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(54) Title: LIPOCALIN MUTEINS AS VEGF ANTAGONISTS FOR USE IN TREATING DISEASES CAUSED OR PROMOTED BY INCREASED VASCULARIZATION

(57) Abstract: The present invention relates to novel uses of muteins or fragments (or variants thereof) derived from a lipocalin, e.g., a human tear lipocalin, that inhibit the binding of vascular endothelial growth factor (VEGF) to a VEGF receptor. Uses include methods of treating a disease or disorder caused or promoted by increased vascularisation in a subject, such as a wide range of cancer. The mutein may be a pharmaceutical composition, for example, and the pharmaceutical composition is safe and well tolerated when administered to a subject in an amount that is sufficient to yield a satisfactory therapeutic readout in said subject.

LIPOCALIN MUTEINS AS VEGF ANTAGONISTS FOR USE IN TREATING DISEASES CAUSED OR PROMOTED BY INCREASED VASCULARIZATION

FIELD OF THE INVENTION

The present invention relates to novel uses of muteins or fragments (or variants thereof) derived from a lipocalin, e.g., a human tear lipocalin, that inhibit the binding of vascular endothelial growth factor (VEGF) to a VEGF receptor. Uses include methods of treating a disease or disorder caused or promoted by increased vascularisation in a subject, such as a wide range of cancer. The mutein of the present invention may be in the form of a pharmaceutical composition optionally comprising a pharmaceutically acceptable carrier, excipient and/or diluent as described herein, for example, and the pharmaceutical composition is safe and well tolerated when administered to a subject in an amount that is sufficient to yield a satisfactory therapeutic readout in said subject.

BACKGROUND

[0001] The members of the lipocalin protein family (Pervaiz, S., and Brew, K. (1987) *FASEB J.* **1**, 209-214) are typically small, secreted proteins which are characterized by a range of different molecular-recognition properties: their ability to bind various, principally hydrophobic molecules (such as retinoids, fatty acids, cholesterols, prostaglandins, biliverdins, pheromones, tastants, and odorants), their binding to specific cell-surface receptors and their formation of macromolecular complexes. Although they have, in the past, been classified primarily as transport proteins, it is now clear that the lipocalins fulfill a variety of physiological functions. These include roles in retinol transport, olfaction, pheromone signaling, and the synthesis of prostaglandins. The lipocalins have also been implicated in the regulation of the immune response and the mediation of cell homoeostasis (reviewed, for example, in Flower, D.R. (1996) *Biochem. J.* **318**, 1-14 and Flower, D.R. et al. (2000) *Biochim. Biophys. Acta* **1482**, 9-24).

[0002] The lipocalins share unusually low levels of overall sequence conservation, often with sequence identities of less than 20%. In strong contrast, their overall folding pattern is highly conserved. The central part of the lipocalin structure consists of a single eight-stranded anti-parallel β-sheet closed back on itself to form a continuously hydrogen-bonded β-barrel. One end of the barrel is sterically blocked by both the N-terminal peptide segment that runs across its bottom and three peptide loops connecting the β-strands. The other end of the β-barrel is open to the solvent and encompasses a target-binding site, which is formed by four peptide loops. It is this diversity of the loops in the otherwise rigid lipocalin scaffold that gives rise to a variety of different binding modes each capable of accommodating targets of different size, shape, and chemical character (reviewed, e.g., in Flower, D.R. (1996), *supra*; Flower, D.R. et al. (2000), *supra*, or Skerra, A. (2000) *Biochim. Biophys. Acta* **1482**, 337-350).

[0003] Human tear pre-albumin, now called tear lipocalin (TLPC or Tlc), is one example of a lipocalin and was originally described as a major protein of human tear fluid (approximately one third of the total protein content) but has also been identified in several other secretory tissues including prostate, nasal mucosa and tracheal mucosa. Homologous proteins have been found in rat, pig, dog, and horse. "Human tear lipocalin" as used herein refers to the human tear lipocalin with the SWISS PROT Data Bank Accession Number P31025 (SEQ ID No: 15) as well as to the mature human tear lipocalin (SEQ ID No: 16). “Mature” means that amino acids 1-18 of the human tear lipcalin protein as, e.g., deposited with the SWISS PROT Data Bank Accession Number P31025 (SEQ ID NO. 15) are not present in a human tear lipocalin protein; see SEQ ID No: 16 (mature human tear lipocalin). Exemplary muteins of a mature human tear lipocalin are shown in SEQ ID NO: 1 or 10. Tear lipocalin is an unusual lipocalin member because of its high promiscuity for relative insoluble lipids and binding characteristics that differ from other members of this protein family (reviewed in Redl, B. (2000) *Biochim. Biophys. Acta* **1482**, 241-248). A remarkable number of lipophilic compounds of different chemical classes such as fatty acids, fatty alcohols, phospholipids, glycolipids and cholesterol are endogenous ligands of this protein. Interestingly, in contrast to other lipocalins the strength of ligand (target)

binding correlates with the length of the hydrocarbon tail both for alkyl amides and fatty acids. Thus, tear lipocalin binds most strongly the least soluble lipids (Glasgow, B.J. et al. (1995) *Curr. Eye Res.* **14**, 363-372; Gasymov, O.K. et al. (1999) *Biochim. Biophys. Acta* **1433**, 307-320).

[0004] In tear fluid, human tear lipocalin appears to be most important for the integrity of the tear film by removing lipids from the mucous surface of the eye to the liquid phase (reviewed in Gasymov, O.K. et al. (1999), *supra*). However, it displays additional activities *in vitro* that are very unusual among lipocalins, namely inhibition of cysteine proteinases as well as non-specific endonuclease activity (van't Hof, W. et al. (1997) *J. Biol. Chem.* **272**, 1837-1841; Yusifov, T.N. et al. (2000) *Biochem. J.* **347**, 815-819). It has been demonstrated, for example, that tear lipocalin is able to bind several lipid peroxidation products *in vitro* resulting in the hypothesis that it might function as a physiological oxidative-stress-induced scavenger of potentially harmful lipophilic molecules (Lechner, M. et al. (2001) Biochem. J. 356, 129-135).

[0005] Various PCT publications (e.g., WO 99/16873, WO 00/75308, WO 03/029463, WO 03/029471 and WO 2005/19256) disclose how muteins of various lipocalins (e.g., human tear lipocalin and human lipocalin 2 (or hNGAL lipocalin)) can be constructed to exhibit a high affinity and specificity against a target that is different than a natural ligand of a wild type lipocalin. This can be done, for example, by mutating one or more amino acid positions of at least one of the four peptide loops.

[0006] PCT publication WO 2008/015239 discloses a method for the generation of human tear lipocalin muteins that possess improved binding properties for a given target, including vascular endothelial growth factor (VEGF), and inhibit the binding of vascular endothelial growth factor (VEGF) to the VEGF receptor.

[0007] The recitation of any reference in this application is not an admission that the reference is prior art to this application.

BRIEF DESCRIPTION OF THE FIGURES

[0008] **Figure 1** shows plasma pharmacokinetics after the first single dose of a variant of a lipocalin mutein (SEQ ID NO: 1), wherein the first four N-terminal amino acid residues (His, His, Leu, Ala) are deleted from the amino acid sequence of the lipocalin mutein (SEQ ID NO: 1), over the course of 21 days. Plasma concentrations of active drug were determined in an electrochemoluminescence-based ELISA. Concentrations declined in a biphasic manner characterized by an initial distribution phase and a terminal elimination phase setting in between eight and twenty-four hours following drug administration. Note that the graph representing 0.1 mg/ml is "lowest" graph, while the graph representing 10.0 mg/ml is at the top. Graphs representing other values are in between these two graphs.

[0009] **Figure 2** shows the pharmacokinetic parameters for said variant of a lipocalin mutein (SEQ ID NO: 1). In this regard, Fig. 2 shows a linear increase in exposure (AUC 0-∞) with cohort doses 0.1 mg/kg, 0.5 mg/kg, 1.5 mg/kg, 3.0 mg/kg, 6.0 mg/kg and 10.0 mg/kg. Also, the volume of distribution around 50ml/kg suggests drug resides primarily in the blood compartment. Fig. 2 further indicates a terminal half-life of 3.6, 5.0, 5.5, 5.9 and 6.6 days for the different dose cohorts. The drug displays an elimination half-life of 6 days as determined by Non-compartmental PK analysis based on nominal sampling times. The exposure (AUC) increases with dose in a linear fashion as evidenced by a Dose Proportionality Factor (DPF) of approximately one.

[0010] **Figure 3** shows the repeat dose PK: C_{min} during the weekly dosing period for said variant of a lipocalin mutein (SEQ ID NO: 1). Differing cohort doses 0.1 mg/kg, 0.5 mg/kg, 1.5 mg/kg, 3.0 mg/kg, 6.0 mg/kg and 10.0 mg/kg achieved an increasing C_{min} of 1.0 μg/ml, 7.1 μg/ml, 20.5 μg/ml, 69.9 μg/ml, 106.5 μg/ml and 144.5 μg/ml, respectively, through measuring the level immediately prior to the 4th weekly administration. PK was followed during the weekly administration stage by measuring plasma trough levels (C_{min}). Steady-State was generally achieved after 3rd to 4th dose

and PK analysis suggests that the drug follows linear PK throughout the repeat dose phase.

[0011] **Figure 4** shows the target engagement of said variant of a lipocalin mutein (SEQ ID NO: 1). Free VEGF-A was undetectable after dosing at 0.5 mg/kg or above for three weeks. The levels of the VEGF-A-drug complex were measured in a sandwich ELISA and are plotted on the left Y-axis in molar. Free drug levels (unbound) are plotted on the second Y-axis on the right hand side. Free drug levels remain in excess over the complex over the three-week duration following the initial single dose and during the repeat dose stage (note different scale on the two Y-axes). Free VEGF-A levels, which were detectable pre-dose in eight out of twenty-two patients, became undetectable 15 min subsequent to a single dose of 0.5 mg/kg and above and remained undetectable over the three-week period (Lower Level of Quantification: five pg/ml).

[0012] **Figure 5** shows the serum MMP2 levels when dosing said variant of a lipocalin mutein (SEQ ID NO: 1) to subjects. A reduction in the levels of soluble MMP2 was observed at a dose level of 0.5 mg/kg, 1.5 mg/kg, 3.0 mg/kg, 6.0 mg/kg, and 10.0 mg/kg. When analysed across cohorts, the effect was highly significant ($p<0.01$ by test with Benjamini & Hochberg correction for multiple comparisons).

DETAILED DESCRIPTION

[0013] The present disclosure relates to novel uses of muteins or fragments (or variants thereof) derived from a lipocalin, e.g., a human tear lipocalin, that inhibit the binding of vascular endothelial growth factor (VEGF) to a VEGF receptor. The mutein may be a pharmaceutical composition, for example. Generally, when a method of treatment is described herein which comprises administering a lipocalin, for example, in the form of a pharmaceutical composition, as described herein, such a disclosure is equal to (i) a lipocalin, for example, in the form of a pharmaceutical composition, for use in a method of treatment of any of the diseases described herein or (ii) the use of a lipocalin for the preparation of a pharmaceutical composition/medicament for the treatment of any of the diseases described herein. Put differently, all aspects,

embodiments, etc. described herein in the context of a method of treatment, whereby a lipocalin, e.g., in the form of a pharmaceutical composition, as described herein is applied for the tretament of a disease are also disclosed as lipocalin for example, in the form of a pharmaceutical composition, for use in a method of treatment of any of the diseases described herein or (ii) the use of a lipocalin for the preparation of a pharmaceutical composition/medicament for the treatment of any of the diseases described herein. Accordingy, all embodiments, aspects, etc. described herein in the context of a method of treatment are equally applicable to (i) and (ii), see above, *mutatis mutandis*.

[0014] In one aspect, the disclosure relates to a method of treating a disease or disorder caused or promoted by increased vascularisation in a subject. The subject preferably is a human (e.g., a patient), but may also include other mammals such as a dog, a mouse, a rat, a pig, or an ape such as cymologous. Therapeutic methods disclosed herein involve a step of administering to the subject an amount of a pharmaceutical composition containing a lipocalin mutein that has a 70% or greater sequence identity or homology to a sequence depicted in a SEQ ID NO: 1, 2, 3, 4, 5, 10, 11, 12, 13, or 14. Processes for determining sequence identity and homology are well known to the worker skilled in the art. The lipocalin mutein applied in the methods and/or uses of the invention acts as a VEGF antagonist by inhibiting the binding of VEGF to its receptor (e.g., VEGF-R1, VEGF-R2, and/or Neuropilin-I).

[0015] It is preferred that the 70% or greater identity or homology of a lipocalin mutein sequence acting as VEGF antagonist as described herein to a sequence depicted in SEQ ID NO: 1, 2, 3, 4, 5, 10, 11, 12, 13, or 14 is between the amino acid stretches outside of the four loops that confine binding. This means that a lipocalin mutein having 70% or greater identity or homology to a lipocalin mutein as described herein has preferably the same loop regions as the lipocalin mutein shown in SEQ ID NOs: 1, 2, 3, 4, 5, 10, 11, 12, 13, or 14, but has a 70% or greater identity or homology to the lipocalin mutein shown in SEQ ID NOs: 1, 2, 3, 4, 5, 10, 11, 12, 13, or 14 in regions outside the four loop regions. The skilled person can easily determine the loop regions within a lipocalin mutein protein. For example, WO 2008/015239 or WO 2005/19256 describes the four loop regions of human tear lipocalin.

[0016] However, it is more preferred that the 70% or greater identity or homology of a lipocalin mutein sequence acting as VEGF antagonist as described herein to a sequence depicted in SEQ ID NO: 1, 2, 3, 4, 5, 10, 11, 12, 13, or 14 is over the entire length of the reference lipocalin mutein.

[0017] As used herein, a "mutein," a "mutated" entity (whether protein or nucleic acid) or "mutant" refers to the exchange, deletion, or insertion of one or more nucleotides or amino acids, respectively, compared to the naturally occurring (wild-type) nucleic acid or protein "reference" scaffold. The preferred (wild-type) reference scaffold has the amino acid sequence deposited with SWISS PROT under accession number P31025 (SEQ ID No: 15), preferably lacking amino acids 1-18. This amino acid sequence is shown in SEQ ID No: 16. Hence, also the amino acid sequence shown in SEQ ID No: 16 is a preferred reference scaffold. Preferably, the number of nucleotides or amino acids, respectively, that is exchanged, deleted or inserted in a lipocalin mutein in relation to a reference lipocalin is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more such as 25, 30, 35, 40, 45 or 50 or more in relation to human tear lipocalin, preferably mature human tear lipocalin. Thus, apart from the lipocalin mutein of SEQ ID No: 1-5, 10-14 any other human tear lipocalin mutein that acts as a VEGF antagonist can be applied. Such other lipocalin muteins may have changes, deletions or insertions as described above in relation to the reference lipocalin scaffold as described herein. They can be prepared in accordance with the disclosure of, e.g., WO 2005/19256.

[0018] The amount of a pharmaceutical composition that can be administered to a subject is that which is not only safe and well tolerated in the subject, but also of a sufficient amount to yield a satisfactory therapeutic readout in said subject. As used herein, a "safe and well tolerated amount" is an amount that does not cause any unacceptable serious adverse event in the subject, which preferably include the absence of any detectable anti-drug antibodies subsequent to the administration of pharmaceutical composition to the subject (single or repeat doses). A "satisfactory therapeutic readout" can be any one or more of the following: (i) stable disease, (ii) regression of disease, (iii) the measurement of a biomarker (either positively or negatively regulated) indicating a sufficient amount of target antagonization is

occurring to treat disease (here, antagonization of VEGF, preferably VEGF-A, to its receptor(s)), (iv) reduction of circulating free target (here VEGF, preferably VEGF-A) in the subject below quantifiable amounts within a range of several minutes to several days after the administration of the pharmaceutical composition to the subject; and (v) a hypertension event that is treatable with standard of care.

[0019] The quantitative amount of a pharmaceutical composition that can be administered to a subject can span a wide range and frequency. For example, the amount of administered pharmaceutical composition may be at least 3 mg/kg in the subject and the frequency of administration may be not less frequent than 3 mg/kg at each dose every four weeks over a period of at least four weeks. Preferably, the amount at each dose is selected from the group consisting of: at least 4 mg/kg, at least 5 mg/kg, at least 6 mg/kg, at least 7 mg/kg, at least 8 mg/kg, at least 9 mg/kg, and at least 10 mg/kg in the subject, while the frequency of administration may be not less frequent than a period of time selected from the group consisting of: every three weeks, every two weeks, every week, and twice per week.

[0020] A biomarker whose expression level in the subject indicate target antagonization is occurring in an amount to treat disease include any of the following: MCP1, IL8, KC, MMP2, MMP3, MMP9, IL6, MMP1, RANTES, IL1B, Apolipoprotein A1, Apolipoprotein E, DCN, CILP and COMP. Also included can be the measurement of soluble VEGF in the subject, where reduction of VEGF levels below a level of quantification in the subject with a sensitive assay is indicative that a sufficient amount of target antagonization is occurring to treat disease.

[0021] The pharmaceutical composition may be dosed to a subject in a variety of methods, including via any parenteral or non-parenteral (enteral) route that is therapeutically effective for proteinaceous drugs. Parenteral application methods comprise, for example, intracutaneous, subcutaneous, intramuscular or intravenous injection and infusion techniques, e.g., in the form of injection solutions, infusion solutions, or tinctures. Where administration is via intravenous infusion, the pharmaceutical composition can be administered over a period of time selected from

the group consisting of: up to fifteen minutes, up to thirty minutes, up to one hour, up to two hours, and up to three hours.

[0022] A disease or disorder to be treated in accordance with the therapeutic methods disclosed herein include cancer and preferably a cancer selected from the group consisting of carcinomas of the gastrointestinal tract, rectum, colon, prostate, ovaries, pancreas, breast, bladder, kidney, endometrium, and lung, leukaemia, and melanoma.

[0023] A pharmaceutical composition provided for herein contains a lipocalin mutein that binds VEGF with a high affinity and specificity. Preferably, the KD of such a lipocalin mutein represents an affinity selected from the group consisting of: 200 nM or less, 100 nM or less, 20 nM or less, and 1 nM or less.

[0024] A pharmaceutical composition provided for herein contains a lipocalin mutein that is represented by SEQ ID NOs: 1-5, 10, 11, 12, 13, or 14 or a variant thereof. Preferably, the conservative variant has a sequence identity or homology of at least 70%, 75%, 80%, 85%, 90%, or 95% to the amino acid represented by SEQ ID NOs: 1-5, 10, 11, 12, 13, or 14.

[0025] The skilled worker will appreciate methods useful to prepare lipocalin muteins contemplated by the present disclosure but whose protein or nucleic acid sequences are not explicity disclosed herein. As an overview, such modifications of the amino acid sequence include, e.g., directed mutagenesis of single amino acid positions in order to simplify sub-cloning of a mutated lipocalin gene or its parts by incorporating cleavage sites for certain restriction enzymes. In addition, these mutations can also be incorporated to further improve the affinity of a lipocalin mutein for VEGF. Furthermore, mutations can be introduced to modulate certain characteristics of the mutein such as to improve folding stability, serum stability, protein resistance or water solubility or to reduce aggregation tendency, if necessary. For example, naturally occurring cysteine residues may be mutated to other amino acids to prevent disulphide bridge formation.

[0026] Accordingly, the disclosure also includes functional fragments or variants of lipocalin muteins disclosed herein, which have a threshold sequence identity or sequence homology to a reference lipocalin or lipocalin mutein, preferably the reference (wild-type) lipocalin has the amino acid sequence deposited with SWISS PROT having accession number P31025 (SEQ ID NO. 15) or has the amino acid sequence shown in SEQ ID NO: 16 (mature human tear lipocalin) and the reference lipocalin mutein is shown in any one of SEQ ID Nos: 1-5, 10, 11, 12, 13, or 14, respectively. By "identity" or "sequence identity" as used in the present disclosure, it is meant a property of sequences that measures their similarity or relationship. The term "sequence identity" or "identity" as used in the present disclosure means the percentage of pair-wise identical residues - following (homologous) alignment of a sequence of a polypeptide of the disclosure (e.g., any lipocalin mutein of the disclosure or the wide-type human tear lipocalin) with a sequence in question - with respect to the number of residues in the longer of these two sequences. Percent identity is determined by dividing the number of identical residues by the total number of residues and multiplying the product by 100. The term "homology" is used herein in its usual meaning and includes identical amino acids as well as amino acids which are regarded to be conservative substitutions (for example, exchange of a glutamate residue by an aspartate residue) at equivalent positions in the linear amino acid sequence of a polypeptide of the disclosure (e.g., any lipocalin mutein of the disclosure or the wide-type human tear lipocalin).

[0027] The percentage of sequence homology or sequence identity can, for example, be determined herein using the program BLASTP, version blastp 2.2.5 (November 16, 2002; cf. Altschul, S. F. et al. (1997) Nucl. Acids Res. 25, 3389-3402). In this embodiment the percentage of homology is based on the alignment of the entire polypeptide sequences (matrix: BLOSUM 62; gap costs: 11.1; cutoff value set to 10-3) including the propeptide sequences, preferably using the wild type protein scaffold as reference in a pairwise comparison, e.g. the lipocalin protein scaffold whose amino acid sequence is deposited with SWISS PROT accession number P31025 (SEQ ID NO: 15), more preferably using mature wild-type protein scaffold as reference (SEQ ID NO. 16). Likewise, any of the lipocalin muteins shown in SEQ ID

NOs: 1-5 and 10-14 can serve as reference. Homology or identity, respectively, is calculated as the percentage of numbers of "positives" (homologous amino acids) indicated as result in the BLASTP program output divided by the total number of amino acids selected by the program for the alignment.

[0028] The term "fragment" as used in the present disclosure in connection with the lipocalin muteins of the disclosure relates to proteins or peptides derived from full-length mature human tear lipocalin that are N-terminally and/or C-terminally shortened, i.e., lacking at least one of the N-terminal and/or C-terminal amino acids. Such fragments comprise preferably at least 10, more preferably 20, most preferably 30 or more consecutive amino acids of the primary sequence of mature human tear lipocalin and are usually detectable in an immunoassay of mature human tear lipocalin.

[0029] The term "variant" as used in the present disclosure relates to derivatives of a protein or peptide that comprise modifications of the amino acid sequence, for example by substitution, deletion, insertion or chemical modification. Preferably, such modifications do not reduce the functionality of the protein or peptide. Such variants include proteins, wherein one or more amino acids have been replaced by their respective D-stereoisomers or by amino acids other than the naturally occurring 20 amino acids, such as, for example, ornithine, hydroxyproline, citrulline, homoserine, hydroxylysine, and norvaline. However, such substitutions may also be conservative, i.e., an amino acid residue is replaced with a chemically similar amino acid residue. Examples of conservative substitutions are the replacements among the members of the following groups: 1) alanine, serine, and threonine; 2) aspartic acid and glutamic acid; 3) asparagine and glutamine; 4) arginine and lysine; 5) isoleucine, leucine, methionine, and valine; and 6) phenylalanine, tyrosine, and tryptophan. On the other hand, it is also possible to introduce non-conservative alterations in the amino acid sequence. Functionality of a lipocalin mutein variant, lipocalin mutein homolog, lipocalin mutein that shares identity to a reference wild-type lipocalin or lipocalin mutein, or fragment thereof as described herein means that the variant, homolog, identical lipocalin, or fragment thereof still acts as a VEGF antagonist by inhibiting the binding of VEGF to its receptor. As a reference lipocalin mutein any one of the

lipocalin muteins shown in SEQ ID Nos: 1, 2, 3, 4, 5, 10, 11, 12, 13, or 14 can be used to determine as to whether a lipocalin mutein variant, lipocalin mutein homolog, lipocalin mutein that shares identity to a reference wild-type lipocalin or lipocalin mutein, or fragment thereof as described herein, acts as a VEGF antagonist. For example, it can be measured as to whether a lipocalin mutein variant, lipocalin mutein homolog, lipocalin mutein that shares identity to a reference wild-type lipocalin or lipocalin mutein, or fragment thereof as described herein competes with the reference lipocalin mutein (which acts as a VEGF antagonist) for inhibiting the binding of VEGF to its receptor. In addition, instead of replacing amino acid residues, it is also possible to either insert or delete one or more continuous amino acids of the primary structure of the lipocalin muteins of the disclosure as long as these deletions or insertion result in a stable folded/functional mutein (for example, human tear lipocalin muteins with truncated N- and C-terminus). In such mutein, for instance, one or more amino acid residues are added or deleted at the N- or C-terminus of a polypeptide of the disclosure (e.g., any lipocalin mutein of the disclosure or the wide-type human tear lipocalin). Generally such a mutein may have about at least 70 %, including at least about 80%, such as at least about 85 % amino acid sequence identity, with the amino acid sequence of the mature human tear lipocalin. As an illustrative example, any one amino acid residue of the first four N-terminal amino acid residues (His, His, Leu, Ala) can be deleted in a tear lipocalin mutein of the disclosure without affecting the biological function of the protein, e.g., in SEQ ID NOs: 1-3.

[0030] Additionally, a lipocalin mutein provided for herein may be modified to alter the pharmacokinetics in a subject. For example, the terminal half-life of a lipocalin mutein may contain a PEGylated moiety ranging from 10 kiloDalton to 40 kiloDalton or even greater. Preferably, a lipocalin mutein contains a PEGylated moiety that is 40 kiloDalton in molecular weight. The half-life of a pharmaceutical composition disclosed herein (as modified to increase its half-life) preferably is at least about four, five, six, or seven days in the subject. VEGF-binding lipocalin muteins as well as PEGylated variants thereof are disclosed in WO 2011/015634

[0031] The “subject” as used in the present disclosure (e.g., being or to be administered with the pharmaceutical composition of the disclosure) may be a

mammal, such as a human (e.g., a patient), a dog, a mouse, a rat, a pig, and ape such as cymologous to name only a few illustrative examples.

[0032] A pharmaceutical composition of the present invention may contain an additional agent that inhibits activity of VEGF such as a compound having anti-angiogenesis activity. The additional agent may be administered together with the pharmaceutical composition of the invention, i.e. simultaneously, or subsequently, i.e., before or after the pharmaceutical composition of the invention is/was administered.

[0033] The disclosure is further illustrated by the following non-limiting Examples and the attached drawings. However, these Examples should not be construed so as to limit the disclosure. Rather, they are merely exemplary embodiments.

EXAMPLES

[0034] The inventors have found that a lipocalin mutein specific for VEGF is well-tolerated by the subject when administered, e.g., as a 2 hour infusion at doses up to 10mg/kg. In this vein, a 10 mg/kg dose is not a maximum tolerated dose (MTD); nor do doses at this concentration demonstrate cumulative toxicity. Further, no ADAs were observed when administered to about twenty-five patients for up to seventeen doses and at a concentration of up to 10 mg/kg.

[0035] In this context, patients were administered pharmaceutical composition on days 1, 22, 29, 36, and 43 and, provided the patients exhibited stable disease on Day 43, further bi-weekly administrations until tumor progression (evaluated by RECIST) was observed. Patients who received pharmaceutic composition were suffering from the following typs of cancer: colorectal (12), Melanoma (3), Pancreatic adenocarcinoma (2), GI Neuroendocrine (2), HCC (2), prostate (1), breast (1), leiomyosarcoma (1), esophagus (1), adenoid cystic (1). Prior Therapeutic Regimens were a median of four, ranging from one to eleven.

[0036] PK was assessed after the first administration, pre dose, 15 and 30 minutes, and 1, 2, 4, 8, 24, 48, 96, 336, and 504 hours, and also assessed pre dose, on days, 22, 29, 36, 43 and 71 during the weekly repeat dose stage. Anti-drug

antibodies and PD markers including target engagement (drug-target complex), free plasma VEGF-A, serum MMP-2 levels, and DCE-MRI were assessed repetitively. Tumor response assessment was conducted through standard imaging assessments at baseline, day 43, and every 8 weeks until progressive disease, evaluated by RECIST.

[0037] The inventors also observed numerous satisfactory therapeutic readouts following the administration of a lipocalin mutein specific for VEGF. For example, hypertension (e.g., Grade 3 hypertension) was observed in greater than 10% of subjects, an expected (and manageable) on-target effect of angiogenesis inhibitors.

[0038] In addition, following the administration of as little as a single dose of 0.5 mg/kg of a pharmaceutical composition, soluble VEGF-A became undetectable within a time period of less than about thirty minutes and remained undetectable (i.e., below levels of quantification (below 5pg/ml)) over a period of about three weeks; in parallel the complex between drug and VEGF target was measurable, clearly demonstrating target engagement by the lipocalin mutein. Lipocalin mutein plasma PK samples were used to determine levels of free VEGF-A and VEGF-A-drug complex (Fig. 4 VEGF-A Target Engagement and Free Drug Levels). For the PK assay, free drug was captured on VEGF-coated plates and was detected by a biotinylated, affinity purified anti- drug antiserum. The drug-target complex was captured on plates coated with a non-competitive anti VEGF-A antibody and was detected by a biotinylated, affinity purified non-competitive anti-drug antiserum. Free VEGF-A captured on VEGF R2-coated plates was detected by a non-competitive, biotinylated anti-VEGF-A antibody. Free VEGF-A levels were detectable at baseline in 8/22 pts, becoming undetectable after about 15 minutes subsequent to the infusion of lipocalin mutein, and remained so for at least 21 days in all patients dosed at 0.5 mg/kg and above. Drug-target complex formation was detectable for up to 3 weeks at all dose levels (22/22 pts) including patients with no detectable free VEGF-A at baseline. The significant excess of free lipocalin mutein over complex during dosing period suggests no unbound VEGF-A activity is available. Time-dependent increase of drug-target complex after dosing the drug likely reflects restricted clearance of the complex when compared to free target, while compensatory VEGF-A upregulation may also occur (similar to other

VEGF inhibitors). Free VEGF-A was undetectable after dosing the lipocalin mutein at 0.5 mg/kg or above for 3 weeks (Lower Level of Quantification: 5 pg/ml), confirming lack of unbound VEGF-A activity in circulation, namely target saturation.

[0039] Furthermore, decreased serum levels of Matrix Metalloprotease-2 (MMP2) were observed, serving as a pharmacodynamic biomarker of VEGF-A inhibition under treatment. In this respect, expression of MMP2 is positively regulated down-stream of VEGF receptor activitation via VEGF ligand. To quantify biomarker levels, e.g., MMP2, a bead-based multiplex approach was used to interrogate potential changes in a panel of oncology-related biomarkers. A reduction in the levels of soluble MMP2 was observed at a dose level of 0.5 mg/kg and above (Fig. 5 Serum MMP2 Levels). VEGF-A is known to positively regulate MMP2 transcription, secretion, and activity (Ispanovic, *Am J Physiol Cell Physiol* 2008). MMP2 activity mediates invasive processes, and its systemic reduction has been documented for cediranib-treated recurrent Glioblastoma patients (phase 1) (Batchelor, *JCO* 2010). The observed reduction in circulating MMP2 levels under treatment suggests that MMP2 reduction serves as a pharmacodynamics biomarker. Thus, MMP2 levels are pharmacodynamic biomarkers for a lipocalin mutein provided for herein.

[0040] Further, stable disease (SD) was observed in 9 patients, including one melanoma patient with SD for 10 months. The median duration of SD was 3 months when median time on study for all patients was 6 weeks.

[0041] PD/PK modeling supports, e.g., weekly, bi-weekly or every-three weekly dosing of a pharmaceutical composition disclosed herein. In particular, dosing required to reach effective AUC or trough levels (C_{min}) was extrapolated from preclinical data where PK information on effective doses in mouse models was combined with PK information in humans. PK/PD modeling was performed with the aim to predict clinically effective doses of the pharmaceutical composition, predicting a set forth in Table 1, for example:

Dosing interval (Days)	Projected efficacious dose (mg/kg)	
	Based on AUC	Based on C_{min}
7	3	1
14	6	3
21	9	8

***

[0042] The following disclosure is not limited to the above Example. When used in above invented methods, it may be advantageous to use the said muteins of human tear lipocalin in the form of fusion proteins. In some embodiments, the said mutein of human tear lipocalin is fused at its N-terminus or its C-terminus to a protein, a protein domain or a peptide such as a signal sequence and/or an affinity tag. As is evident from the above disclosure, the muteins used in the present disclosure or a fusion protein or a conjugate thereof can be employed in all applications disclosed above.

[0043] A pharmaceutical composition disclosed herein comprises the said mutein of human tear lipocalin or a fusion protein or conjugate thereof and a pharmaceutically acceptable excipient. Accordingly, the said mutein of human tear lipocalin can be formulated into compositions using pharmaceutically acceptable ingredients as well as established methods of preparation (Gennaro, A.L. and Gennaro, A.R. (2000) *Remington: The Science and Practice of Pharmacy*, 20th Ed., Lippincott Williams & Wilkins, Philadelphia, PA). To prepare the pharmaceutical compositions, pharmaceutically inert inorganic or organic excipients can be used. To prepare e.g. pills, powders, gelatine capsules or suppositories, for example, lactose, talc, stearic acid and its salts, fats, waxes, solid or liquid polyols, natural and hardened oils can be used. Suitable excipients for the production of solutions, suspensions, emulsions, aerosol mixtures or powders for reconstitution into solutions or aerosol mixtures prior to use include water, alcohols, glycerol, polyols, and suitable mixtures thereof as well as vegetable oils.

[0044] As used in in the present disclosure, VEGF may be selected from the group consisting of VEGF-A, VEGF-B, VEGF-C, and VEGF-D and preferably is VEGF-A. The amino acids sequence for VEGF-A, VEGF-B, VEGF-C, and VEGF-D are depicted, respectively, in SEQ ID NOs: 6-9 and may have the amino acid sequences set forth in SWISS PROT Data Bank Accession Numbers P15692, P49765, P49767, and O43915.

[0045] As used herein, "polypeptide", "peptide" and "protein" are used interchangeably and include reference to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The terms also apply to polymers containing conservative amino acid substitutions such that the protein remains functional.

[0046] The term "residue" or "amino acid residue" or "amino acid" includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "peptide"). The amino acid can be a naturally occurring amino acid and, unless otherwise limited, can encompass known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

[0047] It must be noted that as used herein, the singular forms “a”, “an”, and “the”, include plural references unless the context clearly indicates otherwise. Thus, for example, reference to “a reagent” includes one or more of such different reagents and reference to “the method” includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

[0048] All publications and patents cited in this disclosure are incorporated by reference in their entirety. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material.

[0049] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the disclosure described herein. Such equivalents are intended to be encompassed by the present disclosure.

[0050] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term “comprising” can be substituted with the term “containing” or sometimes when used herein with the term “having”.

[0051] When used herein “consisting of" excludes any element, step, or ingredient not specified in the claim element. When used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim. In each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms.

[0052] As used herein, the conjunctive term “and/or” between multiple recited elements is understood as encompassing both individual and combined options. For instance, where two elements are conjoined by “and/or”, a first option refers to the applicability of the first element without the second. A second option refers to the applicability of the second element without the first. A third option refers to the applicability of the first and second elements together. Any one of these options is understood to fall within the meaning, and therefore satisfy the requirement of the term “and/or” as used herein. Concurrent applicability of more than one of the options is also understood to fall within the meaning, and therefore satisfy the requirement of the term “and/or” as used herein.

[0053] Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific

publications, manufacturer's specifications, instructions, etc.), whether supra or infra, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the disclosure is not entitled to antedate such disclosure by virtue of prior disclosure.

[0054] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention. The invention has been described broadly and generically herein. All patents, patent applications, textbooks and peer-reviewed publications described herein are hereby incorporated by reference in their entirety. Furthermore, where a definition or use of a term in a reference, which is incorporated by reference herein is inconsistent or contrary to the definition of that term provided herein, the definition of that term provided herein applies and the definition of that term in the reference does not apply. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. Further embodiments of the invention will become apparent from the following claims.

CLAIMS

1. A pharmaceutical composition comprising a lipocalin mutein with at least a 70% sequence identity or homology to SEQ ID NOs: 1-5, 10, 11, 12, 13, or 14 for use in a method of treating a disease or disorder caused or promoted by increased vascularisation in a subject, said method comprising administering to the subject an amount of said pharmaceutical composition comprising said lipocalin, wherein said lipocalin mutein acts as a VEGF antagonist by inhibiting the binding of VEGF to its receptor, and wherein said amount of said composition is a safe and well tolerated amount in said subject and is sufficient to yield a satisfactory therapeutic readout in said subject.

2. The pharmaceutical composition according to the use of claim 1, wherein said amount is at least 3 mg/kg in the subject and the frequency of administration is no less frequent than 3 mg/kg at each dose every four weeks over a period of at least four weeks.

3. The pharmaceutical composition according to the use of claim 2 wherein said amount at each dose is selected from the group consisting of: at least 4 mg/kg, at least 5 mg/kg, at least 6 mg/kg, at least 7 mg/kg, at least 8 mg/kg, at least 9 mg/kg and at least 10 mg/kg in the subject.

4. The pharmaceutical composition according to the use of claim 2 or 3, wherein the frequency of administration is no less frequent than a period of time selected from the group consisting of: every three weeks, every two weeks, every week and twice per week.

5. The pharmaceutical composition according to the use of any one of claims 1-4, wherein the therapeutic readout is selected from the group consisting of: (i) reduced levels of MCP1, IL8, KC, MMP2, MMP3, MMP9, IL6, MMP1, RANTES, IL1B, Apolipoprotein A1, Apolipoprotein E, DCN, CILP and/or COMP in the subject; and (ii) reduced soluble VEGF levels below a level of quantification in the subject.

6. The pharmaceutical composition according to the use of claims 1-5, wherein each dose of the pharmaceutical composition is administered intravenously over a period of time slected from the group consisting of: up to fifteen minutes, up to thirty minutes, up to one hour, up to two hours and up to three hours.

7. The pharmaceutical composition according to the use of the preceding claims, wherein the disease or disorder is a cancer selected from the group consisting of carcinomas of the gastrointestinal tract, rectum, colon, prostate, ovaries, pancreas, breast, bladder, kidney, endometrium, and lung, leukaemia, and melanoma.

8. The pharmaceutical composition according to the use of any of the preceding claims, wherein the receptor for VEGF is selected from the group consisting of VEGF-R1, VEGF-R2, and Neuropilin-I.

9. The pharmaceutical composition according to the use of any of the preceding claims, wherein the mutein binds VEGF with a KD of an affinity selected from the group consisting of: 200 nM or less, 100 nM or less, 20 nM or less and 1 nM or less.

10. The pharmaceutical composition according to the use of any of the preceding claims, wherein the mutein has a sequence identity or homology of at least a 75%, 80%, 85%, 90% or 95% to SEQ ID NOs: 1-5, 10, 11, 12, 13, or 14.

11. The pharmaceutical composition according to the use of the preceding claims, wherein the mutein has an amino acid sequence as set forth in any one of SEQ ID NOs: 1-5, 10, 11, 12, 13, or 14 or of a fragment or variant thereof.

12. The pharmaceutical composition according to the use of any of the preceding claims, wherein the pharmaceutical composition has a half-life in the subject of about four, five, six or seven days.

Figure 1

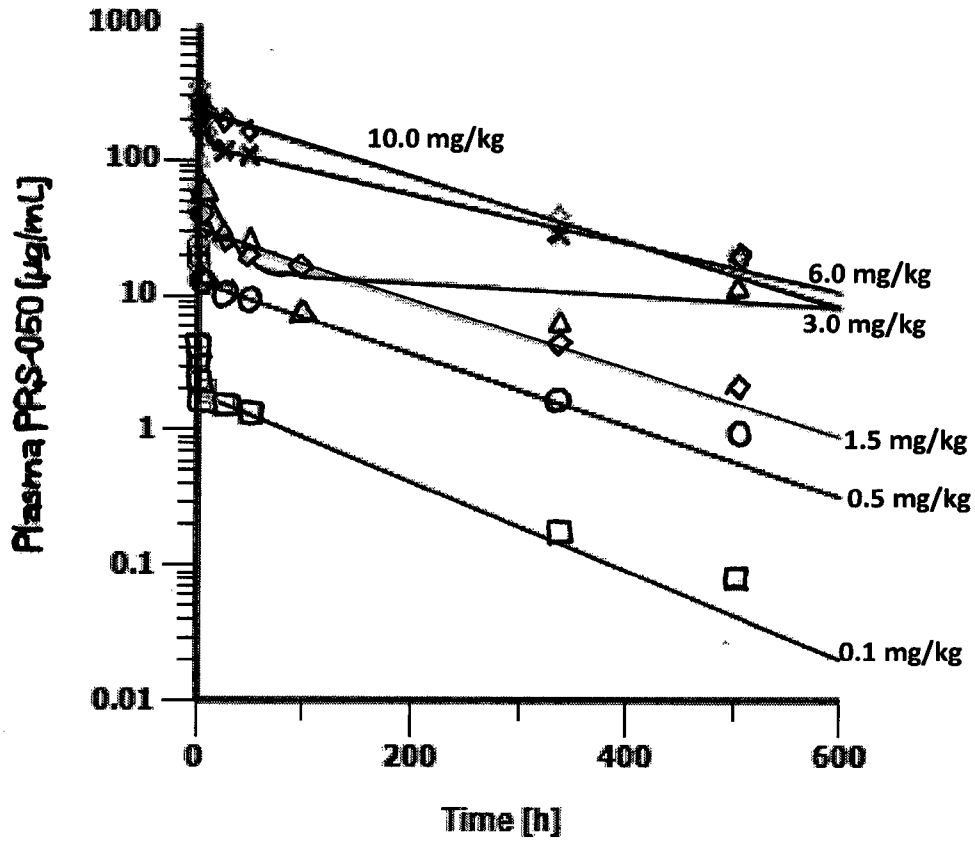

1000
100
10
1
0.1
0.01
Plasma PRS-050 [µg/mL]
10.0 mg/kg
6.0 mg/kg
3.0 mg/kg
1.5 mg/kg
0.5 mg/kg
0.1 mg/kg
0
200
400
600
Time [h]
0.1 mg/kg
0.5 mg/kg
1.5 mg/kg
3.0 mg/kg
6.0 mg/kg
10.0 mg/kg

Figure 2

Cohort (mg/kg)	Cmax (µg/ml)	$AUC_{0-\infty}$ (µg*h/ml)	DPF* $AUC_{0-\infty}$	CL (ml/min/kg)	$T_{½}$ (days)
0.1 n=3	4.3	262	-	0.0088	3.6
0.5 n=4	23.3	2,120	1.8	0.0042	5.0
1.5 n=6	48.6	5,020	0.8	0.0056	5.5
3.0 n=3	83.4	10,800	1.1	0.0046	6.6
6.0 n=3	228	26,600	1.2	0.0042	5.5
10.0 n=5	396	43,000	1.0	0.0040	5.9

* DPF= Dose Proportionality Factor: Ratio of fold increase in AUC divided by fold increase in dose

Figure 3

Cohort (mg/kg)	C_{min}* (µg/ml)
0.1 n=3	1.0
0.5 n=4	7.1
1.5 n=3	20.5
3.0 n=2	69.9
6.0 n=3	106.5
10.0 n=4	144.5

*Trough level immediately prior to the 4th weekly administration (Day 43)

Figure 4

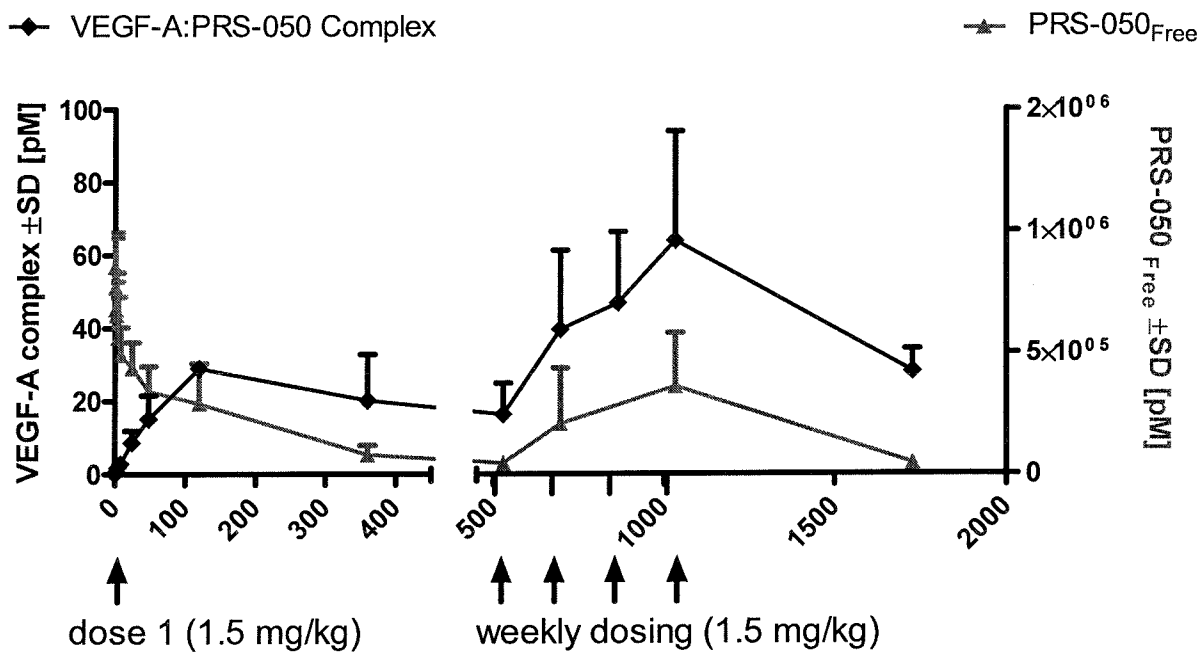
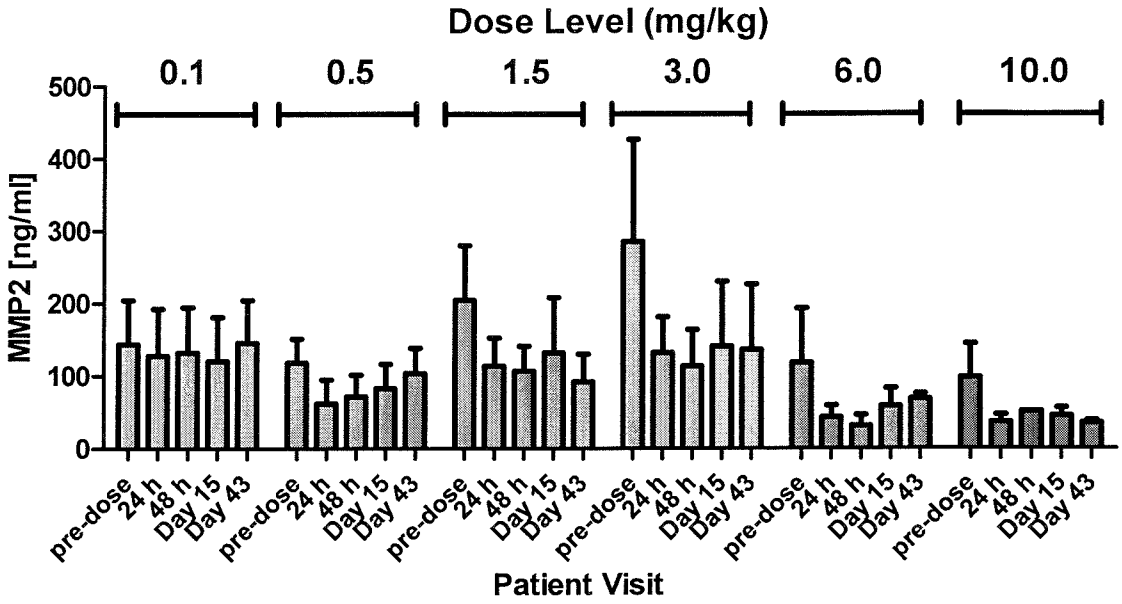

VEGF-A:PRS-050 Complex
PRS-050Free
VEGF-A complex ±SD [pM]
PRS-050 Free ±SD [pM]
100
80
60
40
20
0
2×10^06
1×10^06
5×10^05
0
0
100
200
300
400
500
1000
1500
2000
dose 1 (1.5 mg/kg)
weekly dosing (1.5 mg/kg)

Figure 5


Dose Level (mg/kg)
0.1
0.5
1.5
3.0
6.0
10.0
MMP2 [ng/ml]
500
400
300
200
100
0
pre-dose
24 h
48 h
Day 15
Day 43
Patient Visit

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/072406

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17 A61P35/00
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)
A61K

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, EMBASE, CHEM ABS Data, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2012/136685 A1 (PIERIS AG [DE]; AUDOLY LAURENT [US]) 11 October 2012 (2012-10-11) the whole document	1,7-12
X	WO 2011/015634 A2 (PIERIS AG [DE]; HOHLBAUM ANDREAS [DE]; HUELSMEYER MARTIN [DE]; GILLE H) 10 February 2011 (2011-02-10) page 34, paragraph 4 - page 36, paragraph 3; claims 1,25,34,60-62	1-12

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

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 2013	24/04/2013

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	Greif, Gabriela

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/015239 A2 (PIERIS AG [DE]; JENSEN KRISTIAN [DE]; HUELSMEYER MARTIN [DE]; SCHLEHUB) 7 February 2008 (2008-02-07) page 19, line 1 - page 21, line 15; claims 1,14,34,35 page 24, line 17 - page 26, line 8 page 36, line 13 - page 40, line 20; figures 21-25 page 50, line 18 - page 51, line 30 examples 14-27,31-40,52	1-12
A	Z. TONG ET AL: "Neutrophil Gelatinase-Associated Lipocalin: A Novel Suppressor of Invasion and Angiogenesis in Pancreatic Cancer", CANCER RESEARCH, vol. 68, no. 15, 1 August 2008 (2008-08-01), pages 6100-6108, XP055059140, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-08-0540 the whole document	1-12
Y	LINKOV F ET AL: "Early detection of head and neck cancer: Development of a novel screening tool using multiplexed immunobead-based biomarker profiling", CANCER EPIDEMIOLOGY, BIOMARKERS AND PREVENTION, PHILADELPHIA, PA, US, vol. 16, no. 1, 1 January 2007 (2007-01-01), pages 102-107, XP002595784, ISSN: 1055-9965, DOI: 10.1158/1055-9965.EPI-06-0602 the whole document	5
Y	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; October 2011 (2011-10), LIANG HUASHENG ET AL: "Diagnostic Value of 16 Cellular Tumor Markers for Metastatic Thyroid Cancer: An Immunohistochemical Study", XP002695238, Database accession no. PREV201100680444 abstract & ANTICANCER RESEARCH, vol. 31, no. 10, October 2011 (2011-10), pages 3433-3440, ISSN: 0250-7005(print)	5

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1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KNIPPING G ET AL: "Quantification of apolipoprotein D by an immunoassay with time-resolved fluorescence spectroscopy", JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 202, no. 1, 1 January 1997 (1997-01-01), pages 85-95, XP004056206, ISSN: 0022-1759, DOI: 10.1016/S0022-1759(96)00240-2 abstract -----	5

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/072406

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		KR 20120099371 A	10-09-2012
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