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(71) Applicant *(for all designated States except US)*: **PIERIS AG** [DE/DE]; Lise-Meitner-Strasse 30, 85354 Freising-Weihenstephan (DE).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **AUDOLY, Laurent** [US/US]; 2279 Nash Court, Mahwah, New Jersey 07430 (US).

(74) Agents: **WEINZIERL, Gerhard** et al.; Schiweck Weinzierl Koch, European Patent Attorneys, Landsberger Str. 98, 80339 Munich (DE).

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(54) Title: METHODS AND COMPOSITIONS FOR ANTI-VEGF AND ANTI-C-MET THERAPY

(57) Abstract: Efforts at treating a host of cancers have, separately, focused on antagonizing VEGF, a soluble ligand implicated in angiogenesis by binding to VEGF-receptor, and c-Met, a receptor tyrosine kinase that is mutated or differentially expressed in various tumors. Although these two targets are involved in different signaling pathways, the present inventors have surprisingly discovered that co-administration of specific-binding therapeutic proteins targeting VEGF and c-Met, respectively, has yielded a synergistic result in both *in vitro* and *in vivo* models, compared to administering these therapeutic proteins individually. The present invention, therefore, focuses on therapeutic methods resulting from this discovery and compositions for implementing the same.

Methods and Compositions for anti-VEGF and anti-c-Met therapy

TECHNICAL FIELD OF THE INVENION

[0001] Efforts at treating a host of cancers have, separately, focused on antagonizing VEGF, a soluble ligand implicated in angiogenesis by binding to VEGF-receptor, and c-Met, a receptor tyrosine kinase that is mutated or differentially expressed in various tumors. Although these two targets are involved in different signaling pathways, the present inventors have surprisingly discovered that co-administration of specific-binding therapeutic proteins targeting VEGF and c-Met, respectively, has yielded a synergistic result in both *in vitro* and *in vivo* models, compared to administering these therapeutic proteins individually. The present invention, therefore, focuses on therapeutic methods resulting from this discovery and compositions for implementing the same.

BACKGROUND OF THE INVENTION

[0002] Cancer is a major cause of death worldwide, being the second-leading cause of death in developed countries, and even the number one cause of death in e.g. Australia, Japan, Korea, Singapore and the male population of the UK and Spain. The number of people who develop cancer each year is increasing. Nevertheless cancer therapy has not managed to decrease cancer mortality in the last three decades.

[0003] Hepatocyte growth factor (HGF), also known as scatter factor, which is widely distributed in the extracellular matrix of most tissues, and/or its transmembrane tyrosine kinase receptor, c-Met, can be over-expressed in a large variety of solid tumours. Typically, both are expressed at higher levels in the aggressive phenotype of various carcinomas, such as of the prostate, the stomach, pancreas, thyroid, liver, pancreas, lung, kidney, bladder, ovary, brain, prostate, gallbladder, breast and myeloma tumors.

[0004] In most tumours, c-Met is transcriptionally induced by hypoxia and inflammatory cytokines or pro-angiogenic factors that are abundant in the reactive stroma of full-blown tumours. Thus, activation of c-Met can be a late event that in the onset of cancer, aggravating the intrinsic malignant properties of already transformed cells by conveying proliferative, anti-apoptotic and pro-migratory signals. HGF/c-Met signaling has also been found to be activated in angiogenesis (You, W.-K., & McDonald, D.M., BMB reports (2008) 41, 12, 833-839).

[0005] Angiogenesis, which is essential for solid tumor growth, is mediated by a number of regulators, of which the vascular endothelial growth factor VEGF-A (also known as VEGF) seems to be of particular importance. Experiments with neutralizing antibodies and other inhibitors have shown that blockade of the VEGF-A pathway can be sufficient to significantly suppress angiogenesis associated with solid tumor growth in many models.

[0006] Recently, however, research on targeted therapy has revealed effects of VEGF blockade that may even promote a more invasive cellular phenotype and tumor cell dissemination (Casanovas, Cancer Cell (2005, 8(4), 299-309; Paez-Ribes, Cancer Cell (2009) 15, 220-231). One theory that accounts for this effect relies on severe restriction of the oxygen supply to the tumor that occurs when anti-angiogenic agents are used, creating a state of hypoxia. Hypoxia can lead to the transcriptional activation of a number of genes through the stabilization of the HIF-1α transcription factor which, in the presence of oxygen, is earmarked for proteosomal destruction by oxygen-dependent prolyl-hydroxylases. Targeted genes for activation include VEGF-A, itself, which would, in a normal situation, promote angiogenesis in order to overcome the hypoxia.

[0007] Apart from the c-MET receptor, itself, which is induced in hypoxia, HIF1α also induces protease activities that are required to convert pro-HGF to the mature active HGF factor (Michieli, P., Cell Cycle (2009) 8, 20, 3291-3296). c-Met activation is known to confer proliferative and invasive properties to cells, which may result in enhanced tumor growth and contribute to tumor escape from anti-VEGF therapy. c-Met activation may also favor tumor cell migration to distant sites, thus enabling cells to move away from the hypoxic site and driving metastasis.

[0008] Although antagonizing each of these targets, individually, has yielded promising results in the industry, there is room to generate superior results, especially compared to VEGF monotherapy.

[0009] The use of a c-Met antagonist and a VEGF receptor antagonist in the treatment of cancer has previously been disclosed in international patent application WO 2010/045344. According to WO 2010/045344, the c-met antagonist and the VEGF receptor antagonist can both be an immunoglobulin. WO 2010/045344 reports about promising results with the immunoglobulins and is thus completely silent about a need for alternative formats of binding agents such as Anticalins. Thus, in view of what WO 2010/045344 provides, there is no need, so to say, to change the format of an immunoglobulin binding agent to a distinct protein moiety such as a lipocalin mutein.

FIGURES

[0010] **Figure 1**: The effect of dual inhibition of VEGF(A) and c-Met *in vitro* using HUVEC cells

[0011] **Figure 2**: The benefit of co-administered anti-VEGF(A) and anti-c-Met lipocalin muteins in a U87-MG xenograft model

DETAILED DESCRIPTION OF THE DISCLOSURE

[0012] The present inventors hypothesized that antagonizing VEGF and c-Met concomitantly with lipocalins specific for VEGF and c-Met, respectively, could overcome some of the hypoxia-mediated effects that VEGF monotherapy might induce. To their surprise, the studies performed by the present inventors led to a synergistic result in a tumor setting when antagonizing VEGF and c-Met simultaneously compared to antagonizing each of these targets separately. Such synergistic effect could not have been expected and came as a surprise to the inventors. The present disclosure, therefore, contemplates in one aspect an improved method to treat a subject suffering from a tumour. This method includes a step of administering to the subject a therapeutically effective amount of (i) a first lipocalin mutein specific for VEGF and (ii) a second lipocalin mutein specific for c-Met. The present disclosure also relates to a composition comprising a first lipocalin mutein specific for VEGF and (ii) a second lipocalin mutein specific for c-Met for use in a method of treatment or diagnosis as disclosed herein.

[0013] The present disclosure relates to a combination of a first lipocalin mutein and a second lipocalin mutein. One of these lipocalin muteins can bind to VEGF as a given non-natural

target with detectable affinity. The other lipocalin mutein can bind to c-Met as a given non-natural target with detectable affinity. For example, the first lipocalin mutein can bind to VEGF and the second lipocalin mutein can bind to c-Met, or vice versa. The combination of the first lipocalin mutein and the second lipocalin mutein may be provided in various forms.

[0014] In another aspect the disclosure provides for a kit of parts. The kit includes a first and a second container. The first container includes the first lipocalin mutein and the second container includes the second lipocalin mutein.

[0015] The disclosure also provides a combination of the first lipocalin mutein and the second lipocalin mutein for use in therapy and/or for use in diagnosis. The disclosure further relates to the combination of the first lipocalin mutein and the second lipocalin mutein for use in the treatment and/or diagnosis of neoplasia such as a tumour. Further, the disclosure relates to a method of treating a subject suffering from cancer. The method includes a step of administering to the subject the first lipocalin mutein and the second lipocalin mutein. The disclosure also relates to a composition that includes one or more lipocalin muteins. The one or more lipocalin muteins bind to VEGF and to c-Met. In some embodiments the composition includes a first lipocalin mutein that binds to VEGF and a second lipocalin mutein that binds to c-Met. The composition may be a pharmaceutical formulation or be included in such a pharmaceutical formulation. Such a pharmaceutical formulation may further include a pharmaceutically acceptable excipient. It may include a therapeutically effective amount of the one or more lipocalin muteins.

[0016] The present disclosure is generally directed to methods and compositions for treating neoplasia including cancer and metastasis. The compositions are preferably for use in one or more methods of treatment or diagnosis as disclosed herein. A method according to the disclosure may be a method of reducing tumour growth, including a method for treating a tumour in a subject. By the term “subject” is meant any living organism. The subject in need of such a treatment may in some embodiments be a mammal, such as a human, a dog, a cat, a mouse, a rat, a rabbit, a squirrel, a hamster, cattle, a sheep, a pig, a goat, a horse, an ape such as cynomolgus, an orang-utan, a chimpanzee, a woolly monkey, a marmoset, a leopard, a platypus and a human. In some embodiments the subject is a transgenic non-human animal. The methods and uses according to the disclosure concern the prophylactic or therapeutic treatment of a cell

proliferative disorder, such as a tumour or cancer. Any tumour or cancer may be selected for treatment.

[0017] Examples of tumours include haematological malignancies and solid tumours. Solid tumours include a sarcoma, arising from connective or supporting tissues, a carcinoma, arising from the body's glandular cells and epithelial cells or a lymphoma, a cancer of lymphatic tissue, such as the lymph nodes, spleen, and thymus. Examples of a solid tumour include breast cancer, lung cancer, a brain tumour, a neuroblastoma, colon cancer (including colorectal cancer), rectal cancer, bladder cancer, a liver tumour, a pancreatic tumour, ovarian cancer, prostate cancer, cancer of the kidney, cancer of the esophagus, the gall-bladder, ovary, pancreas, stomach, cervix, thyroid, prostate, head and neck cancer, including Glioblastoma Multiformae (GBM), and a melanoma. In some embodiments, the neoplasia to be treated is a brain tumour such as GBM. In some embodiments, the neoplasia to be treated is lung cancer including non-small lung cancer.

[0018] In some embodiments the neoplasia to be treated is a hematopoietic tumour of lymphoid lineage such as leukaemia, acute lymphocytic leukaemia, acute lymphoblastic leukaemia, B-cell lymphoma, T-cell-lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, hairy cell lymphoma and Burkett's lymphoma. In some embodiments the neoplasia to be treated is a hematopoietic tumour of myeloid lineage such as acute and chronic myelogenous leukaemia, myelodysplastic syndrome and promyelocytic leukaemia, or a tumour of mesenchymal origin such as fibrosarcoma and rhabdomyosarcoma or a sarcoma of soft tissue or bone. In some embodiments the neoplasia to be treated is a tumour of the central and/or peripheral nervous system such as astrocytoma, neuroblastoma, glioma and schwannomas. In some embodiments the neoplasia to be treated is a melanoma, seminoma, teratocarcinoma, osteosarcoma, xeroderoma pigmentosum, keratoctanthoma, thyroid follicular cancer or Kaposi's sarcoma. In one method of the disclosure, a first lipocalin mutein and a second lipocalin mutein are administered as defined below.

[0019] As mentioned, the present disclosure is based on the surprising finding that a combination of a lipocalin mutein directed against c-Met and a lipocalin mutein directed against VEGF shows a stronger effect in treating a tumour than the theoretical effect that could be expected on the basis of the use of the individual lipocalin muteins.

[0020] Lipocalins are proteinaceous binding molecules that have naturally evolved to bind ligands. Lipocalins occur in many organisms, including vertebrates, insects, plants and bacteria.

The members of the lipocalin protein family (Pervaiz, S., & Brew, K. (1987) FASEB J. 1, 209-214) are typically small, secreted proteins and have a single polypeptide chain. They 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 signalling, 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).

[0021] 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. This β-barrel forms a central cavity. One end of the barrel is sterically blocked by the N-terminal peptide segment that runs across its bottom as well as 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 flexible 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).

[0022] International patent application WO 99/16873 discloses polypeptides of the lipocalin family with mutated amino acid positions in the region of the four peptide loops, which are arranged at the end of the cylindrical β-barrel structure encompassing the binding pocket, and which correspond to those segments in the linear polypeptide sequence that includes the amino acid positions 28 to 45, 58 to 69, 86 to 99, and 114 to 129 of the bilin-binding protein of Pieris brassicae. Members of the lipocalin family have been reported to be post-translationally modified, e.g. phosphorylation and glycosylation of tear lipocalin (e.g. You, J., et al. (2010)

Electrophoresis 31, 1853-1861). Nevertheless no post-translational modification is required for their molecular recognition properties.

[0023] International patent application WO 00/75308 discloses muteins of the bilin-binding protein, which specifically bind digoxigenin, whereas the international patent applications WO 03/029463 and WO 03/029471 relate to muteins of the human neutrophil gelatinase-associated lipocalin (hNGAL) and apolipoprotein D, respectively. In order to further improve and fine tune ligand affinity, specificity as well as folding stability of a lipocalin variant various approaches using different members of the lipocalin family have been proposed (Skerra, A. (2001) Rev. Mol. Biotechnol. 74, 257-275; Schlehuber, S., and Skerra, A. (2002) Biophys. Chem. 96, 213-228), such as the replacement of additional amino acid residues. As a further example, the PCT publication WO 2006/56464 discloses muteins of human neutrophil gelatinase-associated lipocalin with binding affinity for CTLA-4 in the low nanomolar range.

[0024] International patent application WO 2005/19256 discloses muteins of tear lipocalin with at least one binding site for different or the same target ligand and provides a method for the generation of such muteins of human tear lipocalin. According to this PCT application, certain amino acid stretches within the primary sequence of tear lipocalin, in particular the loop regions that include amino acids 7-14, 24-36, 41-49, 53-66, 69-77, 79-84, 87-98, and 103-110 of mature human tear lipocalin, are subjected to mutagenesis in order to generate muteins with binding affinities. The resulting muteins have binding affinities for the selected ligand, expressed as the K_D value, i.e. the dissociation constant of the complex formed between the respective mutein and its target/ligand, in the nanomolar range, in most cases >100 nM. International patent application WO 2008/015239 discloses muteins of human tear lipocalin with a least one binding site for a non-natural ligand, including the IL-4 receptor, VEGF and the VEGF receptor, and provides a method for the production of such lipocalin muteins. Binding affinities are in the nanomolar range, reaching as low as almost 1×10^{-10} M in surface plasmon resonance experiments.

[0025] A lipocalin mutein of the combination according to the present disclosure may be a mutein of any desired lipocalin. Examples of suitable lipocalins (also sometimes designated as "protein 'reference' scaffolds" or simply "scaffolds") of which a mutein may be used include, but are not limited to, tear lipocalin (lipocalin-1, von Ebner gland protein), retinol binding protein, neutrophil, lipocalin-type prostaglandin D-synthase, β-lactoglobulin, bilin-binding protein (BBP),

apolipoprotein D (APO D), neutrophil gelatinase associated lipocalin (NGAL), tear lipocalin (TLPC), α2-microglobulin-related protein (A2m), 24p3/uterocalin (24p3), von Ebners gland protein 1 (VEGP 1), von Ebners gland protein 2 (VEGP 2), and Major allergen Can fl precursor (ALL-1). In related embodiments, the lipocalin mutein is selected from the group consisting of human neutrophil gelatinase associated lipocalin (hNGAL), human tear lipocalin (hTLPC or hTLC), human apolipoprotein D (APO D) and the bilin-binding protein of Pieris brassicae. A lipocalin mutein based on hTLC or hNGAL is preferred in the context of the embodiments of the present disclosure.

[0026] When used herein in the context of the lipocalins of the present disclosure that bind to VEGF or c-Met, the term "specific for" includes that the lipocalin is directed against, binds to, or reacts with VEGF or c-Met, respectively. Thus, being directed to, binding to or reacting with includes that the lipocalin specifically binds to VEGF or c-Met, respectively. The term "specifically" in this context means that the lipocalin reacts with a VEGF or c-Met protein as described herein, but essentially not with another protein. The term "another protein" includes any non-VEGF or non-c-Met protein, respectively, including proteins closely related to or being homologous to VEGF or c-Met against which the lipocalins disclosed herein are directed to. However, VEGF or c-Met proteins, fragments and/or variants from species other than human such as those described in the context of the definition "subject" are not excluded by the term "another protein". The term "does not essentially bind" means that the lipocalin of the present disclosure does not bind another protein, i.e., shows a cross-reactivity of less than 30%, preferably 20%, more preferably 10%, particularly preferably less than 9, 8, 7, 6 or 5%. Whether the lipocalin specifically reacts as defined herein above can easily be tested, inter alia, by comparing the reaction of a lipoclin of the present disclosure with VEGF or c-Met and the reaction of said lipocalin with (an) other protein(s). "Specific binding" can also be determined, for example, in accordance with Western blots, ELISA-, RIA-, ECL-, IRMA-tests, FACS, IHC and peptide scans.

[0027] It is preferred in the context of the present disclosure that the lipocalin mutein specific for VEGF preferably binds to VEGF-A, but it is not excluded that it alternatively or also binds to VEGF-B, VEGF-C, VEGF-D and/or PLGF. In one preferred embodiment, said lipocalin mutein binds to VEGF-A with an affinity of at least 1,000 times higher, preferebly, at least 5,000 times higher, more preferably at least 10,000 times higher than to VEGF-B, VEGF-C, VEGF-D

or PLGF. Due to the highly preferential binding to VEGF-A, the signaling of VEGFR3, which modulates of lymph angiogenesis, is preferabvly not interfered with.

[0028] The present disclosure provides a combination of a mutein of a lipocalin that has a particularly high affinity to VEGF-A and a mutein of a lipocalin that has a particularly high affinity to c-Met. The respective lipocalin mutein thus binds to VEGF-A and to c-Met, respectively, as a given non-natural ligand. The term "non-natural ligand" refers to a compound, which does not bind to the corresponding lipocalin under physiological conditions.

[0029] The lipocalin muteins of the disclosure are able to bind to VEGF-A and to c-Met, respectively, with detectable affinity, i. e. with an affinity constant of generally at least about 10^{-5} M. Lower affinities are generally no longer measurable with common methods such as ELISA and therefore of secondary importance. Binding affinities of muteins according to the disclosure may in some embodiments be of a K_D below 0.1 nM, in some embodiments be of a K_D below 10 picomolar (pM) and in some embodiments about 1 pM.

[0030] The first lipocalin mutein of the disclosure is accordingly able to bind VEGF-A with detectable affinity, i.e. with a dissociation constant of at least 200 nM, including about 100 nM, about 50 nM, about 25 nM or about 15 nM. The second lipocalin mutein of the disclosure is accordingly able to bind c-Met with detectable affinity, i.e. with a dissociation constant of at least 200 nM including about 100 nM, about 50 nM, about 25 nM or about 15 nM. In some embodiments a lipocalin mutein of the combination according to the disclosure binds VEGF-A or c-Met, respectively, with a dissociation constant for VEGF-A or c-Met of at least about 10 nM, about 1 nM, about 0.1 nM, about 10 pM or even less.

[0031] The binding affinity of the respective mutein to VEGF-A and c-Met, respectively, can be measured and thereby K_D values of a mutein-ligand complex be determined by a multitude of methods known to those skilled in the art. Such methods include, but are not limited to, fluorescence titration, competition ELISA, calorimetric methods, such as isothermal titration calorimetry (ITC), and surface plasmon resonance (BIAcore).

[0032] c-Met (i.e. the Hepatocyte Growth factor (HGF) receptor) may be the human protein with the amino acid sequence of SWISS PROT Data Bank Accession No. P08581 or of fragments thereof. c-Met may also be the bovine protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q769I5, the mouse protein with the amino acid sequence of SWISS PROT Data Bank Accession No. P16056, the rat protein with the amino acid sequence of

SWISS PROT Data Bank Accession No. P97523, the rabbit protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q09YN5, the pig protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q2QLE0, the chimpanzee protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q2QLF1, the Aotus nancymaae (Ma's night monkey) protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q07DV8, the horse protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q2QLA9, the lowland gorilla protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q2QLA9, the protein of the Duckbill platypus with the amino acid sequence of SWISS PROT Data Bank Accession No. Q07E01, or of a fragment of any of these proteins. As a few further examples, c-Met may also be the protein of the Middle-African hedgehog (Four-toed hedgehog) with the amino acid sequence of SWISS PROT Data Bank Accession No. Q00PJ8, the sheep protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q09YI9, the protein of the clouded leopard with the amino acid sequence of SWISS PROT Data Bank Accession No. Q07E37, or of a fragment of the respective protein. The term "c-Met" thus includes full-length c-Met, but also includes fragments of c-Met and/or variants such as splice variants of c-Met. Examples of c-Met proteins are described herein. Preferably, said fragments or variants are functional, i.e., they have c-Met activity/function as described herein. Accordingly, when one of the lipocalin muteins of the disclosed combination is referred to as being specific for c-Met, it means that such lipocalin muteincan bind c-Met, (a) fragment(s) thereof and/or (a) variant(s) thereof.

[0033] VEGF-A may be the human protein with the amino acid sequence of SWISS PROT Data Bank Accession No. P15692, the hamster protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q99PS1, the bovine protein with the amino acid sequence of SWISS PROT Data Bank Accession No. P15691, the pig protein with the amino acid sequence of SWISS PROT Data Bank Accession No. P49151, the horse protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q9GKR0, the sheep protein with the amino acid sequence of SWISS PROT Data Bank Accession No. P50412, the mouse protein with the amino acid sequence of SWISS PROT Data Bank Accession No. Q00731, the rat protein with the amino acid sequence of SWISS PROT Data Bank Accession No. P16612, the chicken protein with the amino acid sequence of SWISS PROT Data Bank Accession No. P67964, the guinea pig protein with the amino acid sequence of SWISS PROT Data Bank Accession No. P26617, or of a

fragment of the respective protein. The term "VEGF" as mentioned herein includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and/or PLGF. Examples of VEGF proteins are described herein. Preferably, said term, when used in the context of the disclosure and in particular when used in the context of one of the lipocalin muteins of the disclosed combination, refers to VEGF-A. The term "VEGF" thus includes full-length VEGF, but also includes fragments of VEGF, preferably of VEGF-A, and/or variants such as splice variants of VEGF, preferably VEGF-A. Preferably, said fragments or variants are functional, i.e., they have VEGF, preferably VEGF-A activity/function as described herein. Accordingly, when one of the lipocalin muteins of the disclosed combination is referred to as being specific for VEGF, it means that such lipocalin muteincan bind VEGF, preferably VEGF-A, (a) fragment(s) of VEGF, preferably VEGF-A and/or (a) variant(s) of VEGF-A, preferably VEGF-A.

[0034] In general, the term "fragment", as used herein with respect to the corresponding protein ligand VEGF (preferably VEGF-A) or c-Met of a lipocalin mutein of the combination according to the disclosure, relates to N-terminally and/or C-terminally shortened protein or peptide ligands, which retain the capability of the full length ligand to be recognized and/or bound by a mutein according to the disclosure.

[0035] In general, the term "variant", as used herein with respect to the corresponding protein ligand VEGF (preferably VEGF-A) or c-Met of a lipocalin mutein of the combination according to the disclosure, relates to a VEGF protein or fragment thereof or c-Met protein or fragment thereof, respectively, that has one or more such as 1, 2, 3, 4 ,5 ,6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 40, 50, 60, 70, 80 or more amino acid substitutions, deletions and/or insertions in comparison to a wild-type VEGF or c-Met protein, respectively, such as a VEGF or c-Met reference protein as deposited with GenBank or SwissProt as described herein. A VEGF or c-Met variant, respectively, has preferably an amino acid identity of at least 50%, 60%, 70%, 80%, 85%, 90% or 95% with a wild-type VEGF or c-Met protein, respectively, such as a VEGF or c-Met reference protein as deposited with GenBank or SwissProt as described herein.

[0036] Where VEGF-A is the human growth factor with a full length of 232 amino acids as defined in SWISS PROT Data Bank Accession No P15692, the term includes the respective secreted human growth factors of 165 amino acids, including isoform VEGF165B, of 206 (SWISS PROT Data Bank identifiers: P15692-1 and P15692-14), of 189 (SWISS PROT Data Bank identifiers: P15692-2 and P15692-13), of 183 (SWISS PROT Data Bank identifier:

P15692-3), of 165 (SWISS PROT Data Bank identifiers: P15692-4, P15692-8 and P15692-11), of 148 (SWISS PROT Data Bank identifier: P15692-5), of 145 (SWISS PROT Data Bank identifier: P15692-6) of 121 (SWISS PROT Data Bank identifiers: P15692-9 and P15692-12), and of 111 (SWISS PROT Data Bank identifier: P15692-10) amino acids (see also Leung et al. Science, (1989) 246, 1306, or Houck et al. Mol. Endocrin. (1991) 5, 1806), as well as the naturally occurring allelic and processed forms thereof. SWISS PROT Data Bank Accession No P15692 discloses that at present 13 isoforms produced by alternative promoter usage, alternative splicing and alternative initiation are confirmed and that further isoforms appear to exist.

[0037] As a further example, where VEGF-A is the mouse growth factor with a full length of 214 amino acids as defined in SWISS PROT Data Bank Accession No Q00731, the term includes the respective secreted mouse growth factors VEGF-1 of 164 amino acids length (SWISS PROT Data Bank identifier: Q00731-2), VEGF-1L of 140 amino acids length (SWISS PROT Data Bank identifier: Q00731-6), VEGF-2 of 120 amino acids length (SWISS PROT Data Bank identifier: Q00731-3), VEGF-4 of 115 amino acids length (SWISS PROT Data Bank identifier: Q00731-4), VEGF-5 of 102 amino acids length (SWISS PROT Data Bank identifier: Q00731-5) and the isoform termed VEGF-3, which is immobilized at the cell membrane and has a length of 188 amino acids (SWISS PROT Data Bank identifier: Q00731-1).

[0038] A lipocalin mutein binding c-Met may act as an antagonist of Hepatocyte growth factor or as an inverse agonist of Hepatocyte growth factor. An inverse agonist binds to the same binding-site as an agonist for a particular receptor and reverses constitutive activity of the respective receptor. In this regard, c-Met has been reported to possess intrinsic kinase activity, i.e. kinase activity even though no Hepatocyte growth factor is bound thereto. Accordingly, a lipocalin mutein that acts as an inverse agonist at the c-Met receptor binds to the same receptor binding-site as Hepatocyte growth factor, thereby, however, causing the basal activity of c-Met to decrease. In contrast to an inverse agonist an agonist simply increases the activity, activation or function of another molecule.

[0039] The term "antagonist" refers to a compound that decreases the activity, activation or function of another molecule. A receptor antagonist binds to a receptor at the same or a different site as the natural ligand without inducing a biological response. Thereby the receptor antagonist prevents or disrupts the interaction of the natural ligand with the receptor, thus inhibiting its function at the receptor. A lipocalin mutein that acts as an antagonist at the c-Met

receptor thus prevents or stops the actions of Hepatocyte growth factor at the c-Met receptor. In some embodiments, a respective lipocalin mutein competes with HGF for binding at the c-Met receptor. A lipocalin mutein that is capable of binding to VEGF forms a complex with the same and thereby prevents VEGF from binding to one of its receptors, e.g. VEGFR1 or VEGFR2. Such a lipocalin mutein prevents or stops the actions of VEGF at a VEGF receptor, even though it does not bind to the respective receptor.

[0040] The amino acid sequence of a lipocalin mutein of the combination according to the disclosure has a high sequence identity to respective lipocalin when compared to sequence identities with another lipocalin (see also above). In this general context the amino acid sequence of a lipocalin mutein of the combination according to the disclosure is at least substantially similar to the amino acid sequence of the corresponding lipocalin (the wild-type or reference lipocalin). A respective sequence of a lipocalin mutein of the combination according to the disclosure, being substantially similar to the sequences of the corresponding lipocalin, has in some embodiments at least 65%, at least 70%, at least 75%, at least 80%, at least 82%, at least 85%, at least 87%, at least 90% identity, including at least 95% identity to the sequence of the corresponding lipocalin. In this regard, a lipocalin mutein of the combination of course may contain, in comparison to the wild-type (or reference) lipocalin, one or more amino acid substitutions as described herein which renders the lipocalin mutein capable of binding to VEGF or c-Met, respectively. By "identity" is meant a property of sequences that measures the similarity or relationship of the mutein and the corresponding lipocalin. Identity is measured by dividing the number of identical residues by the total number of residues and multiplying the product by 100. Typically a mutein of a lipocalin includes one or more mutations – relative to the native sequence lipocalin – of amino acids in the four loops at the open end of the ligand binding site of the lipocalin (cf. above). As explained above, these regions are essential in determining the binding specificity of a lipocalin mutein for a desired target. As an illustrative example, a mutein derived from a polypeptide of tear lipocalin or a homologue thereof, may have one, two, three, four or more mutated amino acid residues at any sequence position in the N-terminal region and/or in the three peptide loops BC, DE, and FG arranged at the end of the β-barrel structure that is located opposite to the natural lipocalin binding pocket.

[0041] By a “native sequence” lipocalin is meant a lipocalin that has the same amino acid sequence as the corresponding polypeptide derived from nature. Thus, a native sequence lipocalin

can have the amino acid sequence of the respective naturally-occurring lipocalin from any organism, in particular a mammal. Such native sequence polypeptide can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence" polypeptide specifically encompasses naturally-occurring truncated or secreted forms of the lipocalin, naturally-occurring variant forms such as alternatively spliced forms and naturally-occurring allelic variants of the lipocalin. A polypeptide "variant" means a biologically active polypeptide having at least about 50%, 60%, 70%, 80% or at least about 85% amino acid sequence identity with the native sequence polypeptide. Such variants include, for instance, polypeptides in which one or more amino acid residues are added or deleted at the N- or C- terminus of the polypeptide. Generally a variant has at least about 70 %, including at least about 80%, such as at least about 85 % amino acid sequence identity, including at least about 90% amino acid sequence identity or at least about 95% amino acid sequence identity with the native sequence polypeptide. As an illustrative example, the first 4 N-terminal amino acid residues (HHLA) can be deleted in a tear lipocalin mutein of the disclosure without affecting the biological function of the protein, e.g. SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

[0042] "Gaps" are spaces in an alignment that are the result of additions or deletions of amino acids. Thus, two copies of exactly the same sequence have 100% identity, but sequences that are less highly conserved, and have deletions, additions, or replacements, may have a lower degree of identity. Those skilled in the art will be aware of the fact that several computer programs are available for determining sequence identity using standard parameters, for example Blast (Altschul, et al. (1997) Nucleic Acids Res. 25, 3389-3402), Blast2 (Altschul, et al. (1990) J. Mol. Biol. 215, 403-410), and Smith-Waterman (Smith, et al. (1981) J. Mol. Biol. 147, 195-197).

[0043] Percent (%) sequence identity" with respect amino acid sequences disclosed herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a reference sequence, i.e. a lipocalin of the present disclosure, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publically available computer software such as BLAST, ALIGN, or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any

algorithms needed to achieve maximum alignment over the full length of the sequences being compared. The same is true for nucleotide sequences disclosed herein.

[0044] The terms "mutated", "mutant" and "mutein" in reference to a nucleic acid or a polypeptide refers to the exchange, deletion, or insertion of one or more nucleotides or amino acids, respectively, compared to the naturally occurring (the wild-type) nucleic acid or polypeptide, i.e. to the protein 'reference' scaffold that can be taken to define the wild-type. A lipocalin mutein of the combination according to the disclosure includes one or more, such as two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen or even twenty substitutions in comparison to the corresponding native lipocalin, provided that such a lipocalin mutein should be capable of binding to VEGF or c-Met, respectively. For example, a lipocalin mutein can have a substitution at a position corresponding to a distinct position (i.e. at a corresponding position) of the wild-type anticalin having the wild-type sequence of, for example, tear lipocalin, hNGAL, or any other lipocalin disclosed herein. In some embodiments a lipocalin mutein of the combination according to the disclosure includes at least two amino acid substitutions, including 2, 3, 4, 5, or more amino acid substitutions of a native amino acid by an arginine residue. Accordingly, the nucleic acid of a protein 'reference' scaffold as described herein is subject to mutagenesis with the aim of generating a lipocalin mutein which is capable of binding to VEGF or c-Met, respectively. Examples of lipocalin muteins that are capable of binding to VEGF are described in WO 2008/015239 and examples of lipocalin muteins that are capable of binding to c-Met are described in WO 2009/095447.

[0045] A lipocalin mutein of the present disclosure may also have further amino acid substitutions such as one which introduces a Cys residue in order to pegylate such lipocalin mutein. Examples are shown in SEQ ID NO: 1 and SEQ ID NO: 9.

[0046] Also, a lipocalin mutein of the present disclosure can comprise a heterologous amino acid sequence at its N-or C-Terminus, preferably C-terminus, such as a Strep-tag, e.g., Strep II tag (for example, SEQ ID NO: 2 or SEQ ID NO: 12) without affecting the biological activity (binding to its target VEGF or c-Met, respectively) of the lipocalin mutein.

[0047] Likewise, a lipocalin mutein of the present disclosure may lack 1, 2, 3, or 4 amino acids at its N-terminal end in comparison to the respective wild-type lipocalin; for example, SEQ ID NO: 1 or SEQ ID NO: 13).

[0048] The term "mutagenesis" as used herein means that the experimental conditions are chosen such that the amino acid naturally occurring at a given sequence position of a lipocalin, such as human tear lipocalin or hTLC (Swiss-Prot data bank entry P31025) or human neutrophil gelatinase associated lipocalin or hNGAL (Swiss-Prot data bank entry P80188) can be substituted by at least one amino acid that is not present at this specific position in the respective natural polypeptide sequence. The term "mutagenesis" also includes the (additional) modification of the length of sequence segments by deletion or insertion of one or more amino acids. Thus, it is within the scope of the disclosure that, for example, one amino acid at a chosen sequence position is replaced by a stretch of three random mutations, leading to an insertion of two amino acid residues compared to the length of the respective segment of the wild type protein. Such an insertion of deletion may be introduced independently from each other in any of the peptide segments that can be subjected to mutagenesis in the disclosure. In one exemplary embodiment of the disclosure, an insertion of several mutations may be introduced into the loop AB of the chosen lipocalin scaffold (cf. International Patent Application WO 2005/019256 which is incorporated by reference its entirety herein).

[0049] The term "position" when used in accordance with the disclosure means the position of either an amino acid within an amino acid sequence depicted herein or the position of a nucleotide within a nucleic acid sequence depicted herein. The term "corresponding" as used herein also includes that a position is not only determined by the number of the preceding nucleotides/amino acids. Accordingly, the position of a given amino acid in accordance with the disclosure which may be substituted may very due to deletion or addition of amino acids elsewhere in a (mutant or wild-type) lipocalin. Similarly, the position of a given nucleotide in accordance with the present disclosure which may be substituted may vary due to deletions or additional nucleotides elsewhere in a mutein or wild type lipocalin 5'-untranslated region (UTR) including the promoter and/or any other regulatory sequences or gene (including exons and introns).

[0050] Thus, under a "corresponding position" in accordance with the disclosure it is preferably to be understood that nucleotides/amino acids may differ in the indicated number but may still have similar neighbouring nucleotides/amino acids. Said nucleotides/amino acids which may be exchanged, deleted or added are also comprised by the term "corresponding position".

[0051] Specifically, in order to determine whether an amino acid residue of the amino acid sequence of a lipocalin (mutein) different from a wild-type lipocalin corresponds to a certain position in the amino acid sequence of a wild-type lipocalin, a skilled artisan can use means and methods well-known in the art, e.g., alignments, either manually or by using computer programs such as BLAST2.0, which stands for Basic Local Alignment Search Tool or ClustalW or any other suitable program which is suitable to generate sequence alignments. Accordingly, a wild-type lipocalin can serve as "subject sequence" or "reference sequence", while the amino acid sequence of a lipocalin different from the wild-type lipocalin described herein serves as "query sequence". The terms "reference sequence" and "wild type sequence" are used interchangeably herein.

[0052] In some embodiments a substitution (or replacement) is a conservative substitution. Nevertheless, any substitution - including non-conservative substitution or one or more from the "exemplary substitutions listed in Table 1, below - is envisaged as long as the lipocalin mutein retains its capability to bind to VEGF-A and c-Met, respectively, and/or it has an identity to the then substituted sequence in that it is at least 60%, such as at least 65%, at least 70%, at least 75%, at least 80%, at least 85 % or higher identical to the "original" sequence.

[0053] Conservative substitutions are generally the following substitutions, listed according to the amino acid to be mutated, each followed by one or more replacement(s) that can be taken to be conservative: Ala → Gly, Ser, Val; Arg → Lys; Asn → Gln, His; Asp → Glu; Cys → Ser; Gln → Asn; Glu → Asp; Gly → Ala; His → Arg, Asn, Gln; Ile → Leu, Val; Leu → Ile, Val; Lys → Arg, Gln, Glu; Met → Leu, Tyr, Ile; Phe → Met, Leu, Tyr; Ser → Thr; Thr → Ser; Trp → Tyr; Tyr → Trp, Phe; Val → Ile, Leu. Other substitutions are also permissible and can be determined empirically or in accord with other known conservative or non-conservative substitutions. As a further orientation, the following eight groups each contain amino acids that can typically be taken to define conservative substitutions for one another:

1) Alanine (Ala), Glycine (Gly);

2) Aspartic acid (Asp), Glutamic acid (Glu);

3) Asparagine (Asn), Glutamine (Gln);

4) Arginine (Arg), Lysine (Lys);

5) Isoleucine (Ile), Leucine (Leu), Methionine (Met), Valine (Val);

6) Phenylalanine (Phe), Tyrosine (Tyr), Tryptophan (Trp);

7) Serine (Ser), Threonine (Thr); and

8) Cysteine (Cys), Methionine (Met)

[0054] If such substitutions result in a change in biological activity, then more substantial changes, such as the following, or as further described below in reference to amino acid classes, may be introduced and the products screened for a desired characteristic. Examples of such more substantial changes are: Ala → Leu, Ile; Arg → Gln; Asn → Asp, Lys, Arg, His; Asp → Asn; Cys → Ala; Gln → Glu; Glu → Gln; His → Lys; Ile → Met, Ala, Phe; Leu → Ala, Met, Norleucine; Lys → Asn; Met → Phe; Phe → Val, Ile, Ala; Trp → Phe; Tyr → Thr, Ser; Val → Met, Phe, Ala.

[0055] Substantial modifications in the biological properties of the lipocalin are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties: (1) hydrophobic: norleucine, met, ala, val, leu, ile; (2) neutral hydrophilic: cys, ser, thr; (3) acidic: asp, glu; (4) basic: asn, gln, his, lys, arg; (5) residues that influence chain orientation: gly, pro; and (6) aromatic: trp, tyr, phe.

[0056] Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Any cysteine residue not involved in maintaining the proper conformation of the respective lipocalin also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond (s) may be added to the lipocalin to improve its stability.

[0057] Any mutation, including an insertion as discussed above, can be accomplished very easily on the nucleic acid, e.g. DNA level using established standard methods. Illustrative examples of alterations of the amino acid sequence are insertions or deletions as well as amino acid substitutions. Such substitutions may be conservative, i.e. an amino acid residue is replaced with an amino acid residue of chemically similar properties, in particular with regard to polarity as well as size. 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) iso-leucine, 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. In addition, instead of replacing single amino acid residues, it is also possible to either insert or delete one or more continuous amino acids of the primary structure of tear lipocalin as long as these deletions or insertion result in a stable folded/functional mutein.

[0058] Modifications of the amino acid sequence include directed mutagenesis of single amino acid positions in order to simplify sub-cloning of the 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 a given target. Furthermore, mutations can be introduced in order 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. It is also possible to deliberately mutate other amino acid sequence position to cysteine in order to introduce new reactive groups, for example for the conjugation to other compounds, such as polyethylene glycol (PEG), hydroxyethyl starch (HES), biotin, peptides or proteins, or for the formation of non-naturally occurring disulphide linkages. The generated thiol moiety may be used to PEGylate or HESylate the mutein, for example, in order to increase the serum half-life of a respective tear lipocalin mutein.

[0059] The nucleic acid molecule encoding the lipocalin mutein can be expressed using any suitable expression system. The obtained mutein or muteins is/are enriched by means of selection and/ or isolation.

[0060] In some embodiments the lipocalin mutein with a detectable affinity for VEGF is a mutein of human tear lipocalin, which has the sequence of the SWISS-PROT Data Bank Accession Number P31025. In this regard, the lipocalin mutein has, in comparison to the wild-type human tear lipocalin, one or more amino acid substitutions. In one embodiment a respective mutein of human tear lipocalin that binds VEGF has one or more of the amino acid substitutions: Glu 27 → Gly, Phe 28 → Ala, Pro 29 → Leu, Glu 30 → Arg, Met 31 → Cys, Asn 32 → Leu, Leu 33 → Ala, Glu 34 → Gly, Asp 80 → Ile, Lys 83 → Ile, Glu 104 → Cys, and Lys 108 → Val.

[0061] In one embodiment, the mutein of human tear lipocalin that binds VEGF has one of the following sets of amino acid substitutions (in relation to human tear lipocalin):

(1) Arg 26 → Ser; Glu 27 → Gly; Phe 28 → Ala; Pro 29 → Leu; GIu 30 → Arg; Met 31 → Cys; Asn 32 → Leu; Leu 33 → Ala; GIu 34 → GIy; Leu 56 → His; Ser 58 → Lys; Asp 80 → He; Lys 83 → He; GIu 104 → Cys; His 106 → Asn; Lys 108 → Val;

(2) Arg 26 → Pro; Glu 27 → Gly; Phe 28 → Ala; Pro 29 → Leu; GIu 30 → Arg; Met 31 → Cys; Asn 32 → Leu; Leu 33 → Ala; Glu 34 → Gly; Leu 56 → His; Ser 58 → Glu; Asp 80 → He; Lys 83 → He; Glu 104 → Cys; His 106 → Ser; Lys 108 → Val; (3) Arg 26 → Pro; Glu 27 → Gly; Phe 28 → Ala; Pro 29 → Leu; GIu 30 → Arg; Met 31 → Cys; Asn 32 → Leu; Leu 33 → Ala; Glu 34 → Gly; Leu 56 → His; Ser 58 → Lys; Asp 80 → He; Lys 83 → Phe; GIu 104 → Cys; His 106 → Asn; Lys 108 → Val;

(4) Arg 26 → Pro; Glu 27 → Gly; Phe 28 → Ala; Pro 29 → Leu; GIu 30 → Arg; Met 31 → Cys; Asn 32 → Leu; Leu 33 → Ala; Glu 34 → Gly; Leu 56 → Arg; Ser 58 → Lys; Asp 80 → He; Lys 83 → Phe; Glu 104 → Cys; His 106 → Ser; Lys 108 → Val;

(5) Arg 26 → Pro; Glu 27 → Gly; Phe 28 → Ala; Pro 29 → Leu; Glu 30 → Arg; Met 31 → Cys; Asn 32 → Leu; Leu 33 → Ala; Glu 34 → GIy; Leu 56 → His; Ser 58 → Lys; Asp 80 → Ile; Lys 83 → Ile;Glu 104 → Cys; His 106 → Ser; Lys 108 → Val;

(6) Arg 26 → Ser; GIu 27 → Gly; Phe 28 → Ala; Pro 29 → Leu; GIu 30 → Arg; Met 31 → Cys; Asn 32 → Leu; Leu 33 → Ala; Glu 34 → GIy; Leu 56 → His; Ser 58 → Lys; Asp 80 → He; Lys 83 → He; Glu 104 → Cys; His 106 → Ser; Lys 108 → Val;

(7) Arg 26 → Val; Glu 27 → Gly; Phe 28 → Ala; Pro 29 → Leu; Glu 30 → Arg; Met 31 → Cys; Asn 32 → Leu; Leu 33 → Ala; Glu 34 → Gly; Leu 56 → His; Ser 58 → Lys; Asp 80 → He; Lys 83 → He; Glu 104 → Cys; His 106 → Ser; Lys 108 → Val; (8) Arg 26 → Leu; Glu 27 → Gly; Phe 28 → Ala; Pro 29 → Leu; Glu 30 → Arg; Met 31 → Cys; Asn 32 → Leu; Leu 33 → Ala; Glu 34 → Gly; Leu 56 → His; Ser 58 → Lys; Asp 80 → Phe; Lys 83 → Phe; Glu 104 → Cys; His 106 → Ser; Lys 108 → Val; and

(9) Arg 26 → Ile; GIu 27 → Gly; Phe 28 → Ala; Pro 29 → Leu; Glu 30 → Arg; Met 31 → Cys; Asn 32 → Leu; Leu 33 → Ala; Glu 34 → Gly; Leu 56 → His; Ser 58 → Lys; Asp 80 → He; Lys 83 → Phe; Glu 104 → Cys; His 106 → Ser; Lys 108 → Val.

[0062] These and further details on muteins of human tear lipocalin with a detectable affinity for VEGF can be found in international patent application WO 2008/015239.

[0063] In a particularly preferred embodiment, a lipocalin mutein that is specific for VEGF is shown in SEQ ID NO: 1, SEQ ID NO: 8 or SEQ ID NO: 12, while the nucleotide sequence of SEQ ID NO: 1 is shown in SEQ ID NO: 4. Accordingly, in some embodiments, the present disclosure also provides a lipocalin mutein that has the same properties as the lipocalin mutein shown in SEQ ID NO: 1.

[0064] In some embodiments the lipocalin mutein with a detectable affinity for c-Met is a mutein of human tear lipocalin. In one embodiment a respective mutein of human tear lipocalin, which binds c-Met, includes an amino acid sequence with a substitution of at least one or of both of the cysteine residues occurring at each of the sequences positions 61 and 153 by another amino acid. Further, this amino acid sequence has a mutation of at least one amino acid residue at any one of the sequence positions 26-34, 56-58, 80, 83, 104- 106, and 108 of the linear polypeptide sequence of mature human tear lipocalin. The positions 24-36 are included in the AB loop, the positions 53-66 are included in the CD loop, the positions 69-77 are included in the EF loop and the positions 103-110 are included in the GH loop in the binding site at the open end of the β-barrel structure of tear lipocalin. The definition of these four loops is used herein in accordance with Flower (Flower, D.R. (1996) Biochem. J. 318, 1-14; Flower, D.R. et al. (2000) Biochim. Biophys. Acta 1482, 9-24). Usually, such a lipocalin mutein has at least 2, 3, 4, 5, 6, 8, 10, 12, 14, 15, 16, 17 or 18 mutated amino acid residues at the sequence positions 26-34, 56-58, 80, 83, 104-106, and 108 of the linear polypeptide sequence of mature human tear lipocalin. In one embodiment, the mutein has the amino acid substitutions Cys 61 → Ala, Phe, Lys, Arg, Thr, Asn, Tyr, Met, Ser, Pro or Trp and Cys 153 → Ser or Ala. Such a substitution has proven useful to prevent the formation of the naturally occurring disulphide bridge linking Cys 61 and Cys 153, and thus to facilitate handling of the lipocalin mutein.

[0065] In a further embodiment, a respective mutein of human tear lipocalin includes at least one additional amino acid substitution selected from Arg 111 → Pro and Lys 114 → Trp. A mutein of human tear lipocalin with a detectable affinity for c-Met may further have the cysteine at position 101 of the sequence of native mature human tear lipocalin substituted by another amino acid. This substitution may, for example, be the mutation Cys 101 → Ser or Cys 101 → Thr. These and further details of muteins of human tear lipocalin with a detectable affinity for c-Met have been disclosed in international patent application WO/2009/095447.

[0066] In one embodiment, the mutein of human tear lipocalin that binds c-Met has one of the following sets of amino acid substitutions (in relation to human tear lipocalin; SWISS-PROT Data Bank Accession Number P31025):

[0067] Preferably, the mutein that binds c-Met comprises at least 6, 8, 10, 12, 14, 16 or 17 amino acid substitutions with respect to the amino acid sequence of mature human tear lipocalin, which are selected from the group consisting of Arg 26 → Thr, Val, Pro, Ser, GIy; GIu 27 → GIn, GIy, Val, Ser; Phe 28 → Met, Asp; Pro 29 → Leu, Ile, Ala, Trp; Glu 30 -→ Leu, GIy, Arg, Phe; Met 31 → Ser; Asn 32 → Leu, Arg, VaI, Gln; Leu 33 → Tyr, Val, Ile, Thr, Phe; GIu 34 → Val, Arg, Ala; Leu 56 → Asn; Ile 57 → Gln; Ser 58 → Ile, Val; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → GIy.

[0068] Said mutein may preferably further comprise at least one amino acid substitution selected from the group consisting of Thr 37 → Ser; Met 39 → Ile, Leu; Asn 48 → Ser; Lys 52 -→ Thr, Met; Met 55 → Leu; Lys 65 → Arg, Leu; Ala 79 → Leu, Ser; Ala 86 → Thr; and Ile 89 → Ser, Gln, Thr, His.

[0069] In another preferred embodiment, said mutein comprises the amino acid substitutions: Arg 26 → Thr; Glu 27 → Gln; Glu 30 → Leu; Met 31 → Ser; Asn 32→ Leu; Leu 33 → Tyr; Glu 34 → Val; Leu 56 → Asn; Ile 57 → Gln; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → Gly.

[0070] In yet another embodiment, said mutein comprises the amino acid substitutions: Met 31 → Ser; Leu 56 → Asn; Ile 57 → Gln; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → GIy.

[0071] In a still further embodiment, said mutein comprises the amino acid substitutions: Cys 61 → Ser; Cys 101 → Ser; Arg 111 -→ Pro; Lys 114 → Trp; and Cys 153 → Ser.

[0072] More preferred, said mutein comprises one of the following sets of amino acid substitutions:

(1) Arg 26 → Thr; Glu 27 → Gln; Phe 28 → Met; Glu 30 → Leu; Met 31 → Ser; Asn 32→ Leu; Leu 33 → Tyr; Glu 34 → Val; Leu 56 → Asn; He 57 → Gln; Ser 58 → Ile; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → Gly;

(2) Arg 26 → Thr; Glu 27 → Gln; Phe 28 → Asp; Glu 30 → Leu; Met 31 → Ser; Asn 32→ Leu; Leu 33 → Tyr; Glu 34 → Val; Leu 56 → Asn; Ile 57 → Gln; Ser 58 → Val; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 -→ Trp; and Lys 108 → Gly;

(3) Arg 26 → Thr; Glu 27 → Gln; Phe 28 → Asp; Glu 30 → Leu; Met 31 → Ser; Asn 32→ Leu; Leu 33 → Tyr; Glu 34 → VaI; Leu 56 -→ Asn; Ile 57 → Gln; Ser 58 → Ile; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → Gly;

(4) Arg 26 → VaI; Glu 27 → Gly; Phe 28 → Asp; Pro 29 → Leu; Glu 30 → Gly; Met 31 → Ser; Asn 32→ Arg; Leu 33 → VaI; Glu 34 → VaI; Leu 56 → Asn; He 57 → Gln; Ser 58 → Ile; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → Gly;

(5) Arg 26 → Pro; Glu 27 → Gly; Phe 28 -→ Asp; Pro 29 → Ile; Glu 30 → Arg; Met 31 → Ser; Asn 32→ Leu; Leu 33 → Ile; Glu 34 → Val; Leu 56 → Asn; He 57 → Gln; Ser 58 → Ile; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → Gly;

(6) Arg 26 → Ser; Phe 28 → Asp; Pro 29 → Ala; Glu 30 → Phe; Met 31 → Ser; Asn 32→ Val; Leu 33 → Thr; Glu 34 -→ VaI; Leu 56 → Asn; Ile 57 → Gln; Ser 58 → Ile; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → Gly;

(7) Arg 26 → Val; Glu 27 → Val; Phe 28 → Asp; Pro 29 → Trp; Glu 30 → Arg; Met 31 → Ser; Asn 32→ Gln; Leu 33 → Val; Glu 34 → Arg; Leu 56 → Asn; Ile 57 → Gln; Ser 58 → Ile; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 → Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → Gly; and

(8) Arg 26 → Gly; Glu 27 → Ser; Phe 28 → Asp; Pro 29 → Trp; Met 31 → Ser; Asn 32-→ Val; Leu 33 → Phe; Glu 34 → Ala; Leu 56 → Asn; Ile 57 → Gln; Ser 58 → Ile; Asp 80 → Tyr; Lys 83 → Ala; Glu 104 -→ Asp; Leu 105 → Thr; His 106 → Trp; and Lys 108 → Gly.

[0073] Preferably, said mutein further comprises at least one of the mutations relative to the sequence of the mature wild type selected from the group consisting of Thr 40→ Cys, GIu 73→ Cys, Arg 90→ Cys, Asp 95→ Cys, Lys 121→ Cys, Asn 123→ Cys and GIu 131→ Cys.

[0074] In a particular preferred embodiment, the lipocalin mutein that is specific for c-Met is shown in SEQ ID NO: 2, SEQ ID NO: 9 or SEQ ID NO: 13. The nucleotide sequence of SEQ ID NO: 2 is shown in SEQ ID NO: 5. Accordingly, in some embodiments the present disclosure also provides a lipocalin mutein that has the same properties as the lipocalin mutein shown in SEQ ID NO: 2.

[0075] A mutein of the combination according to the disclosure may in some embodiments be conjugated to a compound that extends the serum half-life of the mutein (in this

regard see also PCT publication WO 2006/56464 where such conjugation strategies are described with references to muteins of human neutrophil gelatinase-associated lipocalin with binding affinity for CTLA-4). The compound that extends the serum half-life may be a polyalkylene glycol molecule, such as polyethylene (PEG) or an activated derivative thereof.

[0076] In another embodiment, in order to provide suitable amino acid side chains for conjugating one of the above compounds to a mutein of the combination according to the present disclosure artificial amino acids may be introduced by mutagenesis. Generally, such artificial amino acids are designed to be more reactive and thus to facilitate the conjugation to the desired moiety. One example of such an artificial amino acid that may be introduced via an artificial tRNA is para-acetyl-phenylalanine.

[0077] The first lipocalin mutein and the second lipocalin mutein may be administered in combination, including concomitantly or in series, but as part of the same overall therapeutic regimen. In some embodiments the first lipocalin mutein and the second lipocalin mutein may be included in a composition that may be administered. The pharmaceutical composition may include a therapeutically effective amount of the first and the second lipocalin mutein as active ingredients, in association with at least one pharmaceutically acceptable adjuvant, diluent or carrier. The first lipocalin mutein and the second lipocalin mutein may also be administered independent from each other, including at individual intervals at independent points of time.

[0078] As used herein, the term "effective amount" refers to an amount that is sufficient to achieve the desired result or to have an effect on an undesired condition. For example, a "therapeutically effective amount", when used in the context of lipocalin muteins with a detectable affinity to c-Met and/or VEGF, refers to an amount that is sufficient to stop an increase in tumour volume or to effect a reduction in tumour volume, but is generally insufficient to cause adverse side effects. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the type of tumour being treated and the severity of the disorder, the specific composition employed, the age, body weight, general health, sex and diet of the patient, the time of administration, the route of administration, the rate of excretion/degradation of the lipocalin muteins employed, the duration of the treatment, pharmaceutically effective compounds used in combination or coincidental with the lipocalin muteins employed and other respective factors well known in the medical arts. For example, it is well within the skill of the art to start doses of a compound at levels lower than those required to

achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. If desired, the effective daily dose can be divided into multiple doses for purposes of administration. Consequently, single dose compositions can contain such amounts or submultiples thereof to make up the daily dose. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages of antibodies.

[0079] As explained above, the first and the second lipocalin mutein as defined above, a composition according to the disclosure as well as a kit according to the disclosure are for therapeutic and/or diagnostic purposes. Such use concerns in some embodiments a therapy of cancer. Cancers, to which the use may be directed, include, but are not limited to, mesothelioma, leukemias and lymphomas such as cutaneous T-cell lymphomas (CTCL), noncutaneous peripheral T-cell lymphomas, lymphomas associated with human T-cell lymphotrophic virus (HTLV) such as adult T-cell leukemia/lymphoma (ATLL), B-cell lymphoma, acute nonlymphocytic leukemias, chronic lymphocytic leukemia, chronic myelogenous leukemia, acute myelogenous leukemia, lymphomas, and multiple myeloma, non-Hodgkin lymphoma, acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), Hodgkin's lymphoma, Burkitt lymphoma, adult T-cell leukemia lymphoma, acute-myeloid leukemia (AML), chronic myeloid leukemia (CML), or hepatocellular carcinoma. Further examples include myelodisplastic syndrome, childhood solid tumors such as brain tumors, neuroblastoma, retinoblastoma, Wilms' tumor, a bone tumor and a soft- tissue sarcoma, a common solid tumor of an adult such as head and neck cancer - such as oral, laryngeal, nasopharyngeal and esophageal; genito urinary cancer - such as prostate, bladder, renal, uterine, ovarian, testicular; lung cancer - such as small-cell and non small cell; breast cancer; pancreatic cancer; melanoma and other skin cancers; stomach cancer; a brain tumor; a tumour related to Gorlin's syndrome – such as medulloblastoma or meningioma; and liver cancer. Additional exemplary forms of cancer which may be addressed by a method or a use of the disclosure include, but are not limited to, cancer of skeletal or smooth muscle, stomach cancer, cancer of the small intestine, rectum carcinoma, cancer of the salivary gland, endometrial cancer, adrenal cancer, anal cancer, rectal cancer, parathyroid cancer, and pituitary cancer.

[0080] Exemplary routes of administration of the first and the second lipocalin mutein, or a pharmaceutical composition that includes the same, include oral, transdermal, and parenteral delivery. Suitable routes of administration may, for example, include depot, oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intravenous, intramedullary injections, as well as intrathecal, direct intraventricular, intraperitoneal, intranasal, or intraocular injections. One may also administer the lipocalin mutein in a local rather than systemic manner, for example, via injection of the lipocalin mutein directly into a solid tumour, such as in a depot or sustained release formulation. Furthermore, a lipocalin mutein or pharmaceutical composition may be used in a targeted drug delivery system, for example, in a liposome coated with a tumour-specific binding protein. Such liposomes may for example be targeted to and taken up selectively by a tumour.

[00812] The first lipocalin mutein and the second lipocalin mutein can be used per se, or in a pharmaceutical composition where they may be mixed with other active ingredients, as in combination therapy, and/or a suitable carrier or diluent. In this regard the present disclosure also relates to a pharmaceutical composition treating a tumour such as cancer and/or preventing carcinogenesis in a cell.

[0082] The disclosure also relates to a kit that includes, in one or more containers, separately or in admixture a first lipocalin mutein specific for VEGF in accordance with any of the foregoing. In some embodiments a first container includes a first lipocalin mutein specific for VEGF and the second container includes a second lipocalin mutein specific for c-Met. The disclosure also relates to a kit, in which the lipocalin muteins are included in pharmaceutically acceptable formulations. In some embodiments the kit further includes integrally thereto or as one or more separate documents, information pertaining to the contents or the kit and the use of the lipocalin muteins. The kit includes in some embodiments one or more compositions that are formulated for reconstitution in a diluent. Such a diluent, e.g. a sterile diluent, may also be included in the kit, for example within a container.

[0083] Additional objects, advantages, and features of this disclosure will become apparent to those skilled in the art upon examination of the following examples thereof, which are not intended to be limiting. Thus, it should be understood that although the present disclosure is specifically disclosed by exemplary embodiments and optional features, modification and variation

of the disclosures 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 disclosure.

[0084] SEQ ID NO: 1 is the amino acid sequence of a particularly preferred anti-VEGF lipocalin mutein and SEQ ID NO: 4 is its corresponding nucleotide sequence. Note that in SEQ ID NO : 1 the amino acid Cys (C) at position 91 can also be Asp (D) (see SEQ ID NO : 8), like in the corresponding wild-type lipocalin. The D→C substitution was made in order to pegylate the lipocalin mutein.

[0085] SEQ ID NO: 2 is the amino acid sequence of a particularly preferred anti-c-Met lipocalin mutein and SEQ ID NO: 5 is its corresponding nucleotide sequence. Note that in SEQ ID NO : 2 the amino acid Leu (L) at position 123 can be changed to Cys (C) in order to pegylate the lipocalin mutein (see SEQ ID NO: 9)

[0086] SEQ ID NO: 3 is the amino acid sequence of a lipocalin wild type isotype control and SEQ ID NO: 6 is its corresponding nucleotide sequence.

[0087] Note that, the first 4 N-terminal amino acid residues (HHLA; see SEQ ID NO: 7) can be deleted in a tear lipocalin mutein of the present disclosure without affecting the biological function of the protein, e.g. SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

[0088] SEQ ID NO: 7 represents the first four amino acids of a human tear lipocalin (HHLA).

[0089] SEQ ID NO: 8 corresponds to SEQ ID NO: 1, with the exception of position 91, i.e., in SEQ ID NO: 8 a D is at that position rather than a C as in SEQ ID NO: 1.

[0090] SEQ ID NO: 9 corresponds to SEQ ID NO: 2, with the exception of position 123, i.e., in SEQ ID NO: 9 a C is at that position rather than a L as in SEQ ID NO: 2.

[0091] SEQ ID NO: 10 corresponds to SEQ ID NO: 3, with the exception of position 123 i.e., in SEQ ID NO: 10 a C is at that position rather than a L as in SEQ ID NO: 3.

[0092] Also note that a Strep-tag (e.g. WSHPQFEK; see SEQ ID NO: 11) can be added at the C-terminus of tear lipocalin mutein of the present disclosure without affecting the biological function of the protein, e.g. SEQ ID NO: 2.

[0093] SEQ ID NO: 12 corresponds to SEQ ID NO: 1, with the exception that a Strep-tag is added at the C-terminus.

[0094] SEQ ID NO: 13 corresponds to SEQ ID NO: 9, with the exception that the Strep-tag has been removed from the C-terminus.

[0095] SEQ ID NO: 14 corresponds to SEQ ID NO: 3, with the exception that the Strep-tag has been removed from the C-terminus.

EXAMPLES

[0096] Example 1: The effect of dual inhibition of VEGF(A) and c-Met *in vitro* using HUVEC cells.

The effect of dual inhibition of VEGF-A and HGF was investigated in a cellular using Human Umbilical Vein Endothelial Cells (HUVEC), which previously provided to be responsive to both ligands. These cells were stimulated with both HGF and VEGF-A. Then, lipocalin muteins directed against either VEGF (SEQ ID NO: 1) or c-MET (SEQ ID NO: 2), were titrated either alone or in combination. Treatment with both muteins inhibited cell proliferation more strongly than either lipocalin mutein alone. The experiment demonstrates the validity of the approach of dual inhibition of the two pathways (see Figure 1). In the alternative to the lipocalin mutein shown in SEQ ID NO: 1, also a lipocalin mutein shown in SEQ ID NO: 8 or SEQ ID NO: 12 can be used. Similarly, in the alternative to the lipocalin mutein shown in SEQ ID NO: 2, also a lipocalin mutein shown in SEQ ID NO: 9 or SEQ ID NO: 13 can be used.

[0097] Example 2: The benefit of co-administered anti-VEGF(A) and anti-c-Met lipocalin muteins in a U87-MG xenograft model.

It was sought to test the hypothesis that Met induction and activation constitutes an escape pathway from anti VEGF tumor therapy experimentally in vivo. To this end the U87-MG xenograft model was employed. Cells were implanted s.c. into nude mice (n=10) and tumors were allowed to attain a palpable size (130mm^3) before treatment was initiated with a suboptimal dose of an anti-VEGF drug (Bevacizumab or anti-VEGF lipocalin mutein (SEQ ID NO: 1)), a c-Met-directed lipocalin mutein (SEQ ID NO: 2) or a combination of either Bevacizumab or anti-VEGF lipocalin mutein (SEQ ID NO: 1) with anti-c-Met lipocalin mutein (SEQ ID NO: 2).

In the alternative to the lipocalin mutein shown in SEQ ID NO: 1, also a lipocalin mutein shown in SEQ ID NO: 8 or SEQ ID NO: 12 can be used. Similarly, in the alternative to the lipocalin mutein shown in SEQ ID NO: 2, also a lipocalin mutein shown in SEQ ID NO: 9 or SEQ ID NO: 13 can be used.

Combination treatments conferred added benefit as tumor growth was delayed when compared to single agent groups (see Figure 2). These findings suggest that specific biologics approaches targeting these pathways can be combined to yield superior tumor suppression in this model. The model may underestimate the benefit that can be obtained with this combination because suppression of the HGF/c-Met pathway is also expected to exert an anti-angiogenic effect on tumor endothelial cells. As the anti-c-Met lipocalin mutein does not recognize the murine c-Met receptor expressed on the host endothelial cells such an effect can not be visualized in this model.

[0098] Example 3: multiple dose combinations of co-administered anti-VEGF(A) and anti-c-Met lipocalin muteins in a U87-MG xenograft model.

In one approach, the optimal combination of co-administered anti-VEGF and anti-c-Met lipocalin muteins will be measured on the basis of the time interval until a predefined mean tumor volume, for example 500 mm^3, is reached in the U87-MG model. In cycle therapy experiments, daily i.p. treatment with anti-VEGF lipocalin mutein (SEQ ID NO: 1) at 7.5 mg/kg is carried out until tumor progression. At this point, mice will either be switched to anti-c-Met lipocalin mutein treatment (SEQ ID NO: 2) or anti-c-Met lipocalin mutein (SEQ ID NO: 2) will be added to the anti-VEGF lipocalin mutein (SEQ ID NO: 1) regimen at a dose of 7.5 mg/kg every other day, exploring the optimal schedule to delay tumor growth. A PEGylated or non-PEGylated version, respectively, of wild type lipocalin (tear lipocalin) from which both the anti-c-Met and anti-VEGF lipocalin muteins are derived will be used as a negative control at the combined dose of the antagonists. As an example, the amino acid sequence of a PEGylated version is shown in SEQ ID NO: 10, while the amino acid sequence of a non-PEGylated version is shown in SEQ ID NO: 3 or SEQ ID NO: 14.

[0099] Cell lines suitable for alternative/additional xenograft models may be NCI-H596, KP4, NCI-H441 or EBC-1 cell lines. These cell lines may be selected based on mode of c-met activation and included paracrine (NSCLC cell line NCI-H596), autocrine (pancreatic cell line, KP4), c-met overexpressing (NSCLC cell line, NCI-H441), and focally amplified and overexpressing c-met (NSCLC cell line, EBC-I).

[0100] Example 4: Combinations of anti-VEGF and anti-c-Met muteins in the KP4 xenograft model.

For confirmation of the synergistic effect of the combination approach, nude mice will be implanted with pancreatic KP4 cells. When mean tumor volume has reached 100-200mm^3, mice will be randomized based on tumor volume and treatment with either single anti-VEGF-A (SEQ ID NO: 1, SEQ ID NO: 8 or SEQ ID NO: 12) or anti-c-Met lipocalin muteins (SEQ ID NO: 2, SEQ ID NO: 9 or SEQ ID NO: 13) or the corresponding combination will be initiated at substantially the same doses and regimens as set forth in Examples 2 and 3. Combination treatment is expected to lead to more pronounced tumor growth inhibition and an increased time-to-volume over single agent treatment.

***

[0101] The disclosure 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. Thus, it should be understood that although the present disclosure has been specifically disclosed by exemplary embodiments and optional features, modification and variation of the disclosures 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 disclosure.

[0102] 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.

CLAIMS

1. A first (i) lipocalin mutein specific for VEGF and (ii) a second lipocalin mutein specific for c-Met for use in a method of treating a subject suffering from a tumour.

2. The use of claim 1, wherein said VEGF is full-length VEGF, a fragment or variant thereof.

3. The use of claim 1, wherein said c-Met is full-length c-Met, a fragment or variant thereof.

4. The use of any one of claims 1 to 3, wherein said first lipocalin mutein and said second lipocalin mutein are administered concurrently or as part of the same therapeutic regimen.

5. The use of any one of claims 1 to 4, wherein the second lipocalin mutein is an antagonist or an inverse agonist of Hepatocyte growth factor at c-Met.

6. The use of any one of claims 1 to 5, wherein the first lipocalin mutein is an antagonist of VEGF.

7. The use of any one of claims 1 to 6, wherein the first lipocalin mutein has an affinity to a naturally occurring VEGF defined by a K_D of 50 nm or less.

8. The use of any one of claims 1 to 7, wherein the second lipocalin mutein has an affinity to a naturally occurring c-Met defined by a K_D of 50 nm or less.

9. The use of any one of claims 1 to 8, wherein the first lipocalin mutein comprises the SEQ ID NO: 1.

10. The use of any one of claims 1 to 9, wherein the second lipocalin mutein comprises the SEQ ID NO: 13.

11. The use of any one of claims 1 to 10, wherein the tumour is a cancer.

12. A composition comprising one or more lipocalin muteins, wherein the composition comprises a first lipocalin mutein and a second lipocalin mutein, wherein the first lipocalin mutein is specific for VEGF and the second lipocalin mutein is specific for c-Met.

13. The composition of claim 12, wherein the first lipocalin mutein has an affinity to a naturally occurring VEGF defined by a K_D of 50 nm or less.

14. The composition of claims 12 or 13, wherein the second lipocalin mutein has an affinity to a naturally occurring c-Met defined by a K_D of 50 nm or less.

15. The composition of any one of claims 12 to 14, wherein at least one of the one or more lipocalin muteins is conjugated to a compound that extends the serum half-life of the mutein.

16. A kit of parts comprising a first and a second container, wherein the first container comprises a first lipocalin mutein specific for VEGF and the second container comprises a second lipocalin mutein specific for c-Met.

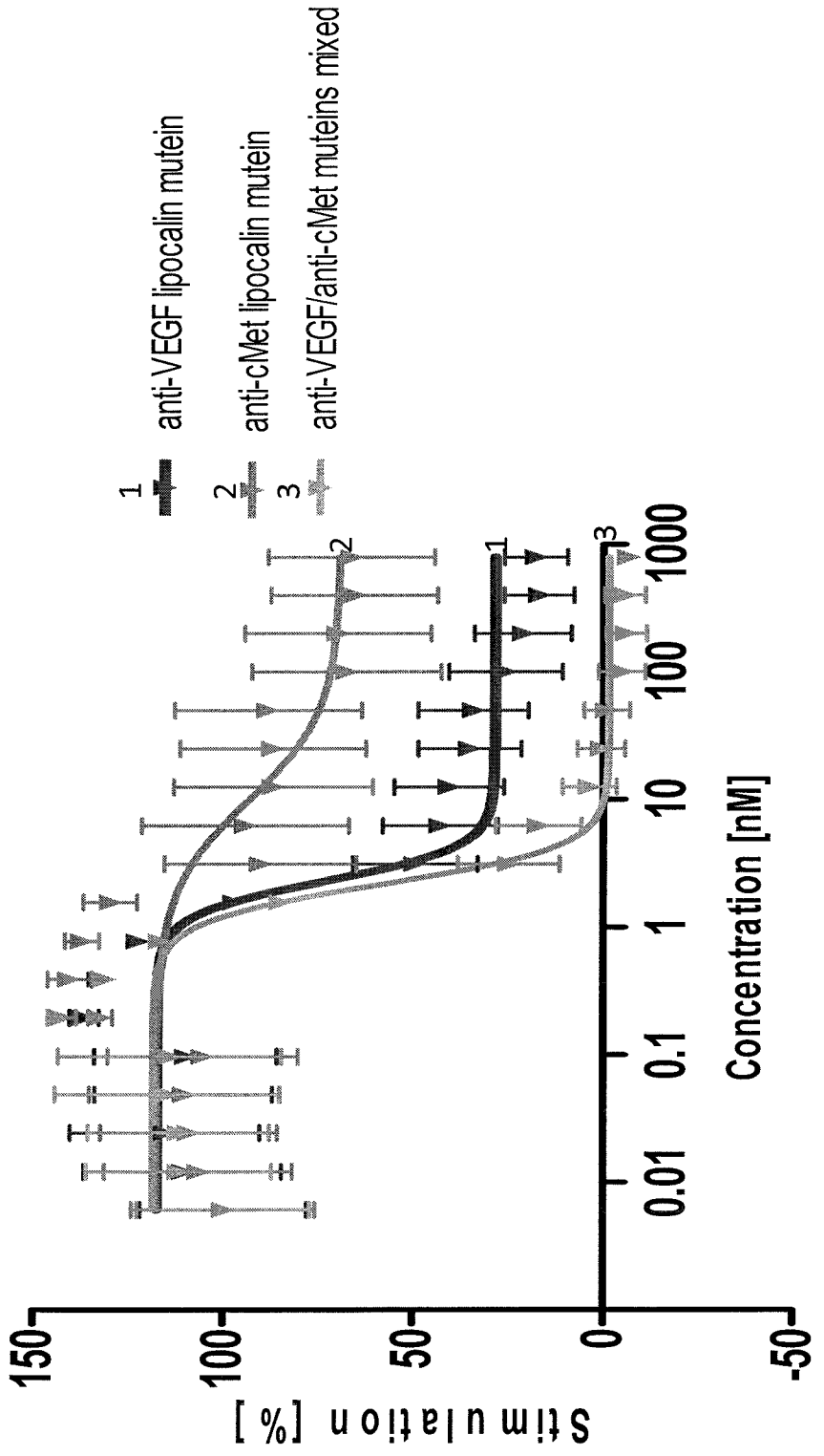
anti-VEGF lipocalin mutein
anti-cMet lipocalin mutein
anti-VEGF/anti-cMet muteins mixed
1
2
3
Stimulation [%]
150
100
50
0
-50
0.01
0.1
1
10
100
1000
Concentration [nM]

Figure 1

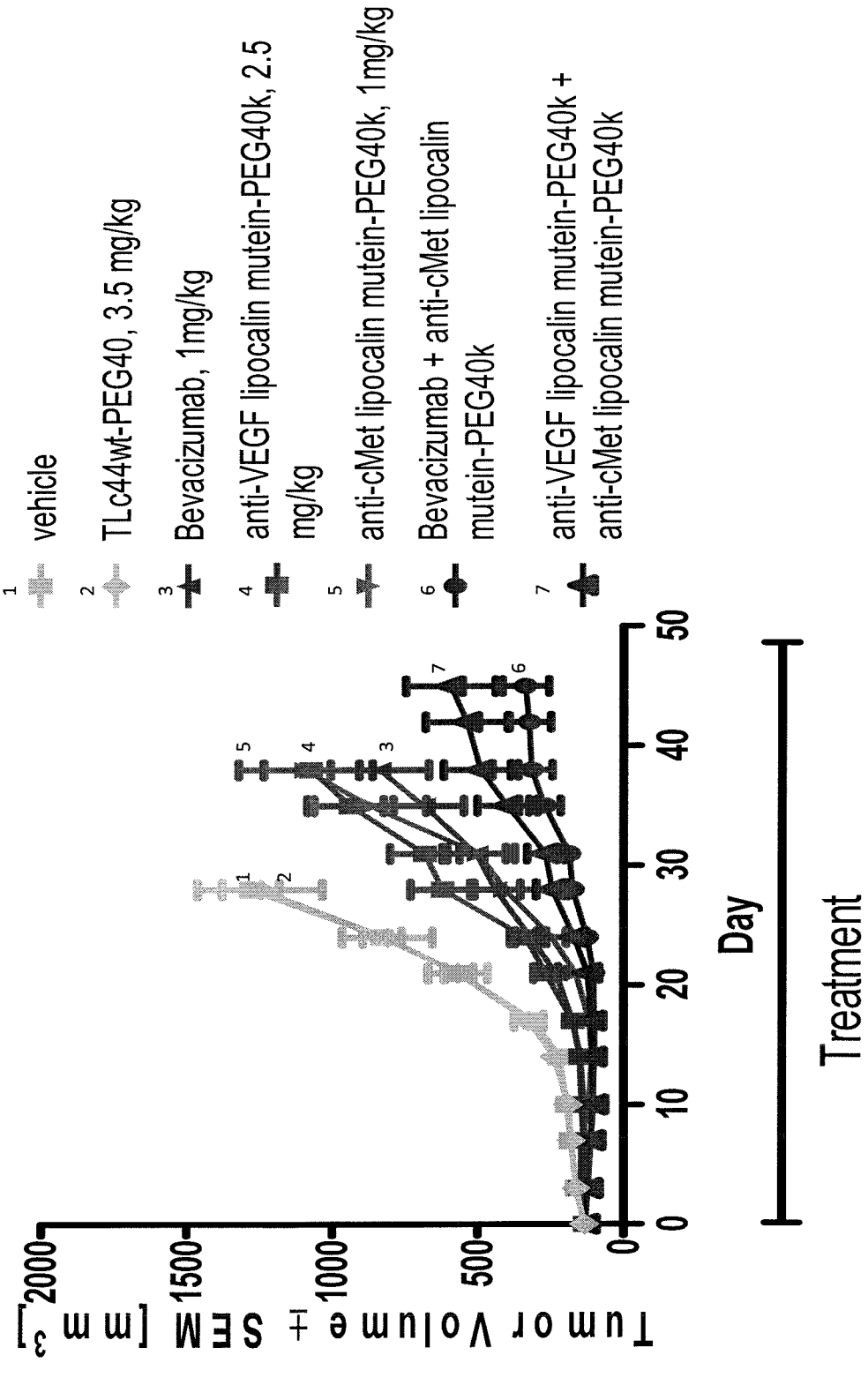
Tumor Volume + SEM [mm³]
2000
1500
1000
500
0
0
10
20
30
40
50
Day
Treatment
1 vehicle
2 TLc44wt-PEG40, 3.5 mg/kg
3 Bevacizumab, 1mg/kg
4 anti-VEGF lipocalin mutein-PEG40k, 2.5 mg/kg
5 anti-cMet lipocalin mutein-PEG40k, 1mg/kg
6 Bevacizumab + anti-cMet lipocalin mutein-PEG40k
7 anti-VEGF lipocalin mutein-PEG40k + anti-cMet lipocalin mutein-PEG40k

Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/056124

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/16 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 A61P

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, Sequence Search

C. 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	WO 99/16873 A1 (SKERRA ARNE [DE]; BESTE GERALD [DE]; SCHMIDT FRANK [DE]; STIBORA THOMA) 8 April 1999 (1999-04-08) claims 1-10 -----	1-16
X	WO 2008/015239 A2 (PIERIS AG [DE]; JENSEN KRISTIAN [DE]; HUELSMEYER MARTIN [DE]; SCHLEHUB) 7 February 2008 (2008-02-07) claims 122-147 page 83; example 45 ----- -/--	1-16

[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
4 July 2012	13/07/2012

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	Rodrigo-Simón, Ana

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/056124

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 2009/095447 A1 (PIERIS AG [DE]; MATSCHINER GABRIELE [DE]; HOHLBAUM ANDREAS [DE]; HUELS) 6 August 2009 (2009-08-06) claim 43 -----	1-16

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/056124

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