the invention with any desired amino acid, i.e. for the modification to generate the novel binding property to VEGF-A; it is not mandatory to take care that the amino acids have a particular chemical property or a side chain, respectively, which is similar to that of the amino acids substituted so that any generally amino acid desired can be used for this purpose provided it enhances the binding affinity to VEGF-A and does not deteriorate the structural integrity of the ubiquitin binding molecule.

In a further embodiment, the amino acid substitutions specifically defined herein are changed by other amino acids with similar chemical properties, so called "conservative substitutions". These substitutions are shown in the table below:

Ala, Val, Leu, Ile, Met, Pro, Phe, Trp: Amino acids with aliphatic hydrophobic side chains
Ser, Tyr, Asn, Gln, Cys: Amino acids with uncharged but polar side chains
Asp, Glu: Amino acids with acidic side chains
Lys, Arg, His: Amino acids with basic side chains
Gly: Neutral side chain

The step of modification of the selected amino acids is performed according to the invention preferably on the genetic level by random mutagenesis, i.e. a random substitution of the selected amino acids. Preferably, the modification of ubiquitin is carried out by means of methods of genetic engineering for the alteration of a DNA belonging to the respective protein. Preferably, expression of the ubiquitin protein is then carried out in prokaryotic or eukaryotic organisms.

In preferred embodiments, the amino acid residues are altered by amino acid substitutions. However, also insertions and deletions are allowable. The number of amino acids which may be added (inserted) is limited to 2 – 15 amino acids in a monomeric ubiquitin subunit, and accordingly 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 amino acids, preferably 5 - 12 amino acids, preferably 7 - 10 amino acids, most preferred 8 amino acids. In one embodiment, amino acid insertions are made in one monomeric subunit of the dimeric VEGF-A ubiquitin based binding protein. It is preferred that the insertion is made within the first (N-terminal) ubiquitin monomer of the hetero-dimeric VEGF-A binding protein. It is further preferred that the insertion is made closely adjacent to the substituted amino acids. It is more preferred that the insertion is close to the fourth beta sheet of ubiquitin in the C-terminal part of the protein. Some exemplary preferred positions for insertion of amino acids are for example positions corresponding to human ubiquitin positions 61 - 62 or 62 - 63 or 63 - 64 or 64 - 65 but also positions 9 - 10, positions 35 - 36, or positions 46 – 47 could be suitable locations for an insertion. However, other positions are possible for insertion of 2 to 15 amino acids in order to generate a binding protein with high affinity to VEGF-A. In one embodiment, no amino acid insertions are made. The number of amino acids to be deleted is 1, 2, 3, 4 or 5 amino acids relating to the monomeric ubiquitin molecule. In a still further embodiment, no deletions have been performed.

Provided that the modified ubiquitin protein of the present invention comprises additionally to said substitutions specified in the claims and explained herein also insertions or deletions of one or more amino acids or provided that e.g. non-human ubiquitin is used as starting protein like ubiquitin of yeast, the amino acid positions given for wild type human ubiquitin (SEQ ID NO: 1) have to be aligned with the modified ubiquitin in order to allot the corresponding proteins and amino acid positions to each other. The numbering (and alignment) of each of the monomeric ubiquitin subunits is done in the same way, i.e. an alignment of, for example, a dimer is started at amino acid position 1 for each respective subunit.

The degree of modification of a modified monomeric ubiquitin according to the invention used as building unit for a hetero- or homo-dimer accounts for minimal 5% to a total up to about 25% or 20% of amino acids (corresponding to minimal about 4 to in total up to about 14 to 19 amino acid residues changed). Considering this, there is a sequence identity to SEQ ID NO: 1 of the modified monomeric ubiquitin protein of at least 75%, in particular if

substitutions and insertions are generating the novel binding property. In further embodiments of the invention, the sequence identity on amino acid level is at least 80%, at least 83%, at least 85%, at least 87% and furthermore at least 90% at least 92% or at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, in particular if only substitutions are made in the ubiquitin monomer. The invention covers also amino acid sequence identities of more than 97% of the modified ubiquitin protein compared to the amino acid sequence of SEQ ID NO: 1.

In a further embodiment of the invention, each of two ubiquitin monomers is a least substituted in 5 or 6 or 7 amino acids selected from and corresponding to positions 6, 8, 62, 63, 64, 65, 66 of SEQ ID NO: 1 and additionally 2-15 amino acids are inserted at position 61-62 of the first (N-terminal) monomer, thus in close proximity to said substituted amino acids. In another embodiment, the ubiquitin monomers to be modified in these positions were already pre-modified which does not effect the binding of targets. For example, further modifications could comprise substitutions at amino acids 75 and 76 or at amino acid 45 to generate better stability or protein-chemical properties. A pre-modified ubiquitin monomer that is particularly well-suited for practicing the present invention might be SEQ ID NO: 38:

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSDY NIQKESTLHLVLRLRAA

A modified ubiquitin monomer is obtainable wherein at least 5 amino acids, but in total up to 9, 10, 11, 12, 13, 14, 15 and a maximum of 16 amino acids, most preferred 5 to 9 amino acids, of the monomeric ubiquitin of SEQ ID NO: 1 are substituted. Additional 2 - 15 amino acids can be inserted into the sequence. According to one embodiment, a modified monomeric ubiquitin could be obtained having 9 substitutions being involved in novel binding to a target and an insertion of 8 amino acids (for example, further 3 amino acids can be modified that does not affect the binding). Based on the total number of amino acids of ubiquitin this corresponds to a percentage of all modifications of about 26% (modifications involved in binding: about 22%). This was extraordinarily surprising and could not be expected since usually a much lower percentage is already sufficient to disturb the folding of the protein.

For the mutagenesis of surface exposed amino acids, these can be identified with respect to the available X-ray crystallographic structure or NMR structure data or related. If no crystal structure is available attempts can be made by means of computer analysis to predict surface-

exposed amino acids and the accessibility of individual amino acid positions with respect to the available primary structure or to model the 3D protein structure and to obtain information about potential surface-exposed amino acids in this manner. Further disclosure thereof can be taken e.g. from Vijay-Kumar et al 1987 J. Mol. Biol., 194(3):531-44.

During the modelling process, surface exposed amino acid positions to be mutagenized are subjected to random mutagenesis and are afterwards re-integrated into the DNA coding for the protein from which they were removed previously. This is followed by a selection process for mutants with the desired binding properties. "Surface-exposed amino acids" are amino acids that are accessible to the surrounding solvent. If the accessibility of the amino acids in the protein is more than 8% compared to the accessibility of the amino acid in the model tripeptide Gly-X-Gly, the amino acids are called "surface-exposed". These protein regions or individual amino acid positions, respectively, are also preferred binding sites for potential binding partners for which a selection shall be carried out according to the invention. In addition, reference is made to Caster et al., 1983 Science, 221, 709 - 713, and Shrake & Rupley, 1973 J. Mol. Biol. 79(2):351-371, which for complete disclosure are incorporated by reference in this application.

In another embodiment of the invention the amino acid positions to be mutagenized within these selected regions are identified. The amino acid positions selected in this way can then be mutagenized on the DNA level either by site-directed mutagenesis, i.e. a codon coding for a specific amino acid is substituted by a codon encoding another previously selected specific amino acid, or this substitution is carried out in the context of a random mutagenesis wherein the amino acid position to be substituted is defined but not the codon encoding the novel, not yet determined amino acid.

Variations of ubiquitin protein scaffold differing by amino acid substitutions or optionally additions amino acid insertions or additionally deletions in the region of the de novo generated artificial binding site from the parental protein and from each other can be generated by a targeted mutagenesis of the respective sequence segments. In this case, amino acids having certain properties such as polarity, charge, solubility, hydrophobicity or hydrophilicity can be replaced or substituted, respectively, by amino acids with respective other properties. Besides substitutions, the terms "mutagenesis" and "modified" and "replaced" comprise also insertions and deletions. On the protein level the modifications can

also be carried out by chemical alteration of the amino acid side chains according to methods known to those skilled in the art.

Methods of mutagenesis of ubiquitin

As a starting point for the mutagenesis of the respective sequence segments, for example the cDNA of ubiquitin which can be prepared, altered, and amplified by methods known to those skilled in the art can be used. For site-specific alteration of ubiquitin in relatively small regions of the primary sequence (about 1-3 amino acids) commercially available reagents and methods are on hand ("Quik Change", Agilent; "Mutagene Phagemid in vitro Mutagenesis Kit", Bio-Rad). For the site-directed mutagenesis of larger regions specific embodiments of e.g. the polymerase chain reaction (PCR) are available to those skilled in the art. For this purpose a mixture of synthetic oligodeoxynucleotides having degenerated base pair compositions at the desired positions can be used for example for the introduction of the mutation. This can also be achieved by using base pair analogs which do not naturally occur in genomic DNA, such as e.g. inosine. Starting point for the mutagenesis of can be for example the cDNA of ubiquitin or also the genomic DNA. Furthermore, the gene coding for the ubiquitin protein can also be prepared synthetically. Different methods known *per se* are available for mutagenesis including methods for site-specific mutagenesis, methods for random mutagenesis, mutagenesis using PCR or similar methods.

In a preferred embodiment of the invention the amino acid positions to be mutagenized are predetermined. The selection of amino acids to be modified is carried out to meet the limitations of present claim 1 with respect to those amino acids which have to be modified. In each case, a library of different mutants is generally established which is screened using methods known *per se*. Generally, a pre-selection of the amino acids to be modified can be particularly easily performed as sufficient structural information is available for the ubiquitin protein to be modified.

Methods for targeted mutagenesis as well as mutagenesis of longer sequence segments, for example by means of PCR, by chemical mutagenesis or using bacterial mutator strains also belong to the prior art and can be used according to the invention.

In one embodiment of the invention the mutagenesis is carried out by assembly of DNA oligonucleotides carrying the amino acid codon NNK. It should be understood, however, that also other codons (triplets) can be used. The mutations are performed in a way that the beta sheet structure is preferably maintained. Generally, the mutagenesis takes place on the outside of a stable beta sheet region exposed on the surface of the protein. It comprises both site-specific and random mutagenesis. Site-specific mutagenesis comprising a relatively small region in the primary structure (about 3-5 amino acids) can be generated with the commercially available kits of Agilent® (QuikChange®) or Bio-Rad® (Mutagene® phagemid in vitro mutagenesis kit) (cf. US 5,789,166; US 4,873,192).

If more extended regions are subjected to site-specific mutagenesis a DNA cassette must be prepared wherein the region to be mutagenized is obtained by the assembly of oligonucleotides containing the mutated and the unchanged positions (Nord et al., 1997 Nat. Biotechnol. 8, 772-777; McConell and Hoess, 1995 J. Mol. Biol. 250, 460-470.). Random mutagenesis can be introduced by propagation of the DNA in mutator strains or by PCR amplification (error-prone PCR) (e.g. Pannekoek et al., 1993 Gene 128, 135 140). For this purpose, a polymerase with an increased error rate is used. To enhance the degree of the mutagenesis introduced or to combine different mutations, respectively, the mutations in the PCR fragments can be combined by means of DNA shuffling (Stemmer, 1994 Nature 370, 389-391). A review of these mutagenesis strategies with respect to enzymes is provided in the review of Kuchner and Arnold (1997) TIBTECH 15, 523-530. To carry out this random mutagenesis in a selected DNA region also a DNA cassette must be constructed which is used for mutagenesis.

Random modification is performed by methods well-established and well-known in the art. A "randomly modified nucleotide or amino acid sequence" is a nucleotide or amino acid sequence which in a number of positions has been subjected to insertion, deletion or substitution by nucleotides or amino acids, the nature of which cannot be predicted. In many cases the random nucleotides (amino acids) or nucleotide (amino acid) sequences inserted will be" completely random" (e. g. as a consequence of randomized synthesis or PCR-mediated mutagenesis). However, the random sequences can also include sequences which have a common functional feature (e. g. reactivity with a ligand of the expression product) or the random sequences can be random in the sense that the ultimate expression product is of completely random sequence with e. g. an even distribution of the different amino acids.

In order to introduce the randomized fragments properly into the vectors, it is according to the invention preferred that the random nucleotides are introduced into the expression vector by the principle of site directed PCR-mediated mutagenesis. However, other options are known to the skilled person, and it is e. g. possible to insert synthetic random sequence libraries into the vectors as well.

To generate mutants or libraries by fusion PCR, for example three PCR reactions may be carried out. Two PCR reactions are performed to generate partially overlapping intermediate fragments. A third PCR reaction is carried out to fuse the intermediate fragments.

The method for construction of the library or mutant variants may include constructing a first set of primers around a desired restriction site (restriction site primer), a forward and reverse restriction primer and a second set of primers, e. g., upstream and downstream of the codon of interest (the mutagenic primers), a forward and reverse mutagenic primer. In one embodiment, the primers are constructed immediately upstream and downstream respectively of the codon of interest. The restriction and mutagenic primers are used to construct the first intermediate and second intermediate fragments. Two PCR reactions produce these linear intermediate fragments. Each of these linear intermediate fragments comprises at least one mutated codon of interest, a flanking nucleotide sequence and a digestion site. The third PCR reaction uses the two intermediate fragments and the forward and reverse restriction primers to produce a fused linear product. The opposite, here to for unattached ends of the linear product are digested with a restriction enzyme to create cohesive ends on the linear product. The cohesive ends of the linear product are fused by use of a DNA ligase to produce a circular product, e. g. a circular polynucleotide sequence.

To construct the intermediate fragments, the design and synthesis of two sets of forward and reverse primers are performed, a first set containing a restriction enzyme digestion site together with its flanking nucleotide sequence, and the second set containing at least one variant codon of interest (mutagenic primer). Those skilled in the art will recognize that the number of variants will depend upon the number of variant amino acid modifications desired. It is contemplated by the inventor that if other restriction enzymes are used in the process, the exact location of this digestion site and the corresponding sequence of the forward and reverse

primers may be altered accordingly. Other methods are available in the art and may be used instead.

Apart from having the randomized fragment of the expression product introduced into a scaffold in accordance with the present invention, it is often necessary to couple the random sequence to a fusion partner by having the randomized nucleotide sequence fused to a nucleotide sequence encoding at least one fusion partner. Such a fusion partner can e. g. facilitate expression and/or purification/isolation and/or further stabilization of the expression product.

Random substitution or insertion of amino acids according to one example of the present invention of amino acids selected from positions 6, 8, 62, 63, 64, 65, 66 of monomeric ubiquitin can be performed particularly easily by means of PCR since the positions mentioned are localized close to the amino or the carboxy terminus of the protein. Accordingly, the codons to be manipulated are at the 5' and 3' end of the corresponding cDNA strand. Thus, the first oligodeoxynucleotide used for a mutagenic PCR reaction apart from the codons at positions 6, and/or 8 to be mutated - corresponds in sequence to the coding strand for the amino terminus of ubiquitin. Accordingly, the second oligodeoxynucleotide - apart from the codons of positions 62, 63, 64, 65, and/or 66 to be mutated - at least partially corresponds to the non-coding strand of the polypeptide sequence of the carboxy terminus. By means of both oligodeoxynucleotides a polymerase chain reaction can be performed using the DNA sequence encoding the monomeric ubiquitin as a template.

Furthermore, the amplification product obtained can be added to another polymerase chain reaction using flanking oligodeoxynucleotides which introduce for example recognition sequences for restriction endonucleases. It is preferred according to the invention to introduce the gene cassette obtained into a vector system suitable for use in the subsequent selection procedure for the isolation of ubiquitin variations having binding properties to a predetermined non-natural target, like VEGF-A or isoforms of VEGF-A.

Regions to be modified in ubiquitin

The regions for modification can be basically selected as to whether they can be accessible for VEGF-A as binding partner and whether the overall structure of the protein will presumably show tolerance to a modification.

Particularly preferred is a substitution of at least 5 of the surface-exposed amino acids in regions 2 to 8 and 62 to 68, in particular selected from amino acid positions 6, 8, 62, 63, 64, 65, 66 of SEQ ID NO: 1 or SEQ ID NO: 38 or of those amino acids corresponding to these positions of a ubiquitin monomer, preferably mammalian (human) ubiquitin. Optionally 5, 6, 7 of said amino acid residues are modified per monomer, optionally in combination with additional amino acid residues, such as an insertion of for example 2 to 15 amino acids, preferably 8 amino acids.

After having made the modifications above, the inventors have found multimeric modified ubiquitin amino acid sequences as described in the examples which bind VEGF-A with very high affinity.

Fusion proteins and protein conjugates

In another preferred embodiment, the invention relates to a fusion protein comprising a binding protein of the invention fused with or conjugated to a therapeutically or diagnostically active component.

The term "fusion protein" relates to a fusion protein comprising a binding or non-binding protein of the invention fused to a functional or an effector component. In one embodiment, the invention relates to a fusion protein comprising a hetero-dimeric binding protein of the invention as targeting moiety fused to a functional or an effector domain. In a still further aspect, the invention relates to a fusion protein or conjugate comprising a multimeric modified ubiquitin binding protein of the invention fused with or conjugated to a diagnostically or therapeutically active component. A fusion protein or conjugate of the invention may comprise non-polypeptide components, e.g. non-peptidic linkers, non-peptidic ligands, e.g. therapeutically or diagnostically relevant radionuclides. It may also comprise small organic or non-amino acid based compounds, e.g. a sugar, oligo- or polysaccharide, fatty acid, etc. Methods for covalently and non-covalently attaching a protein of interest to a support are well known in the art, and are thus not described in further detail here.In one

preferred embodiment of the invention, the multimeric ubiquitin-based VEGF-A binding molecule is covalently or non-covalently conjugated to or fused with a protein or peptide or chemical compound having therapeutically or diagnostically relevant properties.

One embodiment of the invention covers a fusion protein or a conjugate comprising a dimeric modified ubiquitin protein fused with or conjugated to a pharmaceutically or diagnostically active component, wherein said pharmaceutically active component is optionally a cytokine, a chemokine, a cytotoxic compound, an ubiquitin-based binding protein or an enzyme, or wherein said diagnostically active component is selected from a fluorescent compound, a photosensitizer, or a radionuclide.

The term "conjugate" as is used herein describes a multimeric modified ubiquitin which is attached either by covalent bonds or by inter-molecular interactions to a therapeutically or diagnostically molecule, e.g. a protein or a non-protein chemical substance by chemical or other suitable methods. The conjugate molecule can be attached e.g. at one or several sites through a peptide linker sequence or a carrier molecule.

The term "fusion" as is used herein describes a multimeric modified ubiquitin which is fused either by covalent bonds or by molecular interactions with a therapeutically or diagnostically molecule, e.g. a protein or a non-protein chemical substance. Fusion with other protein or peptide molecules preferably takes place by genetic means. However, there is no sharp border line limiting the term "fusion" and "conjugate" so that both may overlap; for these reasons, both terms are used interchangeably.

The following overview gives some examples on how to obtain ubiquitin-based fusion proteins with VEGF-A binding capacity:

a) conjugation of the protein via Lysine residues present in ubiquitin;
b) conjugation of the heterodimeric ubiquitin-based binding protein via Cysteine residues – can be located C-terminal, or at any other position (e.g. amino acid residue 24 or 57); conjugation with maleimid selectable components;
c) peptidic or proteinogenic conjugations – genetic fusions (preferred C- or N-terminal);
d) “Tag”-based fusions - A protein or a peptide located either at the C- or N- terminus of the target protein VEGF-A. Fusion “tags”, e.g. poly-histidine (particularly relevant for radiolabeling).

These and other methods for covalently and non-covalently attaching a protein of interest to a support are well known in the art, and are thus not described in further detail here.

In a further embodiment of the invention the multimeric ubiquitin-based binding protein, in particular hetero-multimeric ubiquitin-based binding protein according to the invention may contain artificial amino acids.

In further embodiments of the fusion protein of the present invention said active component is preferably a component selected from the groups of a radionuclide either from the group of gamma-emitting isotopes, preferably ^{99}Tc, ^{123}I, ^{111}In, or from the group of positron emitters, preferably ^{18}F, ^{64}Cu, ^{68}Ga, ^{86}Y, ^{124}I, or from the group of beta-emitter, preferably ^{131}I, ^{90}Y, ^{177}Lu, ^{67}Cu, or from the group of alpha-emitter, preferably ^{213}Bi, ^{211}At; or a fluorescent dye, preferably Alexa Fluor or Cy dyes (Berlier et al., J. Histochem. Cytochem. 51 (12): 1699-1712, 2003); or a photosensitizer.

A further embodiment relates to fusion proteins according to the invention, further comprising a component modulating serum half-life, preferably a component selected from the group consisting of polyethylene glycol, albumin-binding peptides, and immunoglobulin or immunoglobulin fragments or others.

Binding specificities (Dissociation constants)

The binding specificities of the fusion proteins according to the invention are as defined above for the non-fusion protein given in Kd. In accordance with the invention, the term "Kd" defines the specific binding affinity which is in accordance with the invention in the range of 10^{-7} - 10^{-12} M. A value of 10^{-5} M and below can be considered as a quantifiable binding affinity. Depending on the application a value of 10^{-7} M to 10^{-11} M is preferred for e.g. chromatographic applications or 10^{-9} to 10^{-12} M for e.g. diagnostic or therapeutic applications. Further preferred binding affinities are in the range of 10^{-7} to 10^{-10} M, preferably to 10^{-11} M.

The methods for determining the binding affinities are known per se and can be selected for instance from the following methods: ELISA, Surface Plasmon Resonance (SPR) based

technology (offered for instance by Biacore®), fluorescence spectroscopy, isothermal titration calorimetry (ITC), analytical ultracentrifugation, FACS.

After having made the modifications above, the inventors have found the amino acid modified ubiquitin sequences described in the examples which bind their targets with high affinity (Kd values up to 10^{-10} M).

Dimerization of ubiquitin

A "dimer" is considered as a protein in this invention which comprises two monomeric ubiquitin proteins. If the dimer comprises two identical modified monomers, it is called a "homomeric-dimer" or "homo-dimer". If the dimer comprises two differently modified monomers, it is called a "heteromeric-dimer" or "hetero-dimer". Thus, the "hetero-dimer" of the invention is considered as a fusion of two differently modified monomeric ubiquitin proteins exhibiting a combined binding property for the specific binding partner VEGF-A. It is emphasized that the modified hetero-dimeric VEGF-A binding ubiquitin protein of the invention is not obtained by separately screening each monomeric ubiquitin protein and combining two of them afterwards but by screening for hetero-dimeric proteins consisting of a first and a second monomeric unit binding to VEGF-A. It is to be expected that each of said subunits exhibit a quite limited binding affinity towards VEGF-A while only the combined dimeric modified ubiquitin protein will have the excellent binding properties described herein (see, for example, Figure 3).

Thus, the ubiquitin protein modified in accordance with the invention to efficiently bind VEGF-A is e.g. dimerized. The monomers can be connected directly or via linkers. Many conceivable linkers can be used. Each monomeric ubiquitin shows modifications in at least five of any of amino acids 6, 8, 62, 63, 64, 65, 66, and optionally contain an insert of 2 to 15 amino acids, preferably 8 amino acids. The monomeric proteins are fused to each other. The dimerized molecules can be used for further multimerization. The comments provided for dimerized ubiquitin molecules are *mutatis mutandis* also valid for higher multimerized molecules. Thus, the homo- or hetero-dimers binding to VEGF-A can be used for further multimerization.

Modified ubiquitin multimers bind to VEGF-A

The multimer of ubiquitin according to the invention binding to VEGF-A with Kd = 10^{-7} - 10^{-12} M and exhibiting a binding activity with respect to VEGF-A is selected:

(1) in the N-terminal (first) monomeric unit substitutions of amino acids selected from positions 6, 8, 62, 63, 64, 65, and 66;
(2) in the C-terminal (second) monomeric unit substitutions selected from amino acid positions 6, 8, 62, 63, 64, 65, and 66;
(3) optionally additionally an insert of 2 to15 amino acids, preferably 8 amino acids, located closely adjacent (1, 2 or 3 amino acids) to said substitutions.

The invention also covers homo-multimers of the modified ubiquitin units. These homo-multimers can be formed by fusing at least two of the monomeric ubiquitin units described herein. While the invention is described herein also with respect to specific examples (e.g. specific substitutions) of hetero-dimeric modified ubiquitin proteins, the monomeric units which form those homo-dimers can also be used as building units for forming homo-multimers.

In an embodiment, the fusion protein is a genetically fused hetero-dimer of said ubiquitin monomer having substitutions in positions 6, 8, 62, 62, 64, 65, or 66 of the first ubiquitin monomer, and optionally 2 to 15 amino acids inserted, preferably 8 amino acids, preferably in a loop region of the protein, for example, in close proximity to the substituted amino acids (0, 1, 2, or 3 amino acids from the substituted residues). The insertion is preferred close to the fourth beta sheet. Some exemplary positions for insertion could be between the following amino acid residues corresponding to wildtype human ubiquitin 61 - 62, 62 - 63, 63 - 64 and/or 64 - 65, but other positions are possible.

Generally the following substitutions in the following amino acid positions are preferable. It is most preferred that substitutions in the N-terminal (first) ubiquitin monomer of the multimeric binding protein are L8D and are selected from at least K6Y or K6F or K6W.

Further, within the first ubiquitin monomer of hetero-dimeric modified ubiquitin binding proteins with affinity to VEGF-A or isoforms, substitutions selected from E64M or E64W are preferred.

It is further preferred to have the following substitutions in the first (N-terminal) monomer of hetero-dimeric modified ubiquitin binding proteins:

- L8D or optionally L8S or L8A or L8Q,
- K63W or K63P or K63S or K63A or K63V,
- S65P or S65Q or S65A or S65E, and
- T66A or T66F or T66Y or T66P, and
- optionally the exchange in position 62 is a hydrophilic amino acid, preferably Q62S or Q62E or Q62V or Q62P or Q62Y.

If the modified hetero-meric, preferably hetero-dimeric, binding protein contains an insert, preferably of 8 amino acids, preferably closely adjacent to the fourth beta sheet, the following substitutions are most preferred in the first ubiquitin monomeric ubiquitin unit: K6Y, L8D, Q62S, K63W, E64M, S65P, T66A. It is preferred that said insertion comprises or consists of the amino acid sequence DVAEYLGI SEQ ID NO: 37.

Substitutions in the first ubiquitin monomer can be: K6Y or K6F, L8D or L8S or L8A or L8Q, Q62S or Q62E or Q62V or Q62P or Q62Y or Q62D, K63W, K63P or K63S or K63A or K63V, E64M or E64W or E64A, S65P orS65Q or S65A or S65E, and T66A or T66F or T66Y or T66P.

Substitutions in the second (C-terminal) monomeric unit are much more variable than substitutions in the first monomer. The following substitutions in the second monomeric unit are preferred:
K6A or K6Y or K6H or K6T or K6W or K6Q or K6S or K6L or K6R or K6D or K6N or K6G,
L8D or L8M or L8N or L8T or L8Y or L8H or L8S or L8R or L8E or L8I or L8F or L8A or L8G,
Q62R or Q62E or Q62G or Q62T or Q62S or Q62A or Q62Y or Q62V or Q62N or Q62D or Q62I or Q62M,

K63D or K63Q or K63L or K63V or K63N or K63A or K63R or K63S or K63M or K63G or K63T or K63E or K63W,
E64T or E64S or E64H or E64R or E64A or E64N or E64K or E64Q or E64L or E64D or E64W or E64P,
S65V or S65P or S65Q or S65I or S65T or S65Y or S65F or S65N or S65A or S65G or S65K, and
T66S or T66Q or T66P or T66W or T66L or T66F or T66H or T66Y or T66A or T66V or T66K or T66E or T66M.

Table 1 shows preferred amino acid substitutions in hetero-dimeric ubiquitin-based VEGF-A binding proteins with 8 amino acid insertion in the first monomer (insertion not shown in this table). In positions 6, 8, 62, 63, 64, 65, 66: substitutions in the N-terminal (first) ubiquitin monomer, positions 6', 8', 62', 63', 64', 65', 66': substitutions in the C-terminal (second) ubiquitin monomer of the binding protein. Further substitutions in other positions are not shown but are possible. In addition, substitutions that are not relating to the binding to a non-natural target such as substitutions in position 45, 75, and 76 are not shown. The "-" indicates that there is no substitution in this position; rather the wild-type amino acid remains. Please refer to Figure 2 for the complete sequence information.

Table 1. Substitution of selected amino acids in ubiquitin to create a high affinity binding to VEGF-A.

clone ID	Insert	6	8	62	63	64	65	66	6'	8'	62'	63'	64'	65'	66'
40401	Yes	Y	D	S	W	M	P	A	A	D	R	D	T	V	S
59517	Yes	Y	D	S	W	M	P	A	L	S	T	R	N	Y	H
59649	Yes	Y	D	S	W	M	P	A	L	R	T	S	K	-	S
60423	Yes	Y	D	S	W	M	P	A	R	R	-	N	Q	F	Q
60323	Yes	Y	D	S	W	M	P	A	D	E	E	Q	L	N	W
60397	Yes	Y	D	S	W	M	P	A	A	D	N	D	-	-	A
59507	Yes	Y	D	S	W	M	P	A	S	F	I	D	W	-	Q
59987	Yes	Y	D	S	W	M	P	A	S	R	R	-	H	Y	-
59603	Yes	Y	D	S	W	M	P	A	Y	A	S	E	K	K	K
39975	No	F	D	V	P	W	Q	Y	Y	M	E	Q	S	P	Q
40703	No	Y	D	E	W	M	P	F	H	M	T	L	R	P	P
38505	No	F	S	P	P	W	A	Y	T	N	G	N	H	Q	W
38943	No	F	S	P	S	W	A	Y	W	D	S	D	T	V	L
40685	No	F	A	P	S	W	A	Y	Q	T	S	D	T	I	S
39675	No	F	Q	Y	A	W	-	P	S	Y	A	V	R	T	F

The inventors have found two consensus families for VEGF-A binding proteins which are discussed below:

First consensus family (having an insertion within the first –N-terminal- ubiquitin monomer):

- N-terminal (first) ubiquitin monomer:
  - amino acid positions 6, 8, 63-65 are YD, WMP,
  - position 62 is a hydrophilic amino acid (e.g.. Ser, His, Tyr, Thr, Arg, Lys, Asn, Asp or Glu).
- Second (C-terminal) ubiquitin monomer:
  - preferable amino acids capable of hydrophilic interactions (Ser, Thr, Glu, Asp, Arg, Lys, His, Gln, Asn, Tyr)

Second consensus family (preferred is no insertion of additional amino acids within the first monomer):

- N-terminal (first) ubiquitin monomer:
  - position 6: preferably aromatic amino acids (Phe F, Tyr Y, Trp W),
  - position 8: preferably amino acids capable to form hydrogen bonds (e.g. Glu E, Asp D, Gln Q, Asn N),
  - position 62 preferably Val V or Pro P,
  - position 63 preferably Trp W or Pro P,
  - position 64 preferably Trp W or other aromatic amino acids like Phe F, Tyr Y
  - position 65 preferably amino acids which are not awkwardly shaped, and
  - in position 66 preferably aromatic amino acids (Trp W, Tyr Y, Phe F) or Ala A.

In one embodiment of the invention, the following substitutions are preferred in the N-terminal (first) monomer: 6Y 8D 62E 63W 64M 65P 66F. No insertion is preferred in these binding proteins. For the second monomer, no preferred amino acid positions for substitution can be given. For example, clones 61922, 61206, 61090, 61950, 61222, 61862, 61182.

In another embodiment of the invention, the following substitutions are preferred in the N-terminal (first) monomer: 6F 8D 62V 63P 64W 65Q 66Y. It is preferred that no insertion of

further amino acids is made in the first monomer. For the second monomer, no preferred amino acid positions for substitution can be given. For example, clones 61894 and 1337-C4.

Most preferred are the following modifications to generate binding proteins for VEGF-A (variant 40401) (SEQ ID NO: 14)

(1) Substitutions in the first (N-terminal) monomeric unit selected from K6Y, L8D, Q62S, K63W, E64M, S65P, and T66A;

(2) Insertion of 8 amino acids in the first (N-terminal) monomer of the amino acid residues DVAEYLGI located closely adjacent to said substituted amino acids;

(3) in the second (C-terminal) monomeric unit selected from K6A, L8D, Q62R, K63D, E64T, S65V, and T66S

Also preferred are multimers of variant 40401, for example, homo-dimers or hetero-dimers or multimerized form of those.

The following variants 59517 (SEQ ID NO: 16), 59649 (SEQ ID NO: 17), 60423 (SEQ ID NO: 18), 60323 (SEQ ID NO: 19), 60397 (SEQ ID NO: 21), 59507 (SEQ ID NO: 22), 59987 (SEQ ID NO: 23), 59603 (SEQ ID NO: 24) show the same substitutions in the first monomeric unit and the insertion of 8 amino acids in the first monomeric ubiquitin unit. The following substitutions are preferred in the second monomeric unit of the heterodimeric ubiquitin VEGF-A binding proteins:

variant 59517: selected from K6L, L8S, Q62T, K63R, E64N, S65Y, and T66H
variant 59649: selected from K6L, L8R, Q62T, K63S, E64K, and T66S
variant 60423: selected from K6R, L8R, K63N, E64Q, S65F, and T66Q
variant 60323: selected from K6D, L8E, Q62E, K63Q, E64L, S65N, and T66W
variant 60397: selected from K6A, L8D, E51K, Q62N, K63D, and T66A
variant 59507: selected from K6S, L8F, Q62I, K63D, E64W, and T66Q
variant 59987: selected from K6S, L8R, Q62R, E64H, and S65Y
variant 59603: selected from Q2R, K6Y, L8A, Q62S, K63E, E64K, S65K, T66K.

The following binding proteins for VEGF-A do not contain an insert in the first monomeric ubiquitin unit but still bind VEGF-A with high affinities:

Also preferred are the following modifications to generate binding proteins for VEGF-A (variant 39975) (SEQ ID NO: 27):

(1) Substitutions in the first monomeric unit at least K6F, L8D, Q62V, K63P, E64W, S65Q, and T66Y;

(2) in the second monomeric unit at least K6Y, L8M, Q62E, K63Q, E64S, S65P, and T66Q.

Also preferred are the following modifications to generate binding proteins for VEGF-A (variant 40703) (SEQ ID NO: 25):

(1) Substitutions in the first monomeric unit at least K6Y, L8D, Q62E, K63W, E64M, S65P, and T66F;

(2) in the second monomeric unit at least K6H, L8M, Q62T, K63L, E64R, S65P, and T66P:

Also preferred are the following modifications to generate binding proteins for VEGF-A (variant 38505) (SEQ ID NO: 28):

(1) Substitutions in the first monomeric unit at least K6F, L8S, K11E, K33R, Q62P, K63P, E64W, S65A, and T66Y;

(2) in the second monomeric unit at least K6T, L8N, Q62G, K63N, E64H, S65Q, and T66W.

Also preferred are the following modifications to generate binding proteins for VEGF-A (variant 38943) (SEQ ID NO: 29):

(1) Substitutions in the first monomeric unit at least K6F, L8S, Q62P, K63S, E64W, S65A, and T66Y;

(2) in the second monomeric unit at least K6W, L8D, Q62S, K63D, E64T, S65V, and T66L.

Also preferred are the following modifications to generate binding proteins for VEGF-A (variant 40685) (SEQ ID NO: 30):

(1) Substitutions in the first monomeric unit at least K6F, L8A, Q62P, K63S, E64W, S65A, and T66Y;

(2) in the second monomeric unit at least K6Q, L8T, Q62S, K63D, E64T, S65I, and T66S.

Also preferred are the following modifications to generate binding proteins for VEGF-A (variant 39675) (SEQ ID NO: 26): Substitutions in the first monomeric unit at least K6F, L8Q, Q62Y, K63A, E64W, and T66P; in the second monomeric unit at least K6S, L8Y, Q62A, K63V, E64R, S65T, and T66F; and a deletion of E51

In another embodiment of the invention, the binding protein is a homo-dimer. For example, variant 54644 (SEQ ID NO: 36) consists of the first monomer of variant 40401 combined with a linker with another first monomer of variant 40401 (linker is underlined, insertion is shown in italics):

MQIFVYTDTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSD
YNI*DVAEYLGI*SWMPALHLVLRLRGGGIG
MQIFVYTDTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSD
YNI*DVAEYLG*ISWMPALHLVLRLRAA

Either no linker or any linker can be used to connect the two monomers head-to-tail. Preferred linkers are those of the sequence GIG or SGGGGIG or SGGGGSGGGGIG or (SGGG)nIG or (SGGG)n.

These alternative substitutions in each monomer as shown in Table 1 can be combined with each other without any limitations provided that the resulting modified ubiquitin hetero-dimers show a specific binding affinity to VEGF-A of Kd = 10^{-7} - 10^{-12} M and exhibit a binding activity with respect to VEGF-A and provided that the structural stability of the ubiquitin protein is not destroyed or hampered. The binding affinities for the proteins of the invention are shown in Table 2.

Table 2: Binding Data of the preferred VEGF-A binding proteins of the invention (n.d. = not determined). Shown are affinity data for the VEGF-A isoforms 121 and 165. Data have been obtained from ELISA, Biacore and cell proliferation assays. All assays are further described in the Examples section.

clone ID	insert	Affinity ELISA hVEGF121 [nM]	Affinity ELISA hVEGF165	Affinity Biacore hVEGF121 [nM]	Affinity Biacore hVEGF165 [nM]	Cell proliferation

			[nM]			Assay IC50 [nM]
40401	Yes	2,5	2,2	15	22	8
59517	Yes	2,9	n.d.	n.d.	n.d.	n.d.
59649	Yes	2,8	n.d.	17	9,2	6,6
60423	Yes	3,8	n.d.	14	8	8
60323	Yes	2,3	n.d.	16	14	10
60397	Yes	2,6	n.d.	15	14	7,7
59507	Yes	4,4	n.d.	33	28	27
59987	Yes	2,5	n.d.	9	7	11
59603	Yes	21	n.d.	n.d.	n.d.	n.d.
39975	No	120	n.d.	n.d.	n.d.	n.d.
40703	No	378	n.d.	n.d.	n.d.	n.d.
38505	No	712	n.d.	n.d.	n.d.	n.d.
38943	No	847	n.d.	n.d.	n.d.	n.d.
40685	No	2020	n.d.	n.d.	n.d.	n.d.
39675	No	334	n.d.	n.d.	n.d.	n.d.

In a further aspect of the invention, the present invention covers also polynucleotides which encode for a binding protein or fusion protein or conjugate as described further in this invention. Additionally, vectors comprising said polynucleotides are covered by the invention.

In an additional aspect of the present invention, host cells are covered which comprise a protein or a fusion protein or conjugate described herein and/or a polynucleotide coding for said recombinant protein or fusion protein or conjugate of the invention or a vector containing said polynucleotide.

Vectors, host cells and methods of production of proteins

Vectors may be expression and cloning vectors containing a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 micron plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma,

adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding the modified ubiquitin scaffold protein. Promoters suitable for use with prokaryotic hosts include the phoA promoter, beta-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the modified ubiquitin based scaffold protein.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT - rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Suitable host cells include prokaryotes, yeast, mammalian cells, or bacterial cells. Suitable bacteria include gram negative or gram positive organisms, for example, E. coli or Bacillus spp. Yeast, preferably from the Saccharomyces species, such as S. cerevisiae, may also be used for production of polypeptides. Various mammalian or insect cell culture systems can also be employed to express recombinant proteins. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, (Bio/Technology, 6:47, 1988).

Uses of the proteins of the invention

The specific VEGF-A binding proteins of the invention are to be used for instance for preparing diagnostic means for *in vitro* or *in vivo* use as well as therapeutic means. The proteins according to the invention can be used e.g. as direct effector molecules (modulator, antagonist, agonist) or antigen-recognizing domains.

The pharmaceutical composition of the invention can be used for treatment of cancer, e.g. breast or colon cancers, or any other tumor diseases in which VEGF-A is abundant. In addition, VEGF-A binding proteins can be used for eye diseases, such as age-related macular degeneration (AMD) or diabetic macular edema (DME).

The compositions are adapted to contain a therapeutically effective dose. The quantity of the dose to be administered depends on the organism to be treated, the type of disease, the age and weight of the patient and further factors known per se.

The invention covers a pharmaceutical composition containing a modified multimeric ubiquitin protein or a modified multimeric ubiquitin fusion protein or a conjugate or a combination thereof and a pharmaceutically acceptable carrier. The invention further covers a diagnostic agent comprising a modified ubiquitin protein or a fusion protein or conjugate with a diagnostically acceptable carrier. The compositions contain a pharmaceutically or diagnostically acceptable carrier and optionally can contain further auxiliary agents and excipients known *per se*. These include for example but not limited to stabilizing agents, surface-active agents, salts, buffers, colouring agents etc.

The pharmaceutical composition can be in the form of a liquid preparation, a cream, a lotion for topical application, an aerosol, in the form of powders, granules, tablets, suppositories, or capsules, in the form of an emulsion or a liposomal preparation. The compositions are preferably sterile, non-pyrogenic and isotonic and contain the pharmaceutically conventional and acceptable additives known *per se*. Additionally, reference is made to the regulations of the U.S. Pharmacopoeia or Remington's Pharmaceutical Sciences, Mac Publishing Company (1990).

In the field of human and veterinary medical therapy and prophylaxis pharmaceutically effective medicaments containing at least one dimeric VEGF-A binding ubiquitin protein modified in accordance with the invention can be prepared by methods known per se. Depending on the galenic preparation these compositions can be administered parentally by injection or infusion, systemically, rectally, intraperitoneally, intramuscularly, subcutaneously, transdermally or by other conventionally employed methods of application. For applications for eye disease treatments, the direct application into the eye as drops is preferred. The type of pharmaceutical preparation depends on the type of disease to be treated, the severity of the disease, the patient to be treated and other factors known to those skilled in the art of medicine.

It surprisingly turned out that a VEGF-A specific ubiquitin multimer of the invention can be advantageously applied in therapy. This approach provides a less toxic, but still therapeutically effective concentration. It can be expected that systemic side effects can be remarkably reduced by administering the protein according to the present invention.
A "pharmaceutical composition" according to the invention may be present in the form of a composition, wherein the different active ingredients and diluents and/or carriers are in admixed with each other, or may take the form of a combined preparation, where the active ingredients are present in partially or totally distinct form. An example for such a combination or combined preparation is a kit-of-parts.

In a further embodiment, the pharmaceutical composition is in the form of a kit-of-parts, providing separated entities for the recombinant ubiquitin protein/fusion protein of the invention and for the one or more chemotherapeutic agents.

In a still further aspect the invention discloses diagnostic compositions comprising modified ubiquitins according to the invention specifically binding VEGF-A or its isoforms together with diagnostically acceptable carriers.

Since enhanced VEGF-A expression is correlated with tumor malignancy, it is desirable to develop diagnostics for non-invasive imaging in order to gain information about VEGF-A in patients. Furthermore, VEGF-A imaging could be useful for the assessment of the response of a patient to an anti-angiogenic therapy. Using radiolabelled monoclonal antibodies against VEGF, it has been demonstrated that VEGF imaging in patients is technically feasible and has

the potential to yield valuable information about the physiology of the tumor (for review see Haubner et al., Eur. J. Nucl. Med. Mol. Imaging (2010), 37 (Suppl. 1): S86-S103). However, due to their pharmacokinetic characteristics, intact antibodies are not suitable for routine imaging. Due to their small size and high affinity, radiolabelled proteins based on a ubiquitin scaffold might be much better suited for use as a VEGF imaging diagnostic.

In a further aspect of the invention, a recombinant protein and/or a fusion protein or conjugate is covered for use in a method of medical treatment or diagnosis.

In addition, combination preparations can be used, in particular combinations with cancer therapeutic agents (e.g. cytostatica). In a further embodiment, the pharmaceutical composition is in the form of a kit of parts, providing separated entities for the recombinant ubiquitin protein/fusion protein of the invention and for the one or more cancer therapeutic agents.

Method of production of the hetero-dimeric VEGF-A binding proteins of the invention

VEGF-A binding proteins according to the invention may be prepared by any of the many conventional and well known techniques such as plain organic synthetic strategies, solid phase-assisted synthesis techniques or by commercially available automated synthesizers. On the other hand, they may also be prepared by conventional recombinant techniques alone or in combination with conventional synthetic techniques.

In one aspect of the present invention, a method for generating a recombinant modified ubiquitin protein is provided. The method comprises at least the following steps:

a) providing an ubiquitin protein;

b) providing VEGF-A or its isoforms as potential target;

c) modifying said ubiquitin protein in order to obtain a monomeric protein having an amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 of at least 80%, wherein at least 5 amino acids are modified by substitution of amino acids in positions 6, 8, 62, 63, 64, 65, 66; and wherein optionally 2 to 15 amino acids or 6 to 10 (6, 7, 8, 9, or 10) amino acids, or 7 to 9 (7, 8, or 9) amino acids or 8 amino acids are inserted in at least one of said monomers, said insertions being further closely adjacent to said substituted amino acids of said ubiquitin monomer,

d) optionally fusing two of said monomeric protein units which are modified identically or differently;

e) contacting said modified monomeric or fused dimeric ubiquitin protein with said target;

f) screening for modified ubiquitin proteins which bind to said target with a specific binding affinity of 10^{-7} - 10^{-12} M, and optionally

g) isolating said modified ubiquitin proteins meeting the provisions of f).

In another aspect of the present invention, a method for identifying a recombinant modified ubiquitin protein is provided. The method comprises at least the following steps:

a) providing a population of identically or differently modified multimeric ubiquitin proteins originating from monomeric ubiquitin proteins, said population comprising multimeric ubiquitin proteins comprising one or more modified ubiquitin monomers linked together in a head-to-tail arrangement wherein each monomer of said multimeric protein is identically or differently modified by substitutions of at least 5 amino acids selected from amino acids corresponding to positions 6, 8, 62, 63, 64, 65, 66, of SEQ ID NO: 1, and wherein optionally 2 - 15 amino acids or 6 - 10 amino acids or 7 - 9 amino acids or 8 amino acids are inserted in at least one of said monomers, said insertions being further optionally in a loop region of said ubiquitin monomer,

b) providing VEGF-A or its isoforms as potential ligand;

c) contacting said population of identically or differently modified proteins with said VEGF-A or its isoforms;

d) identifying a modified multimeric ubiquitin protein by a screening process, wherein said modified multimeric ubiquitin protein binds to said VEGF-A or its isoforms with a specific binding affinity of Kd in a range of 10^{-7} - 10^{-12} M and exhibits a binding activity with respect to said VEGF-A or its isoforms, and optionally

e) isolating said modified multimeric ubiquitin protein with said binding affinity.

A further embodiment covers a method for generating a fusion protein or conjugate, comprising the following steps:

a) providing a modified ubiquitin;

b) fusing or conjugating said modified modified ubiquitin protein to a pharmaceutically and/or diagnostically active component.

In another aspect the present invention is directed to a method for the preparation of a VEGF-A binding protein as defined in the first embodiment, said method comprising the following steps:

(a) preparing a nucleic acid encoding a protein as defined in the first aspect;
(b) introducing said nucleic acid into an expression vector;
(c) introducing said expression vector into a host cell;
(d) cultivating the host cell;
(e) subjecting the host cell to culturing conditions under which a fusion protein is expressed from said vector, thereby producing a fusion protein as defined in the first aspect;
(f) optionally isolating the fusion protein produced in step (e).

Optionally, the modification may be performed by genetic engineering on the DNA level and expression of the modified protein in prokaryotic or eukaryotic organisms or in vitro.

In a further embodiment, said modification step includes a chemical synthesis step.

In one aspect of the invention, said population of differently modified proteins is obtained by genetically fusing two DNA libraries encoding each for differently modified monomeric ubiquitin proteins.

In a still further aspect, said method is adapted in order that said modified hetero-dimeric ubiquitin protein is fused with a diagnostic component, or wherein said recombinant modified hetero-dimeric ubiquitin protein is formed via said diagnostic component.

According to the invention, a modified protein can further be prepared by chemical synthesis. In this embodiment the steps c) to d) of claim 1 are then performed in one step.

In a further aspect, the present invention is directed to a library containing DNA encoding for modified monomeric ubiquitin proteins as defined above which form the basis for providing the hetero-dimeric ubiquitin proteins of the invention.

In a still further aspect of the invention, a fusion library containing DNA obtained by fusing two libraries as specified above is provided each library encoding for identically or differently modified monomeric ubiquitin protein units in order to obtain homo- or hetero-multimeric ubiquitin fusion proteins, the monomeric units thereof being linked together in a head-to-tail arrangement, said library encoding for homo- or hetero-multimeric fusion proteins of ubiquitin exhibiting a binding activity with respect to VEGF-A. Said linking together is performed either by using anyone of the linkers known by the skilled artisan or a linker described herein.

Example 1 outlines the production of a complex library. However, care must be taken as regards the quality of such a library. Quality of a library in scaffold technology is in the first place dependent from its complexity (number of individual variants) as well as functionality (structural and protein-chemical integrity of the resulting candidates). Both characteristics, however, may exert negative influences on each other: enhancing the complexity of a library by increasing the number of modified positions on the scaffold might lead to a deterioration of the protein-chemical characteristics of the variants. This might result in a decreased solubility, aggregation and/or low yields. A reason for this is the larger deviation from native scaffolds having an energetically favourable protein packaging.

Therefore, it is a balancing act to construct such a scaffold library suitably between the extreme positions of introducing as many variations as possible into the original sequence in order to optimize it for a target and, on the other hand, of conserving the original primary sequence as much as possible in order to avoid negative protein-chemical effects.

It is noted that the present disclosure encompasses also each conceivable combination of the features described herein in view of the aspects or embodiments of the invention.

Methods of selecting, enriching and characterizing the displayed proteins

Selection of the hetero-multimeric modified ubiquitins with respect to their binding activities to a VEGF-A and its isofomrs with a specific binding affinity of Kd in a range of 10^{-7} - 10^{-12} M can be performed by means of methods known to those skilled in the art. For this purpose, the modified ubiquitins presented e.g. on the ribosomal complexes can be transiently immobilized to VEGF-A bound e.g. on microtiter plates or can be bound to magnetic particles after binding in solution, respectively. Following separation of non-binding variations the

genetic information of variations with binding activity can be specifically eluted in the form of the mRNA by destruction of the ribosomal complex. The elution is preferably carried out with EDTA. The mRNA obtained in this manner can be isolated and reverse transcribed into DNA using suitable methods (reverse transcriptase reaction), and the DNA obtained in this manner can be re-amplified.

By means of successive cycles of in vitro transcription/translation, selection, and amplification of the modified ubiquitins with binding properties for VEGF-A and its isoforms can be enriched.

The further characterization of said modified ubiquitins can be performed in the form of a soluble protein as detailed above after cloning of the corresponding gene cassette into a suitable expression vector. The appropriate methods are known to those skilled in the art or described in the literature.

Preferably, the step of detection of the modified ubiquitin proteins having a binding affinity with respect to a predetermined binding partner is followed by a step of isolation and/or enrichment of the detected protein.

Following the expression of the ubiquitin protein modified according to the invention, it can be further purified and enriched by methods known per se. The selected methods depend on several factors known per se to those skilled in the art, for example the expression vector used, the host organism, the intended field of use, the size of the protein and other factors. For simplified purification the protein modified according to the invention can be fused to other peptide sequences having an increased affinity to separation materials. Preferably, such fusions are selected that do not have a detrimental effect on the functionality of the ubiquitin protein or can be separated after the purification due to the introduction of specific protease cleavage sites. Such methods are also known se to those skilled in the art.

Selection of the modified ubiquitin proteins with binding affinity with respect to the target VEGF-A and determination of the modified amino acids responsible for the binding affinity

After e.g. at least two different DNA libraries encoding for hetero-dimeric modified ubiquitin proteins have been established by differently modifying selected amino acids in each of the monomeric ubiquitin units, these e.g. two libraries are genetically fused by e.g. linker technology to obtain DNA molecules encoding for e.g. hetero-dimeric modified ubiquitin proteins. The DNA of these libraries is expressed into proteins and the modified dimeric proteins obtained thereby are contacted according to the invention with the VEGF-A or its isoforms to enable binding of the potential partners to each other if a binding affinity does exist.

It is a crucial aspect of the invention that the contacting and screening process is performed already with respect to the homo- or hetero-dimeric ubiquitin protein. This process enables screening on those ubiquitin proteins which provide a binding activity to VEGF-A.

Contacting according to the invention is preferably performed by means of a suitable presentation and selection method such as the phage display, ribosomal display, mRNA display or cell surface display, yeast surface display or bacterial surface display methods, preferably by means of the phage display method. For complete disclosure, reference is made also to the following references: Hoess, Curr. Opin. Struct. Biol.. 3 (1993), 572-579; Wells and Lowmann, Curr. Opin. Struct. Biol. 2 (1992), 597-604; Kay et al., Phage Display of Peptides and Proteins-A Laboratory Manual (1996), Academic Press. In the same manner as phage display alternative methods for the presentation on bacteria (bacterial surface display; Daugherty et al., 1998, Protein Eng. 11(9):825-832) or yeast cells (yeast surface display; Kieke et al., 1997 Protein Eng. 10(11):1303-10) or cell-free selection systems such as the ribosome display (Hanes and Plückthun, 1997 Proc. Natl. Acad. Sci. U S A. 94(10):4937-4942; He and Taussig, 1997_Nucleic Acids Res. 25(24):5132-5134) or the cis display (Odegrip et al., 2004 Proc. Natl. Acad. Sci. U S A. 101(9):2806-2810) or the mRNA display can be applied. The methods mentioned above are known to those skilled in the art and can be used according to the invention including modifications thereof.

The determination whether the modified protein has a quantifiable binding affinity with respect to a predetermined binding partner can be performed according to the invention preferably by one or more of the following methods: ELISA, plasmon surface resonance spectroscopy, fluorescence spectroscopy, FACS, isothermal titration calorimetry and analytical ultracentrifugation.

Phage display selection method

One type of phage display procedure adapted to this application is described in the following as an example for a selection procedure according to the invention with respect to variations of ubiquitin which show binding properties to VEGF-A.

In the phage display procedure described herein recombinant variations of ubiquitin are presented on a filamentous phage while the coding DNA of the presented variation is present at the same time packed in a single-stranded form in the phage envelope. Thus, in the frame of an affinity enrichment variations having certain properties can be selected from a library and their genetic information can be amplified by infection of suitable bacteria or added to another cycle of enrichment, respectively. Presentation of the mutated ubiquitin on the phage surface is achieved by genetic fusion to an amino-terminal signal sequence-preferably the PelB signal sequence-and a capsid or surface protein of the phage-preferred is the carboxyterminal fusion to the capsid protein pIII or a fragment thereof. Furthermore, the encoded fusion protein can contain further functional elements such as e.g. an affinity tag or an antibody epitope for detection and/or purification by affinity chromatography or a protease recognition sequence for specific cleavage of the fusion protein in the course of the affinity enrichment. Furthermore, an amber stop codon can be present for example between the gene for the ubiquitin variation and the coding region of the phage capsid protein or the fragment thereof which is not recognized during translation in a suitable suppressor strain partially due to the introduction of one amino acid.

The bacterial vector suitable for the selection procedure in the context of the isolation of ubiquitin variations with binding properties to VEGF-A or isoforms and into which the gene cassette for the fusion protein described is inserted is referred to as phagemid. Among others, it contains the intergenic region of a filamentous phage (e.g. M13 or f1) or a portion thereof which in the case of a superinfection of the bacterial cell carrying the phagemid by means of helper phages such as e.g. M13K07 results in the packaging of a closed strand of phagemid DNA into a phage capsid. The phagemids generated in this manner are secreted by the bacterium and present the respective ubiquitin variation encoded-due to its fusion to the capsid protein pIII or the fragment thereof-on their surface. Native pIII capsid proteins are present in the phagemid so that its ability to re-infect suitable bacterial strains and therefore

the possibility to amplify the corresponding DNA is retained. Thus, the physical linkage between the phenotype of the ubiquitin variation - i.e. its potential binding property - and its genotype is ensured.

Phagemids obtained can be selected with respect to the binding of the ubiquitin variation presented thereon to VEGF-A or its isoforms by means of methods known to those skilled in the art. For this purpose, the presented ubiquitin variations can be transiently immobilized to target substance bound e.g. on microtiter plates and can be specifically eluted after non-binding variations have been separated. The elution is preferably performed by basic solutions such as e.g. 100 mM triethylamine. Alternatively, the elution can be performed under acidic conditions, by proteolysis or direct addition of infected bacteria. The phagemids obtained in this manner can be re-amplified and enriched by successive cycles of selection and amplification of ubiquitin variations with binding properties to VEGF-A.

Further characterization of the ubiquitin variations obtained in this way can be performed in the form of the phagemid, i.e. fused to the phage, or after cloning of the corresponding gene cassette into a suitable expression vector in the form of a soluble protein. The appropriate methods are known to those skilled in the art or described in the literature. The characterization can comprise e.g. the determination of the DNA sequence and thus of the primary sequence of the variations isolated. Furthermore, the affinity and specificity of the variations isolated can be detected e.g. by means of biochemical standard methods such as ELISA or plasmon surface resonance spectroscopy, fluorescence spectroscopy, FACS, isothermal titration calorimetry, analytical ultracentrifugation or others. In view of the stability analysis, for example spectroscopic methods in connection with chemical or physical unfolding are known to those skilled in the art.

Characterization of the VEGF-A-binding proteins

The further characterization of the ubiquitin variations obtained in this manner can be performed in the form of a soluble protein as detailed above after cloning of the corresponding gene cassette into a suitable expression vector. The appropriate methods are known to those skilled in the art or described in the literature. Exemplary methods for characterization of dimeric binding proteins are outlined in the Examples section of this invention.

Preferably, the step of detection of the proteins having a binding affinity with respect to a predetermined binding partner is followed by a step of isolation and/or enrichment of the detected protein.

Following the expression of the ubiquitin protein modified according to the invention, it can be further purified and enriched by methods known per se. The selected methods depend on several factors known per se to those skilled in the art, for example the expression vector used, the host organism, the intended field of use, the size of the protein and other factors. For simplified purification the protein modified according to the invention can be fused to other peptide sequences having an increased affinity to separation materials. Preferably, such fusions are selected that do not have a detrimental effect on the functionality of the ubiquitin protein or can be separated after the purification due to the introduction of specific protease cleavage sites. Such methods are also known per se to those skilled in the art.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows VEGF-A and isoforms.
VEGF-A (accession no. P15692) corresponds to SEQ ID NO: 2
VEGF-A-121 (accession no. P15692-9) corresponds to SEQ ID NO: 3
VEGF-A-165 (accession no. P15692-4) corresponds to SEQ ID NO: 4

Figure 1a shows the sequences of human VEGF-A and of the VEGF-A isoforms VEGF-121, and VEGF-165. The signal peptide is underlined in VEGF-A and its isoforms. Human VEGF-A has a protein length of 232 amino acids (molecular weight 27.042 Da; after cleavage of the signal peptide the molecular weight is 23.895 Da). The processed isoform VEGF-121 without signal peptide has a protein length of 121 amino acids (molecular weight 14.057 Da). The processed isoform VEGF-165 without signal peptide has a protein length of 165 amino acids (molecular weight 19.166 Da).

Figure 1b shows a sequence alignment of VEGF-A (Accession number P15692; 1st line), VEGF-165 (Accession number P15692-4; 2nd line), and VEGF-121 (Accession number P15692-9; 3rd line). The signal peptide is underlined.

Figure 1c shows a sequence alignment of VEGF-121 (Accession number P15692-9; 1st line), VEGF-121 from Humanzyme (VEGF121_HZ; 2nd line), VEGF-165 (Accession number P15692-4; 3rd line) and VEGF-165 from Humanzyme (VEGF165_HZ; 4th line). The signal peptide is underlined.

Figure 1d shows an alignment of the VEGF-A sequences of different mammalian species, e.g. human (accession numbers P15692-4 and P15692-9), mouse (accession number Q00731-3), rat (accession number P16612-2), rabbit (accession number Q866G4), dog (accession number Q9MYV3-3), and guinea pig (*Cavia porcellus*, accession number P26617, referred to as VEGFA_CAVPO in the figure). The alignment shows a high degree of identity between different species. The signal sequences are ommitted; highligthed amino acid residues are identical or similar between mammalian species.

Figure 2 shows different hetero-multimeric, e.g. hetero-dimeric, VEGF-A binding clones. The linker is shown in italics. Not substituted amino acids of the ubiquitin monomers and of the linker are shown in blue; substituted or inserted amino acids are not highlighted.

Figure 2a shows the amino acid sequence of VEGF-A binding protein 40401 (SEQ ID NO: 14). The clone is substituted in positions 6, 8, 62-66 in both ubiquitin units and shows an additional insertion of 8 amino acids.

Figure 2b shows the amino acid sequence of sequence of a heterodimeric ubiquitin sequence used as basis for substitutions to generate high specific binding proteins. Positions 6, 8, 62-66 in both ubiquitin units are marked with an X. A sequence for a linker is not shown since any possible linker known in the art could be used or even no linker could be used. Note that compared to wildtype the ubiquitin is further modified in position 45 in both monomers to Tryptophan. This modification does not influence the binding. At the last C-terminal amino acids in the first monomer, there is either Glycine or an exchange from Glycine to Alanine at the last C-terminal amino acids of the first monomer. These exchanges at positions 45, 75, 76 are optional and do not influence the binding to VEGF-A.

Figure 2c. shows an alignment of 15 sequences of modified hetero-dimeric ubiquitins with binding affinities to VEGF-A.

Figure 3 shows high affinity binding of binding protein 40401 to VEGF-A (closed circles connected by a fitted line). The binding is shown by closed circles connected by a fitted line. The figure shows a concentration dependent ELISA of the binding of the hetero-dimeric ubiquitin variant to human VEGF-A, in particular to isoform 121 and to isoform 165. As negative control, NGF was used (symbol connected by a broken line). Variant 40401 (also referred to as SPVF-11_1211_A1_TsX6 in this figure) shows high affinity binding to VEGF-A 121 (Kd = 2.5 nM = 2.5 x 10^{-9} M) and to VEGF-A 165 (Kd = 2.2 nM = 2.2 x 10^{-9} M). The closed circles show the affinity of the binding of 40401 to VEGF121 and the closed triangles show the affinity of the binding to VEGF165 compared to no binding of this variant to negative control (NGF) (stars connected by a broken line).

Figure 4 shows results of an analysis of the modified hetero-dimeric ubiquitin molecule 40401 via label-free interaction assays using Biacore®. Different concentrations of hetero-dimeric ubiquitin variants were selected (see figure legend: 0-1000 nM) for binding to either VEGF121 or VEGF165 immobilized on a chip (Biacore) to evaluate the interaction between the hetero-dimeric variant 40401 and VEGF-A. Analyzing the association and dissociation curves resulted in a Kd of 2.2 x 10^{-8} M (k_{off} rate of 2.45 x 10^{-3} s^{-1}) to VEGF165 and a Kd of 1.5 x 10^{-8} M (k_{off} rate of 1.76 x 10^{-3} s^{-1}) to VEGF121 which indicates a long half time of a complex of 40401 and VEGF-A.

Figure 5 shows inhibition of VEGF-A-induced proliferation of HUVEC by the binding protein 40401. Different concentrations of 40401 were preincubated with VEGF-A in medium together with a fixed VEGF-AQ concentration and the mixture applied to growing HUVEC. After three days the proliferation of cells was quantified with WST reagent. The dose response curve was fitted and an IC50 of 8.1 nM was calculated.

EXAMPLES

The following Examples are provided for further illustration of the invention. The invention is particularly demonstrated with respect to particular modifications of ubiquitin binding to VEGF-A as an example. The invention, however, is not limited thereto, and the following Examples merely show the practicability of the invention on the basis of the above

description. For a complete disclosure of the invention reference is made also to the literature cited in the application which is incorporated completely into the application by reference.

Example 1. Identification of hetero-dimeric VEGF-A binding proteins based on modified ubiquitin proteins

Library Construction and Cloning

Unless otherwise indicated, established recombinant genetic methods were used, for example as described in Sambrook et al. A random library of human ubiquitin hetero-dimers with high complexity was prepared by concerted mutagenesis of at least 14 selected amino acid positions. The modified amino acids, which were substituted by NNK triplets, comprised amino acids selected from positions 6, 8, 62, 63, 64, 65, 66 within the proximal (first) ubiquitin monomer and amino acids selected from positions 6, 8, 62, 63, 64, 65, 66 within the distal (second) ubiquitin monomer. Both ubiquitin monomers were genetically linked (head to tail) by a Glycine/Serine linker with at least the sequence GIG or at least the sequence SGGGG, for example GIG, SGGGG, SGGGGIG, SGGGGSGGGGIG or SGGGGSGGGG, but any other or no linker is possible.

The target protein VEGF-A

VEGF-A exists in several isoforms. VEGF121 and VEGF165 are naturally abundant isoforms of VEGF-A (accession number p15692). VEGF121 (Accession Number p15692-9) and VEGF165 (accession number p15692-9) were purchased from Humanzyme (order numbers HZ-1206 (VEGF121) and HZ-1153 (VEGF165)). Compared to the data base entry, isoform VEGF165 is 26 amino acids shorter because the signal peptide is not included. Both isoforms were expressed in human cells to ensure a correct glycolysation. The sequences of VEGF-A and the isoforms 165 and 121 are shown in Figure 1a - 1d.

TAT Phage Display Selection

The heterodimeric ubiquitin library was enriched against VEGF-A (see Figure 1) using, for example, TAT phage display as selection system. Other selection methods known in the art can be used. The target can be immobilized nonspecifically onto protein binding surfaces or via biotinylated residues which were covalently coupled to the protein. The immobilization via biotin onto streptavidin beads or neutravidin strips is preferred. The target-binding phages are selected either in solution or on immobilized target; for example, the biotinylated and

immobilized target with phage was incubated followed by washing of the phages bound to the matrix and by elution of matrix-bound phages. In each cycle following target incubation, the beads were magnetically separated from solution and washed several times. In the first selection cycle the biotinylated target was immobilized to neutravidin strips whereas in cycles two to four selections in solution were performed followed by immobilization of target-phage complexes on Streptavidin-coated Dynabeads® (Invitrogen). After washing in the first two selection cycles the phages of target-binding modified ubiquitin molecules were released by elution with acidic solution. In selection cycles three and four elution of phages was carried out by competitive elution with excess target. The eluted phages were reamplified. To direct specificty of binders a protein similar to the target can be included during selection.

Alternatively to TAT phage display selection: Ribosome Display Selection

The ubiquitin library was enriched against the target using, for example, ribosome display as selection system. Other selection methods known in the art can be used. The target was biotinylated according to standard methods and immobilized on Streptavidin-coated Dynabeads® (Invitrogen). Ternary complexes comprising ribosomes, mRNA and nascent ubiquitin polypeptide were assembled using the PURExpress™ *In Vitro* Protein Synthesis Kit (NEB). Up to four primary rounds of selection were performed, wherein ternary complexes were incubated followed by two similar rounds of selection. In each cycle following target incubation, the beads were magnetically separated from solution and washed with ribosome display buffer with increasing stringency. After washing in the first two selection cycles, the beads were again magnetically separated from solution and mRNA of target-binding modified ubiquitin molecules was released from ribosomes by addition of 50 mM EDTA. In selection cycles three and four elution of mRNA complex was carried out by competitive elution with excess target (Lipovsek and Plueckthun, 2004). After each cycle, RNA purification and cDNA synthesis were performed using RNeasy MinElute Cleanup Kit (Qiagen, Germany), Turbo DNA-free Kit (Applied Biosystems, USA) and Transcriptor Reverse Transcriptase (Roche, Germany).

Cloning of Enriched Pools

After the fourth selection cycle the synthesized cDNA was amplified by PCR, cut with suitable restriction nucleases and ligated into an expression vector via compatible cohesive ends.

Single Colony Hit Analysis

After transformation into NovaBlue (DE3) cells (Merck, Germany) ampicillin-resistant single colonies were grown in SOB medium containing 100 µg/ml ampicilin and 20 g/l glucose. Expression of the VEGF-A binding modified ubiquitin was achieved by cultivation in 96-well deep well plates using auto induction medium ZYM-5052 (Studier, 2005). Cells were harvested and subsequently lysed. After centrifugation the resulting supernatants were screened by ELISA using Nunc MediSorp plates (Thermo Fisher Scientific, USA) coated with 4 µg/ml VEGF-A and a ubiquitin-specific Fab fragment conjugated with horseradish peroxidase (POD). As detecting reagent TMB-Plus (KEM-EN-Tec) was used and the yellow colour was developed using 0.2 M H_2SO_4 solution and measured in a plate reader at 450 nm versus 620 nm. Usually, several, for example, four cycles of selection displays versus VEGF-A were carried out. In the last two cycles of selection binding molecules were eluted with an excess of free VEGF-A.

Maturation of selected VEGF-A binding clones with high affinities

In order to enhance the affinity ubiquitin-based dimeric binding proteins to VEGF-A, ubiquitin building units (monomers) of a dimer of selected binding proteins were fused to naïve monomeric ubiquitin libraries. For example, either the N-terminal or C-terminal monomer of a dimeric ubiquitin binding unit was fused to a monomeric ubiquitin library. The selection strategy is outlined below in Table 3. Several, for example, 1 to 10, preferably 3, VEGF-A binding molecules were selected and the N-terminal ubiquitin monomer with substitutions in positions 6, 8, 62, 63, 64, 65, and/or 66, and optionally an insertion at position 61-62, was fused to naïve monomeric ubiquitin libraries with randomized amino acid positions 6, 8, 62, 63, 64, 65, and / or 66 via a suitable amino acid linker, for example GIG. In parallel ubiquitin monomers of the C-terminal region of a hetero-dimeric binding protein having substitutions in positions 6, 8, 62, 63, 64, 65, and/or 66 were fused to naïve monomeric ubiquitin libraries with randomized amino acid positions 6, 8, 62, 63, 64, 65 and/or 66 and/or 42, 44, 68, 70, and 72-74 via a suitable amino acid linker, for example GIG. The resulting dimeric ubiquitin libraries with up to 7 randomized positions were pooled and exhibited a theoretical number of around $1{,}5x10^{10}$ different variants which could be fully displayed in a ribosome display with up to 10-fold presentation of each variant using methods known to somebody skilled in the art. The mixed library was applied to 4 rounds of ribosome display including 3 rounds competitive elution of VEGF-A binding molecules with soluble VEGF121.

VEGF-A binders with high affinity to VEGF-A do not always form complexes which are stable for a longer period of time. Some complexes (ubiquitin-dimer and VEGF-A) have high off-rates, meaning that the binding is strong but the complex differentiates quickly. A lower off-rate as determined e.g. by Biacore assays is desirable. Thus, to differentiate stable VEGF-A binding complexes from variants with high affinities but high off-rates, one round with 16 hrs off-rate selection was performed. The elution is performed under competitive conditions with 1000 x non bound target protein (compared to the target protein which is bound to e.g. streptavidin-beads). All binders which are still bound to the immobilized target protein after 16h are further analyzed. After this selection, pools with VEGF-A binding molecules were subcloned to an expression vector using standard methods known to a skilled person and probed for binding to different types of VEGF-A in hit-screening (e.g. ELISA) as described below.

Some exemplary VEGF-A binding proteins are shown in Figure 2 and in Tables 1 and 2.

Example 2: Binding Analysis of modified Ubiquitin-based binding proteins to human VEGF-A

Example 2A. Binding analysis of modified ubiquitin-based VEGF binding variants by concentration dependent ELISA.

Binding of ubiquitin-based variants to human VEGF-A was assayed by a concentration dependent ELISA. Increasing amounts of purified protein applied to NUNC-medisorp plates coated with human VEGF-A 121 or VEGF-A 165 and NGF as negative control. Antigen coating with 1 to 2,5 µg/ml per well was performed at 4°C overnight. After washing the plates with PBS, 0.1 % Tween 20 pH 7.4 (PBST) the wells were blocked using blocking solution (PBS pH 7.4; 3 % BSA; 0.5% Tween 20) at room temperature for 2 h. Wells were washed again three times with PBST. Different concentrations of modified ubiquitin based VEGF-A binding protein were then incubated in the wells at RT for 1 h (see Figure 3). After washing the wells with PBST, the anti-Ubi fab fragment (a-Ubi-Fab) POD conjugate was applied in an appropriate dilution (for example, 1:6500) in PBST. The plate was washed three times with 300 µl buffer PBST/well. 50 µl TMB substrate solution (KEM-EN-Tec) were added to each well and the plate was incubated. The reaction was stopped by adding 0.2 M H_2SO_4 per well. The ELISA plates were read out using the TECAN Sunrise ELISA-Reader. The photometric absorbance measurements were done at 450 nm using 620 nm as a reference wavelength.

Figure 3a shows clearly the very high affinity binding of 40401 (SEQ ID NO: 14) to VEGF-A with an apparent KD value of 2.2 to 2.5 nM. No variant showed binding to the control (NGF). Further results of other VEGF-A binding proteins are shown in Table 2 (above).

Further examples are shown in Figure 3. Thus, only very few modifications (up to 6 substitutions in each monomer) in the ubiquitin-wildtype result in a high affinity binding to VEGF-A. The binding protein 40401(SEQ ID NO: 14) having an additional insert of 8 amino acids shows the highest affinity binding to VEGF-A.

Example 2B. Binding analysis of modified ubiquitin-based VEGF binding variants by Biacore assays.

Different concentrations of the variant were analyzed (for example, 0-1,000 nM of the variant, preferably 40401) for binding to VEGF immobilized on a CM5-chip (Biacore) using methods known to those skilled in the art. The obtained data were processed via the BIA evaluation software and 1:1-Langmuir-fitting. The K_D of 40401 for VEGF165 was 2.2 x 10^{-8} M, as shown in Figure 4. The K_D of the fusion protein 40401 for VEGF121 was 1.5 x 10^{-8} M, as shown in Figure 4 and in Table 2. Further results of other VEGF-A binding proteins are shown in Table 2 (above).

Example 3: Inhibition of VEGF stimulated cell proliferation by modified hetero-dimeric ubiquitin based binding proteins of the invention

Inhibition of VEGF stimulated HUVEC cell proliferation was assessed with the following assay: HUVEC cells (Promocell) were grown in Hams F-12 Nutrient Mixture (Kaighn's Modification, Gibco) with 10 % FCS, 0.1 mg/ml Heparin, 10 ng/ml b-FGF and passages 5 and 6 were used. On day one, 6000 cells/well were seeded in complete medium in collagen coated 96 well plates. On the following day, cells were preincubated with 100% Hams F12 Nutrient Mixture for 6 h. After this time, the medium was exchanged for the preincubation mix, prepared of medium containing 5% FCS, 0.1 mg/ml Heparin and gentamycin supplemented with dilution series of the VEGF-specific binding protein premixed with 15 ng/ml VEGF121 (Biomol/Humanzyme). The dilution series were prepared n 1:3 steps (starting from 1.5 µM as indicated and incubated 1 h at room temperature. Each concentration was run in triplicate. VEGF-specific therapeutic monoclonal antibody Avastin® (Roche) was used as control (not shown). Viability of the cells was assessed after 3 days with WST reagent

(Roche) according to the manufacturer's instructions. Results of this inhibition assay are shown in Figure 5 and Table 2. Further results of other VEGF-A binding proteins are shown in Table 2 (above). The binding protein of the invention clearly shows a significant inhibition of VEGF-A induced proliferation of HUVEC cells.

5

CLAIMS

1. A multimeric modified ubiquitin protein capable of binding VEGF-A or its isoforms, comprising

at least two monomeric ubiquitin units linked together in a head-to-tail arrangement,

wherein each monomeric unit of said multimeric protein is differently or identically modified at least by substitutions of at least 5 amino acids corresponding to positions 6, 8, 62, 63, 64, 65, 66 of SEQ ID NO: 1, and

wherein optionally at least one monomeric ubiquitin unit contains an insertion of 2 to 15 amino acids within or in close proximity of 1, 2, or 3 amino acids in direction of the N- or C-terminus of said amino acid substitutions, and

wherein said modified monomeric ubiquitin unit having an amino acid identity to SEQ ID NO: 1 of at least one of the group of at least 75%, at least 80%, at least 85%, and at least 90%, said protein having a specific binding affinity to said VEGF-A or its isoforms of Kd = 10^{-7} - 10^{-12} M.

2. The multimeric modified ubiquitin protein according to claim 1, wherein said multimeric ubiquitin protein is a dimeric or trimeric or tetrameric protein comprising two, three, or four differently or identically modified monomeric ubiquitin units ..

3. The multimeric modified ubiquitin protein according to claim 1 to 2, wherein the substitutions in the N-terminal (first) ubiquitin monomer of the multimeric binding protein are L8D and are selected from at least K6Y or K6F or K6W.

4. The multimeric modified ubiquitin protein of claims 1 to 3, wherein 6 to 10 amino acids or 7 to 9 amino acids or 8 amino acids are inserted in at least one monomeric ubiquitin protein.

5. The multimeric modified ubiquitin protein of claim 4, wherein in the first ubiquitin monomer, amino acid positions 6, 8, 63, 64, 65 are independently Y, D, W, M or P, and wherein optionally position 62 is selected from hydrophilic amino acids (S, H, Y, T, R, K, N, D, or E).

6. The multimeric modified ubiquitin protein according to claim 5, wherein said substitutions comprise in the first monomeric unit at least K6Y, L8D, K63W, E64M, S65P, preferably K6Y, L8D, Q62S, K63W, E64M, S65P, and T66A and / or wherein said insertion comprises or consists of the amino acid sequence DVAEYLGI (SEQ ID NO: 37).

7. A fusion protein or conjugate comprising a protein according to anyone of the previous claims fused to or conjugated with a diagnostic component, wherein said diagnostically active component is optionally a fluorescent compound, a photosensitizer, or a radionuclide, or fused to or conjugated with a therapeutically active component, wherein said pharmaceutically active component is optionally a cytokine, a chemokine, a cytotoxic compound, or an enzyme, or a fusion protein or conjugate wherein said multimeric, preferably dimeric modified ubiquitin unit capable of binding VEGF-A or its isoforms is fused with or conjugated to a second multimeric, preferably dimeric modified ubiquitin with different target specificity.

8. A polynucleotide coding for a recombinant protein according to anyone of claims 1 to 6 or fusion protein or a conjugate according claim 7.

9. A vector comprising a polynucleotide according to claim 8.

10. A host cell comprising a protein according to anyone of claims 1 to 6, a fusion protein or conjugate according to claim 7, a vector according to claim 9 and/or a polynucleotide according to claim 8.

11. The protein according to any one of claims 1 to 6 or fusion protein according to claim 7 for use in medicine, preferably for use in the treatment of cancer or eye diseases.

12. A pharmaceutical or diagnostic composition comprising a protein according to anyone of claims 1 to 6 or a fusion protein or a conjugate according to claim 7 with one or more pharmaceutically acceptable carriers or excipients or with one or more diagnostically acceptable carrier.

13. A method of generating a fusion protein or a conjugate according to claim 7, wherein a protein according to anyone of claims 1 to 6 is fused with or conjugated to a diagnostic component or a pharmaceutical component.

14. A recombinant protein according to anyone of claims 1 to 6 or a fusion protein or conjugate according to claim 7 for use in a method of medical treatment or diagnosis.

15. A method for generating a multimeric modified ubiquitin protein according to anyone of claims 1 to 6 comprising the following steps:

a) providing a population of modified multimeric ubiquitin proteins originating from monomeric ubiquitin proteins, said population comprising multimeric ubiquitin proteins comprising differently or identically modified ubiquitin monomers linked together in a head-to-tail arrangement wherein each monomer of said multimeric protein is differently or identically modified at least by substitutions of at least 5 amino acids corresponding to positions 6, 8, 62, 63, 64, 65, and/or 66 of SEQ ID NO: 1 and wherein optionally at least one monomeric ubiquitin unit contains an insertion of 2 to 15 amino acids within or in close proximity of 1, 2 or 3 amino acids in direction of the N- or C-terminus of said amino acid substitutions

b) providing VEGF-A or its isoforms as potential target,

c) contacting said population of modified proteins with VEGF-A;

d) identifying a modified multimeric ubiquitin protein by a screening process, wherein said modified multimeric ubiquitin protein binds to said target with a specific binding affinity of Kd in a range of 10^{-7} - 10^{-12} M, and optionally

e) isolating said modified multimeric ubiquitin protein with said binding affinity.

16. A method for identifying a multimeric modified ubiquitin protein according to anyone of claims 1 to 6 comprising the following steps:

a) providing a population of modified multimeric ubiquitin proteins originating from monomeric ubiquitin proteins, said population comprising multimeric ubiquitin proteins comprising differently or identically modified ubiquitin monomers linked together in a head-to-tail arrangement wherein each monomer of said multimeric protein is differently or identically modified at least by substitutions of at least 5 amino acids corresponding to positions 6, 8, 62, 63, 64, 65, and/or 66 of SEQ ID NO: 1 and wherein optionally at least one monomeric ubiquitin unit contains an insertion of 2 to 15 amino acids within or in close proximity of 1, 2 or 3 amino acids in direction of the N- or C- terminus of said amino acid substitutions

b) providing VEGF-A or its isoforms as potential target,

c) contacting said population of modified proteins with VEGF-A;

d) identifying a modified multimeric ubiquitin protein by a screening process, wherein said modified multimeric ubiquitin protein binds to said target with a specific binding affinity of Kd in a range of 10^{-7} - 10^{-12} M, and optionally

e) isolating said modified multimeric ubiquitin protein with said binding affinity.

17. A method for the preparation of a protein as defined in any one of claims 1 to 6, said method comprising the following steps:

(a) preparing a nucleic acid encoding a protein as defined in any one of claims 1 to 6

(b) introducing said nucleic acid into an expression vector;

(c) introducing said expression vector into a host cell;

(d) cultivating the host cell;

(e) subjecting the host cell to culturing conditions under which a fusion protein is expressed from said vector, thereby producing a fusion protein as defined in any one of claims 1 to 6;

(f) optionally isolating the fusion protein produced in step (e).

FIG. 1

FIG. 1a

VEGF-A (Accession number P15692)

MNFLLSWVHWSLALLLYLHHAKWSQAAPMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLEC
VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQEKKSVRGKGKGQKRKRKKSRYKSWSVYVGARCCLMPWSLPGPHPCGPC
SERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNERTCRCDKPRR

VEGF-121 (Accession number P15692-9)

MNFLLSWVHWSLALLLYLHHAKWSQAAPMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLEC
VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQENCDKPRR

VEGF165 (Accession number P15692-4)
MNFLLSWVHWSLALLLYLHHAKWSQAAPMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLEC
VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQENPCGPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNERTCRCDK
PRR

FIG. 1b

```
P15692_VEGFA          1 mnfllswvhwslalllylhhakwsqaapmaeggggnhhevvkfmdvyqrsychpietlvdifqeypdeie
P15692-4_VEGFA165     1 mnfllswvhwslalllylhhakwsqaapmaeggggnhhevvkfmdvyqrsychpietlvdifqeypdeie
P15692-9_VEGF121      1 mnfllswvhwslalllylhhakwsqaapmaeggggnhhevvkfmdvyqrsychpietlvdifqeypdeie

P15692_VEGFA         71 yifkpscvplmrcggccndeglecvpteesnitmqimrikphqqqhigemsflqhnkcecrpkkdrarqe
P15692-4_VEGFA165    71 yifkpscvplmrcggccndeglecvpteesnitmqimrikphqqqhigemsflqhnkcecrpkkdrarqe
P15692-9_VEGF121     71 yifkpscvplmrcggccndeglecvpteesnitmqimrikphqqqhigemsflqhnkcecrpkkdrarqe

P15692_VEGFA        141 kksvrgkgkgqkrkrkksrykswsvyvgarcclmpwslpgphpcgpcserrkhlfvqdpqtckcsckntd
P15692-4_VEGFA165   141 n----------------------------------------pcgpcserrkhlfvqdpqtckcsckntd
P15692-9_VEGF121    141 k-------------------------------------------------------------------

P15692_VEGFA        211 srckarqlelnertcrcdkprr
15692-4_VEGFA165    170 srckarqlelnertcrcdkprr
P15692-9_VEGF121    142 ----------------cdkprr
```

FIG. 1c

```
P15692-9_VEGF121      1 mnfllswvhwslalllylhhakwsqaapmaeggggnhhevvkfmdvyqrsychpietlvdifqeypdeie
VEGF121_HZ            1 -------------------------apmaeggggnhhevvkfmdvyqrsychpietlvdifqeypdeie
15692-4_VEGFA165      1 mnfllswvhwslalllylhhakwsqaapmaeggggnhhevvkfmdvyqrsychpietlvdifqeypdeie
VEGF165_HZ            1 -------------------------apmaeggggnhhevvkfmdvyqrsychpietlvdifqeypdeie

P15692-9_VEGF121     71 yifkpscvplmrcggccndeglecvpteesnitmqimrikphqqqhigemsflqhnkcecrpkkdrarqe
VEGF121_HZ           45 yifkpscvplmrcggccndeglecvpteesnitmqimrikphqqqhigemsflqhnkcecrpkkdrarqe
15692-4_VEGFA165     71 yifkpscvplmrcggccndeglecvpteesnitmqimrikphqqqhigemsflqhnkcecrpkkdrarqe
VEGF165_HZ           45 yifkpscvplmrcggccndeglecvpteesnitmqimrikphqqqhigemsflqhnkcecrpkkdrarqe

P15692-9_VEGF121    141 ------------------------------------------kcdkprr
VEGF121_HZ          115 ------------------------------------------ncdkprr
15692-4_VEGFA165    141 npcgpcserrkhlfvqdpqtckcsckntdsrckarqlelnertcrcdkprr
VEGF165_HZ          115 npcgpcserrkhlfvqdpqtckcsckntdsrckarqlelnertcrcdkprr
```

FIG. 1 d

```
VEGF165 human   1 apmaeggggnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkpscvplmrcggccndeglecvpteesnitmqimrik
VEGF121 human   1 apmaeggggnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkpscvplmrcggccndeglecvpteesnitmqimrik
VEGFA_MOUSE     1 aptteg-egkshevikfmdvyqrsycrpietlvdifqeypdeieyifkpscvplmrcagccndealecvptsesnitmqimrik
VEGFA_RAT       1 aptteg-egkahevvkfmdvyqrsycrpietlvdifqeypdeieyifkpscvplmrcagccndealecvptsesnvtmqimrik
VEGFA Rabbit    1 apmaeegdnkphevvkfmevyrrsycqpietlvdifqeypdeieyifkpscvplvrcggccndeslecvpteefnvtmqimrik
VEGFA_Dog       1 apma-ggehkphevvkfmdvygrsycrpietlvdifqeypdeieyifkpscvplmrcggccndeglecvpteefnitmqimrik
VEGFA_CAVPO     1 apmaeg-egkpreevkfmdvykrsycrpiemlvdifqeypdeieyifkpscvplmrcggccndeslecvpteefnitmqimrik

VEGF165 human  85 phqgqhigemsflqhnkcecrpkkdrarqenpcgpcserrkhlfvqdpqtckcsckntdsrckarqlelnertcrcdkprr
VEGF121 human  85 phqgqhigemsflqhnkcecrpkkdrarqen----------------------------------------------cdkprr
VEGFA_MOUSE    84 phqsqhigemsflqhsrcecrpkkdrtkpenhcepcserrkhlfvqdpqtckcsckntdsrckarqlelnertcrcdkprr
VEGFA_RAT      84 phqsqhigemsflqhsrcecrpkkdrtkpenhcepcserrkhlfvqdpqtckcsckntdsrckarqlelnertcrcdkprr
VEGFA Rabbit   85 phqgqhigemsflqhnkcecrpk----------------------------------------------------------
VEGFA_Dog      84 phqgqhigemsflqhskcecrpkkdrarqenpcgpcserrkhlfvqdpqtckcsckntdsrckarqlelnertcrcdkprr
VEGFA_CAVPO    84 phqgqhigemsflqhskcecrpkkekarqenpcgpcserrkhlfvqdpqtckcscrntdsrckarqlelnertcrcdkprr
```

FIG. 2

FIG. 2a.

```
  1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdynidvaeylgiswmpalhlvlrlrgg---------gig
 85 mqifvatdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdynirdtvslhlvlrlraa
```

FIG. 2b.

```
1 mqifvxtxtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdynixxxxxlhlvlrlrnn(linker)
  mqifvxtxtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdynixxxxxlhlvlrlraa
```

FIG. 2c.

```
59517_1321-B2      1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
59649_1321-D10     1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
60423_1325-G10     1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
60323_1325-E4      1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
40401_1211-A1      1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
60397_1325-B9      1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
59507_1321-E1      1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwaskqledgrtlsdyn
59987_1323-E7      1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
59603_1321-E7      1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwageqledgrtlsdyn
40703_1212-E8      1 mqifvytdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
39675_1207-B10     1 mqifvftqtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
39975_1208-G4      1 mqifvftdtgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
38505_1201-B1      1 mqifvftstgetitlevepsdtienvkakiqdregippdqqrliwagkqledgrtlsdyn
38943_1203-D4      1 mqifvftstgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn
40685_1212-D11     1 mqifvftatgktitlevepsdtienvkakiqdkegippdqqrliwagkqledgrtlsdyn

59517_1321-B2     61 idvaeylgiswmpalhlvlrlraa---------gigmqifvltstgktitlevepsdtie
59649_1321-D10    61 idvaeylgiswmpalhlvlrlraa---------gigmqifvltrtgktitlevepsdtie
60423_1325-G10    61 idvaeylgiswmpalhlvlrlraa---------gigmqifvrtrtgktitlevepsdtie
60323_1325-E4     61 idvaeylgiswmpalhlvlrlraa---------gigmqifvdtetgktitlevepsdtie
40401_1211-A1     61 idvaeylgiswmpalhlvlrlrgg---------gigmqifvatdtgktitlevepsdtie
60397_1325-B9     61 idvaeylgiswmpalhlvlrlraa---------gigmqifvatdtgktitlevepsdtie
59507_1321-E1     61 idvaeylgiswmpalhlvlrlraa---------gigmqifvstftgktitlevepsdtie
59987_1323-E7     61 idvaeylgiswmpalhlvlrlraa---------gigmqifvstrtgktitlevepsdtie
59603_1321-E7     61 idvaeylgiswmpalhlvlrlraa---------gigmrifvytatgktitlevepsdtie
40703_1212-E8     61 i--------ewmpflhlvlrlraasggggsggggigmqifvhtmtgktitlevepsdtie
39675_1207-B10    61 i--------yawsplhlvlrlrgg---------gigmqifvstytgktitlevepsdtie
39975_1208-G4     61 i--------vpwqylhlvlrlrgg---------gigmqifvytmtgktitlevepsdtie
38505_1201-B1     61 i--------ppwaylhlvlrlraasggg-----gigmqifvttntgktitlevepsdtie
38943_1203-D4     61 i--------pswaylhlvlrlraasggggsggggigmqifvwtdtgktitlevepsdtie
40685_1212-D11    61 i--------pswaylhlvlrlrgg---------gigmqifvqttgktitlevepsdtie

59517_1321-B2    112 nvkakiqdkegippdqqrliwagkqledgrtlsdynitrnyhlhlvlrlraa
59649_1321-D10   112 nvkakiqdkegippdqqrliwagkqledgrtlsdynitsksslhlvlrlraa
60423_1325-G10   112 nvkakiqdkegippdqqrliwagkqledgrtlsdyniqnqfqlhlvlrlraa
60323_1325-E4    112 nvkakiqdkegippdqqrliwagkqledgrtlsdynieqlnwlhlvlrlraa
40401_1211-A1    112 nvkakiqdkegippdqqrliwagkqledgrtlsdynirdtvslhlvlrlraa
60397_1325-B9    112 nvkakiqdkegippdqqrliwagkqlkdgrtlsdynindesalhlvlrlraa
59507_1321-E1    112 nvkakiqdkegippdqqrliwagkqledgrtlsdyniidwsqlhlvlrlraa
59987_1323-E7    112 nvkakiqdkegippdqqrliwagkqledgrtlsdynirkhytlhlvlrlraa
59603_1321-E7    112 nvkakiqdkegippdqqrliwagkqledgrtlsdynisekkklhlvlrlraa
40703_1212-E8    113 nvkakiqdkegippdqqrliwagkqledgrtlsdynitlrpplhlvlrlraa
39675_1207-B10   104 nvkakiqdkegippdqqrliwagkqledgrtlsdyniavrtflhlvlrlraa
39975_1208-G4    104 nvkakiqdkegippdqqrliwagkqledgrtlsdynieqspqlhlvlrlraa
38505_1201-B1    108 nvkakiqdkegippdqqrliwagkqledgrtlsdynignhqwlhlvlrlraa
38943_1203-D4    113 nvkakiqdkegippdqqrliwagkqledgrtlsdynisdtvllhlvlrlraa
40685_1212-D11   104 nvkakiqdkegippdqqrliwagkqledgrtlsdynisdtislhlvlrlraa
```

FIG. 3

FIG. 3a

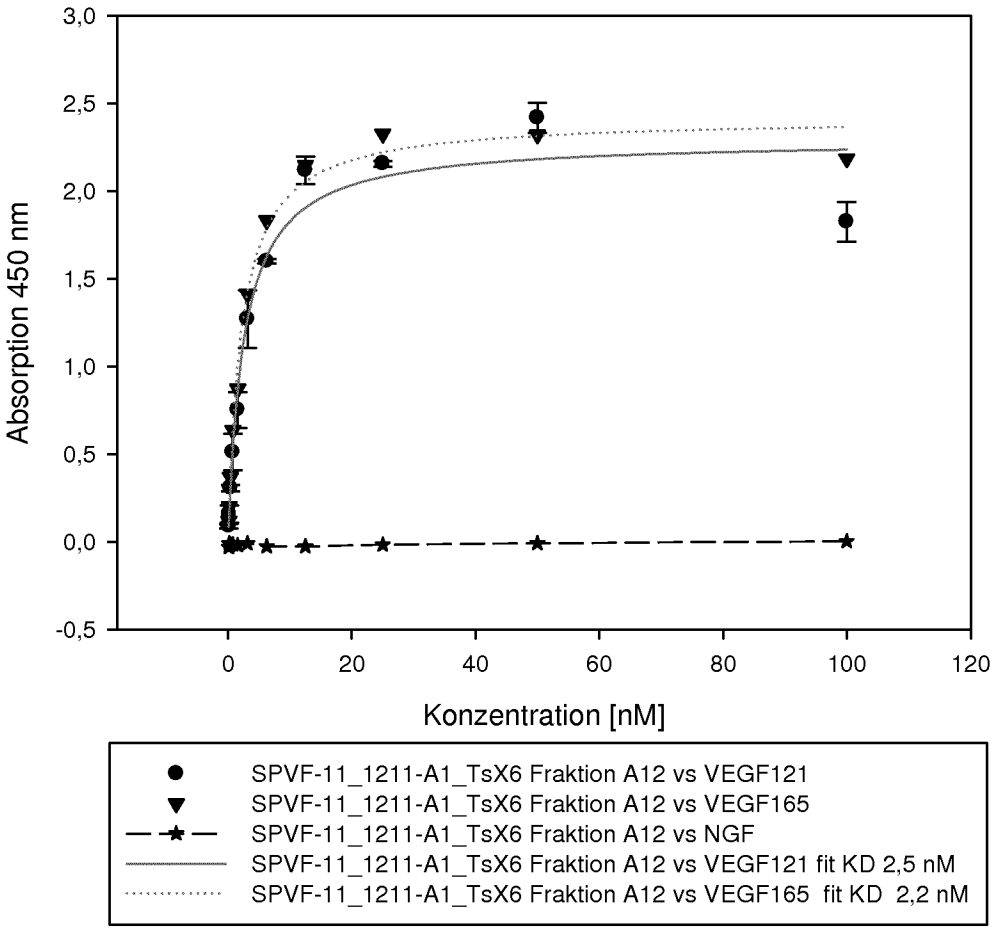
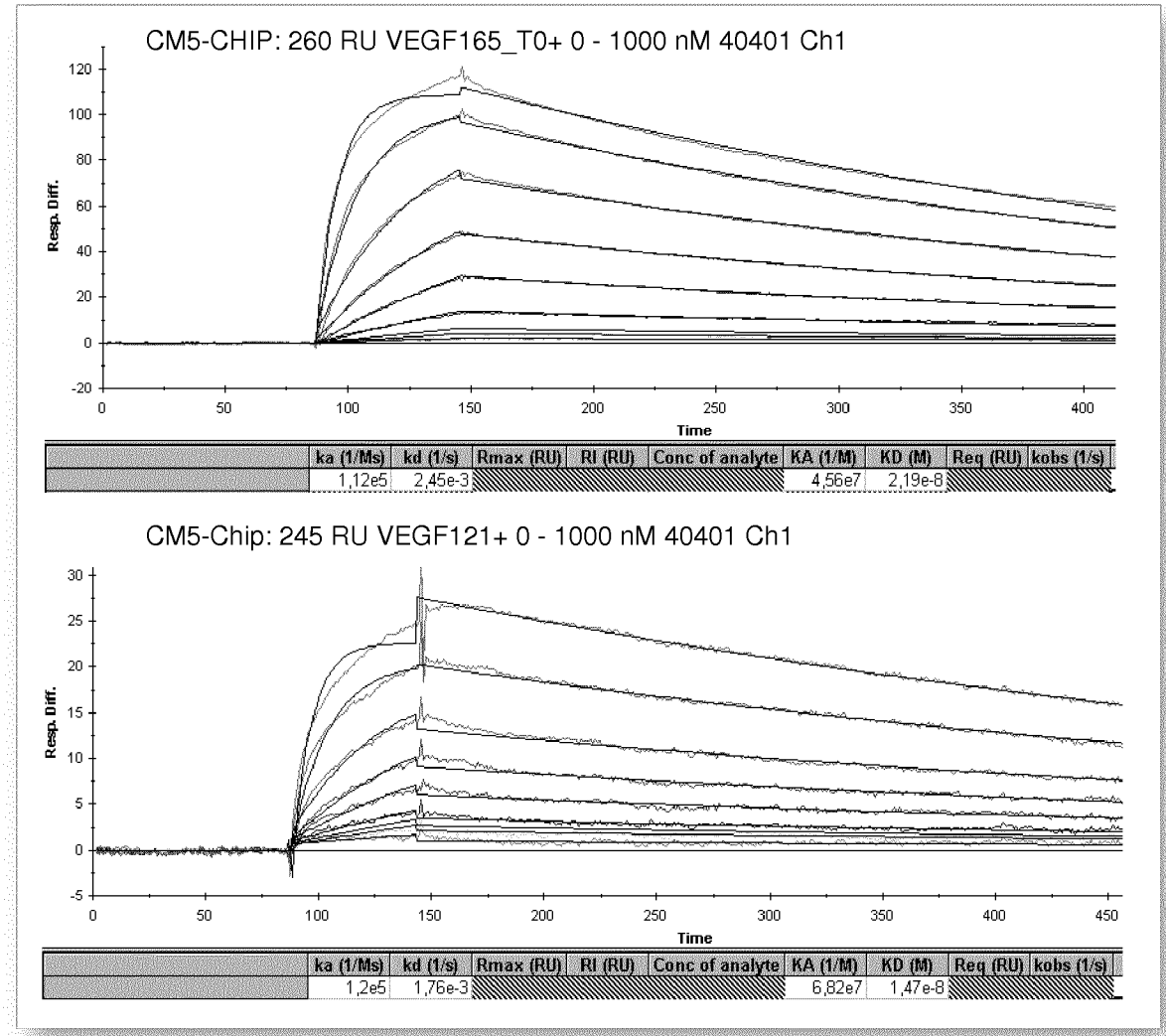

3,0
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
Absorption 450 nm
0
20
40
60
80
100
120
Konzentration [nM]
SPVF-11_1211-A1_TsX6 Fraktion A12 vs VEGF121
SPVF-11_1211-A1_TsX6 Fraktion A12 vs VEGF165
SPVF-11_1211-A1_TsX6 Fraktion A12 vs NGF
SPVF-11_1211-A1_TsX6 Fraktion A12 vs VEGF121 fit KD 2,5 nM
SPVF-11_1211-A1_TsX6 Fraktion A12 vs VEGF165 fit KD 2,2 nM

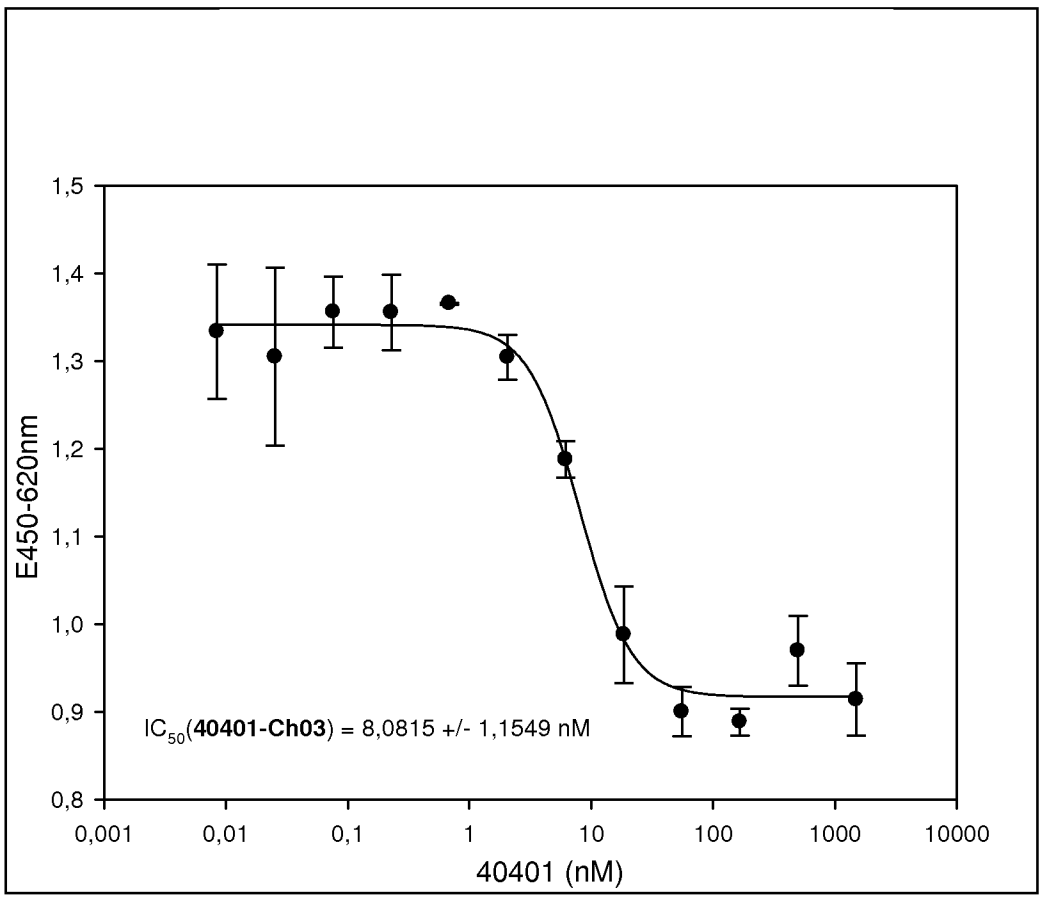
FIG. 4



	ka (1/Ms)	kd (1/s)	Rmax (RU)	RI (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)
	1,12e5	2,45e-3				4,56e7	2,19e-8		

	ka (1/Ms)	kd (1/s)	Rmax (RU)	RI (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)
	1,2e5	1,76e-3				6,82e7	1,47e-8		

FIG. 5


1,5
1,4
1,3
1,2
1,1
1,0
0,9
0,8
E450-620nm
IC50(40401-Ch03) = 8,0815 +/- 1,1549 nM
0,001
0,01
0,1
1
10
100
1000
10000
40401 (nM)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/061454

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/061454

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/475 C12N15/10 C07K14/49
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 C12N

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 practicable, 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 2004/106368 A1 (SCIL PROTEINS GMBH [DE]; FIEDLER MARKUS [DE]; FIEDLER ULRIKE [DE]; RUD) 9 December 2004 (2004-12-09) cited in the application abstract page 6, line 11 - page 9, line 28 page 13, line 15 - page 14, line 5 page 31, line 26 - page 32, line 16 page 36, line 30 - page 39, line 12; claims 1,15,17,20, 27,29,41; examples 5-9; tables 2,3	1-17

-/--

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

[X] See patent family annex.

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

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"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
18 September 2012	24/09/2012

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	Mossier, Birgit

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 2008/096012 A2 (SCIL PROTEINS GMBH [DE]; CELARES GMBH [DE]; KRAEHMER RALF [DE]; LEENDE) 14 August 2008 (2008-08-14) abstract page 26 - page 31	1-17
A	HEY T ET AL: "Artificial, non-antibody binding proteins for pharmaceutical and industrial applications", TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 23, no. 10, 1 October 2005 (2005-10-01), pages 514-522, XP027778157, ISSN: 0167-7799 [retrieved on 2005-10-01] abstract; tables 1-4	1-17
A	SKERRA ET AL: "Alternative non-antibody scaffolds for molecular recognition", CURRENT OPINION IN BIOTECHNOLOGY, LONDON, GB, vol. 18, no. 4, 14 September 2007 (2007-09-14), pages 295-304, XP022244962, ISSN: 0958-1669, DOI: 10.1016/J.COPBIO.2007.04.010 abstract; table 1	1-17

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	JO NOBUO ET AL: "Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular enclothelial growth factor therapy in multiple models of ocular neovascularization", AMERICAN JOURNAL OF PATHOLOGY; [10640], AMERICAN SOCIETY FOR INVESTIGATIVE PATHOLOGY, US, vol. 168, no. 6, 1 June 2006 (2006-06-01), pages 2036-2053, XP002500820, ISSN: 0002-9440, DOI: 10.2353/AJPATH.2006.050588 abstract	1-17
A	LIANG WEI-CHING ET AL: "Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, US, vol. 281, no. 2, 7 November 2005 (2005-11-07), pages 951-961, XP002373804, ISSN: 0021-9258, DOI: 10.1074/JBC.M508199200 abstract	1-17
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-/--

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	WO 2011/073209 A1 (SCIL PROTEINS GMBH; STEUERNAGEL ARND [DE]; FIEDLER ERIK [DE]; FIEDLER) 23 June 2011 (2011-06-23) abstract page 8, line 25 - page 12, line 21 page 20, line 4 - line 15 page 25, line 5 - page 29, line 6; claim 28 -----	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/061454

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