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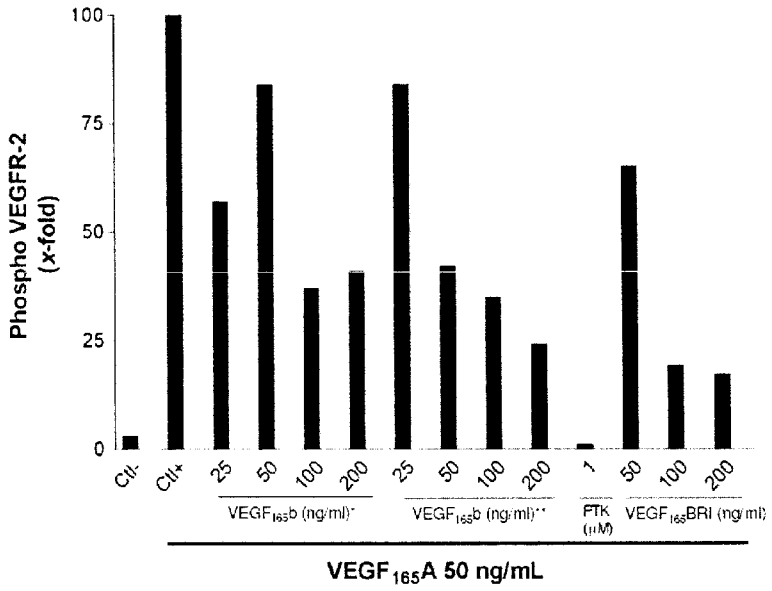


FIG. 4

(57) Abstract: The present invention is directed to a VEGF antagonist based on the sequence of VEGF- A_{165}, and comprising a mutation at residue 159, 161, 162, 163, 164, 165, or a combination thereof. The Cys at residue 160 of VEGF-A_{165} is retained.

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POTENT VEGF ANTAGONISTS

Cross-reference to Related Applications

This application claims the benefit of United States Provisional Patent Application USSN 61/202,568 filed March 12, 2009, the entire contents of which his herein incorporated by reference.

Field of the Invention

The present invention relates to vascular endothelial growth factor (VEGF) antagonists. More specifically, the present invention relates to VEGF antagonists that reduce signal transduction by VEGF receptors.

Background of the Invention

Vascular endothelial growth factors (VEGFs) are key regulators of angiogenesis and vascular homeostasis. In mammals, various VEGFs have been identified, including VEGF-A, -B, -C, and -D; isoforms of each have also been discovered. The VEGFs bind various combinations of VEGF receptors (VEGFR-1, VEGFR-2, and/or VEGFR-3) and co-receptors (including neuropilins and heparan sulphate proteoglycans). These interactions initiate VEGFR signal transduction, leading to normal vascular development.

Among the VEGF family, VEGF-A has been identified as playing a central role in angiogenesis. Numerous naturally-occurring isoforms of this glycoprotein have been identified, including predominant isoforms comprising 121, 145, 165, 189, and 206 amino acids. The isoforms share a common N-terminus, but differ at the C-terminus; the VEGF-A isoforms share the same basic effects on vascular endothelium, however they may differ in their physical properties and abilities to bind VEGFR co-receptors. Consequently, the different VEGF-A isoforms can vary in their *in vivo* bioactivities.

VEGF-A_{165} has been extensively studied, and has been identified in playing a central role in vascular development accompanying cancers, chronic inflammatory diseases, and other human pathologies. This type of endothelial cell proliferation and angiogenesis is referred to as "pathological angiogenesis." It has been shown that VEGF-A_{165} alone can induce the proliferation of endothelial cells and formation of new blood vessels in pathological angiogenesis.

Due to the role of VEGFs and VEGFRs in human pathologies, antagonism of VEGFR signal transduction and/or homeostatic abilities has been an area of intense research. Different approaches have been used to inhibit or modulate the interaction of VEGF with VEGFR, one

being production of VEGF mutants that bind the VEGFR without allowing its activation. A recently discovered VEGF-A splice variant, VEGF-A_{165}b, has such properties (Bates et al (2002); Harper & Bates (2008); Bates et al, US Patent Application Publication No. 2005/0054036).

VEGF-A_{165}b differs from VEGF-A_{165} by its C-terminal 6 amino acids; specifically, the VEGF-A_{165} C-terminus comprises CDKPRR (residues 160-165; SEQ ID NO:1), while VEGF-A_{165}b comprises SLTRKD (SEQ ID NO:2). The loss of Cys160 impairs the formation of an intra-chain disulfide bond between residues 146 and 160 present in the wild-type molecule. Additionally, the DKPRR motif of VEGF-A, shown to bind to neuropilin-1 (Cébe-Suarez et al (2008)), is not present in VEGF-A_{165}b. Cébe-Suarez et al (2006, 2008), Harper and Bates (2008), and Jia et al (2006) indicate that Cys160 is essential for presenting the DKPRR motif in the proper context for binding to neuropilin-1. Thus, the loss of CDKPRR in VEGF-A_{165}b prevents its binding to neuropilin-1 and subsequent formation of stable co-receptor complex. Furthermore, Harper and Bates (2008) indicate that the neutral residues at the C-terminus of VEGF-A_{165}b, coupled with the absence of Cys160 to keep the C-terminus in proximity of the neuropilin binding site, prevent phosphorylation of key tyrosine residues in the VEGFR.

While VEGF-A_{165}b may show promise in anti-angiogenesis therapies, protein stability could be affected and aggregation of the VEGF-A_{165}b following disulfide bond formation (at Cys 146) with itself or other proteins could result. Additionally, it was recently shown that this variant still partially activate the VEGFR2 receptor (Kawamura et al (2008)).

Better VEGF and/or VEGFR antagonists are needed to provide a therapeutic effect while avoiding the disadvantages of the prior art.

Summary of the Invention

The present invention provides an improved VEGF antagonist. In view of the prior art findings that removal of Cys160 results in antagonistic VEGF molecules, the present VEGF are surprising in that they retain Cys160 while exhibiting antagonistic activity.

Accordingly, the present invention provides a VEGF antagonist based on the sequence of VEGF-A_{165} and comprising a mutation at residue 159, 161, 162, 163, 164, 165, or a combination thereof, and wherein the Cys at residue 160 is retained. The wild-type sequence of VEGF-A_{165} upon which the mutant is based may comprise the sequence shown in SEQ ID NO:3.

The VEGF antagonist of the present invention shows improved anti-angiogenic activity compared to VEGF-A_{165}b. Additionally, because the Cys146-Cys160 disulfide bridge is intact in the present VEGF mutant, the mutant shows improved protein stability and lower aggregation compared to VEGF-A_{165}b. The intact disulfide bond also allows the mutant to retain a more wild-type structural conformation, thus minimizing an immunogenic response. Additionally, it is also presently shown that the VEGF mutant of the present invention shows less activation of VEGFR2.

This summary of the invention does not necessarily describe all features of the invention. The invention may also reside in a sub-combination of the described features.

Brief Description of the Drawings

These and other features of the invention will now be described by way of example, with reference to the appended drawings, wherein:

FIGURE 1 shows the sequence alignment of VEGF-A_{165} (SEQ ID NO:3), VEGF-A_{165}b (SEQ ID NO:4), VEGF-A_{165}BRI (SEQ ID NO:5), VEGF-A_{165}c (SEQ ID NO:6), VEGF-A_{165}d (SEQ ID NO:7), VEGF-A_{165}e (SEQ ID NO:8),VEGF-A_{165}f (SEQ ID NO:9) and VEGF-A_{165}g (SEQ ID NO:10). Amino acid differences compared to the VEGF-A_{165} are shown in bold and underlined.

FIGURE 2 is a SDS-PAGE gel showing purified fractions of VEGF165 mutants. Lanes: 1- VEGF-A_{165}b; 2- VEGF-A_{165}BRI; 3- VEGF-A_{165}c; 4- VEGF-A_{165}d; 5- VEGF-A_{165}e; and 6- VEGF-A_{165}f.

FIGURE 3 shows the effect of VEGF-A_{165} and VEGF-A_{165}BRI on VEGFR2 (Flk-1) phosphorylation and ERK activation. As shown, VEGF-A_{165}BRI does not activate VEGFR2, but does activate ERK phosphorylation in HUVEC cells.

FIGURE 4 is a bar graph showing results of VEGFR2 phosphorylation quantification by scanning densitometry. VEGF-A_{165}BRI appears to antagonize VEGF-A_{165} with potency similar to VEGF-A_{165}b in HUVEC cells. VEGF-A_{165}b* and VEGF-A_{165}BRI were produced by transfection of HEK293 cells and purified as described in the Examples, while VEGF-A_{165}b** was obtained from R & D Systems.

FIGURE 5 is a Western blot showing the inhibition of endogenous ERK activity in HBEC AB from microcapillary fraction (20 μm) by VEGF-A_{165}BRI (FIGURE 5A) and VEGF-A_{165}b (FIGURE 5B). These results show that VEGF-A_{165}BRI is more potent than VEGF-A_{165}b at inhibiting endogenous ERK activity in HBEC. FIGURE 5A lanes: 1- Control; 2- VEGF-

A_{165}BRI 5 ng/mL; 3- VEGF-A_{165}BRI 25 ng/mL; 4- VEGF-A_{165}BRI 100 ng/mL; 5- VEGF-A_{165}BRI 200 ng/mL; 6- VEGF-A_{165}BRI 400 ng/mL. FIGURE 5B lanes: 1- Control; 2- VEGF-A_{165}b 5 ng/mL; 3- VEGF-A_{165}b 25 ng/mL; 4- VEGF-A_{165}b 100 ng/mL; 5- VEGF-A_{165}b 200 ng/mL; 6- VEGF-A_{165}b 400 ng/mL.

FIGURE 6 is a Western blot showing the inhibition of VEGF-A_{165}-induced ERK activation in HBEC AB from microcapillary fraction (20 µm) by VEGF-A_{165}BRI and VEGF-A_{165}b. These results show that VEGF-A_{165}BRI is more potent than VEGF-A_{165}b at blocking ERK activation by $VEGF_{165}$. Lanes: 1- Control (DME); 2- VEGF-A_{165} 25 ng/mL; 3- VEGF-A_{165}b 200 ng/mL; 4- VEGF-A_{165}b 200 ng/mL (different batch); 5- VEGF-A_{165}BRI 200 ng/mL; 6- VEGF-A_{165} 25 ng/mL + VEGF-A_{165}b 200 ng/mL; 7- VEGF-A_{165} 25 ng/mL + VEGF-A_{165}b 200 ng/mL (different batch); 8- VEGF-A_{165} 25 ng/mL + VEGF-A_{165}BRI 200 ng/mL.

FIGURE 7 is a Western blot showing the inhibition of endogenous or VEGF-A_{165}-stimulated ERK activity in mouse astrocytes by VEGF-A_{165} mutants. Results show that all mutants prepared inhibits VEGF-induced ERK activation in mouse astrocytes, but mutants "c" and "d" have the least agonistic activity. Lanes: 1- Control (medium alone); 2- VEGF-A_{165} 25 ng/ml; 3- VEGF-A_{165}c 200 ng/mL; 4- VEGF-A_{165}d 200 ng/mL; 5- VEGF-A_{165}e 200 ng/mL; 6- VEGF-A_{165}f 200 ng/mL; 7- VEGF-A_{165}b + VEGF-A_{165} 25 ng/ml; 8- VEGF-A_{165}c + VEGF-A_{165} 25 ng/ml; 9- VEGF-A_{165}d + VEGF-A_{165} 25 ng/ml; 10- VEGF-A_{165}e + VEGF-A_{165} 25 ng/ml; 11- VEGF-A_{165}f + VEGF-A_{165} 25 ng/ml. Experiments were run in duplicate as shown by the upper and lower panels.

FIGURE 8 shows the elution profile of VEGF-A_{165} (FIGURE 8A) and VEGF-A_{165}b (FIGURE 8B) from a gel filtration column. VEGF-A_{165}b shows formation of oligomers.

FIGURE 9 is an image of a 4-14% Bis-Tris gels under reducing (FIGURE 9A) and non-reducing (FIGURE 9B) conditions. Lanes 1 are VEGF-A_{165} and lanes 2 are VEGF-A_{165}b. VEGF-A_{165}b shows formation of oligomers.

Detailed Description of the Invention

The present invention relates to vascular endothelial growth factor (VEGF) antagonists. More specifically, the present invention relates to VEGF antagonists that reduce signal transduction by VEGF receptors.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

In one embodiment of the present invention, there is provided a VEGF antagonist based on the sequence of VEGF-A_{165} comprising a mutation at residue 159, 161, 162, 163, 164, 165, or a combination thereof, and wherein the Cys at residue 160 is retained. The wild-type sequence of VEGF-A_{165} upon which the mutant is based may comprise the sequence shown in SEQ ID NO:3.

The present invention is directed to a VEGF antagonist. By the term "VEGF antagonist" or "vascular endothelial growth factor antagonist" it is meant a polypeptide that shows anti-angiogenic activity. The anti-angiogenic activity may be achieved by modulation or inhibition of the action of VEGF and/or VEGFR by the VEGF antagonist; this may be accomplished in any manner of mechanisms. For example but without wishing to be limiting, the VEGF antagonist may hinder, prevent or block interaction of VEGF to VEGFR; may prevent phosphorylation of the VEGFR; may also prevent binding to/recruitment of neuropilin or HSPGs; or by any other method of action.

The VEGF antagonist of the present invention is based on the sequence of VEGF-A_{165}. The sequence of VEGF-A_{165} is well-known in the art and may comprise the sequence shown in SEQ ID NO:3. The VEGF antagonist may also include an additional N- or C-terminal sequence to, for example, target protein expression or provide ease of purification; for example, but without wishing to be limiting, the sequence may comprise the VEGF signal peptide MNFLLSWVHWSLALLLYLHHAKWSQA (SEQ ID NO:11) at the N-terminus of the VEGF antagonist. The additional sequence may be, for example and without wishing to be limiting, a His tag (His_5/His_6/His_8/His_{10}). Such additional sequences may be cleaved during synthesis in the cell or may be cleaved after secretion/isolation of the VEGF antagonist.

The VEGF antagonist, while based on the sequence of VEGF-A_{165}, comprises a mutation at residue 159, 161, 162, 163, 164, 165, or a combination thereof. Such "mutation" may comprise an amino acid substitution, deletion or addition. The newly added or substituent amino acid may be a basic, neutral, hydrophobic, or acidic amino acid. By the term "basic amino acid" it is meant hydrophilic amino acids having a side chain pK value of greater than 7, which are typically positively charged at physiological pH. Basic amino acids include histidine (His or H), arginine (Arg or R), and lysine (Lys or K). By the term "neutral amino acid" (also "polar amino acid"), it is meant hydrophilic amino acids having a side chain that is uncharged at physiological pH, but which has at least one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Polar amino acids include serine (Ser or S), threonine (Thr or T), cysteine (Cys or C), tyrosine (Tyr or Y), asparagine (Asn or N), and glutamine (Gln or Q). The term "hydrophobic amino acid" (also "non-polar amino acid") is meant to include amino acids exhibiting a hydrophobicity of

greater than zero according to the normalized consensus hydrophobicity scale of Eisenberg (1984). Hydrophobic amino acids include proline (Pro or P), isoleucine (Ile or I), phenylalanine (Phe or F), valine (Val or V), leucine (Leu or L), tryptophan (Trp or W), methionine (Met or M), alanine (Ala or A), and glycine (Gly or G). "Acidic amino acid" refers to hydrophilic amino acids having a side chain pK value of less than 7, which are typically negatively charged at physiological pH. Acidic amino acids include glutamate (Glu or E), and aspartate (Asp or D).

In one embodiment of the present invention, there is provided a VEGF antagonist based on the sequence of VEGF-A_{165} and comprising:

a) a substitution at residue 159 for an acidic or neutral amino acid;

b) a substitution at residue 161 for a basic or neutral amino acid;

c) a substitution at residue 162 for an acidic or neutral amino acid;

d) a substitution at residue 163 for a hydrophobic amino acid;

e) a substitution at residue 164 for an acidic or neutral amino acid;

f) a substitution at residue 165 for an acidic or neutral amino acid; or

g) a combination of a) to f).

The substitution at residue 159 may be, for example, for a isoleucine, glutamate, aspartate, alanine, or glycine; in a non-limiting example, the substitution at residue 159 is for a isoleucine or glutamate. The substitution at residue 161 may be for a glycine, alanine, lysine or arginine; in a specific, non-limiting example, the substitution at residue 161 is for lysine (SEQ ID NO:8). The substitution at residue 162 may be for a glutamate, aspartate, or alanine; for example, but not wishing to be limiting, residue 162 may be mutated to glutamate (SEQ ID NO:6) or alanine (SEQ ID NO:9). The substitution at residue 163 may be for a glycine or alanine; in a non-limiting example, residue 163 may be mutated to glycine. Without wishing to be limiting, the substitution at residue 164 may be for a glutamate, aspartate, alanine, or glycine. The substitution at residue 165 may be for a glutamate, aspartate, alanine, or glycine.

The VEGF antagonist as described above may also comprise a combination of two or more of the mutations listed above. In a specific non-limiting example, the VEGF antagonist comprises two mutations at residues 162 and 163; in a further example, the residue at

position 162 may be mutated to Glu and the residue at position 163 to Gly (SEQ ID NO:5). In an alternative, non-limiting, example, the VEGF antagonist comprises two mutations at residues 161 and 162; in a further example, the residue at position 161 may be mutated to Gly and the residue at position 162 to Ala (SEQ ID NO:7). In yet another alternative example, and without wishing to be limiting, the VEGF antagonist comprises two mutations at residues 159 and 162; in a further example, the residue at position 159 may be mutated to Ile and the residue at position 162 to Glu (SEQ ID NO:10). Alternatively, the VEGF antagonist may comprise a glutamate residue at positions 159 and 162.

Additionally, the VEGF antagonist of the present invention also includes polypeptides that are substantially identical to the VEGF antagonist described herein. The substantially identical polypeptide may comprise one or more conservative amino acid mutation, provided the C-terminal 6 amino acids remain unchanged (i.e., none of the conservative amino acid mutations are found in the 6 C-terminal amino acids). It is known in the art that one or more conservative amino acid mutation to a reference protein may yield a mutant protein with no substantial change in physiological, chemical, or functional properties compared to the reference protein; in such a case, the reference and mutant proteins would be considered "substantially identical" polypeptides. Conservative amino acid mutation may include addition, deletion or substitution of an amino acid; a conservative amino acid substitution is defined herein as the substitution of an amino acid residue for another amino acid residue with similar chemical properties (e.g. size, charge, or polarity). The VEGF antagonist of the present invention also encompasses substantially identical proteins comprising one or more conservative amino acid mutation, provided these mutations are not within the 6 C-terminal amino acids; provided the Cys at position 160 is retained; and provided the anti-angiogenic activity is retained.

Sequence identity is used to evaluate the similarity of two sequences; it is determined by calculating the percent of residues that are the same when the two sequences are aligned for maximum correspondence between residue positions. Any known method may be used to calculate sequence identity; for example, computer software is available to calculate sequence identity. Without wishing to be limiting, one can use NCBI BLAST2 service maintained by the Swiss Institute of Bioinformatics, and as found at http://ca.expasy.org/tools/blast/. The substantially identical sequences of the present invention may be at least 85% identical; in another example, the substantially identical sequences may be at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% homologous at the amino acid level to sequences described herein.

The VEGF antagonists of the present invention shows equal or improved anti-angiogenic activity compared to VEGF-A_{165}b. Additionally, because the Cys146-Cys160 disulfide bridge is intact in the present VEGF mutant, the mutants may exhibit improved protein stability and lower incidence of aggregation compared to VEGF-A_{165}b. The intact disulfide bond may also allow the mutants to retain a more wild-type structural conformation, thus minimizing an immunogenic response. Additionally, it is also presently shown that the VEGF mutant of the present invention shows less activation of VEGFR2.

The present results show that the VEGF-A_{165}BRI polypeptide is a more potent antagonist than VEGF-A_{165}b. This agonist-to-antagonist conversion is not due to the disappearance of C160, nor to the R164K and R165D mutations, as proposed by Cébe-Suarez et al (2008) and Harper and Bates (2008), but rather is due to the mutation at position 162 (to T or E in the case of VEGF-A_{165}b and VEGF-A_{165}BRI, respectively). Without wishing to be bound by theory, the residue at position 162 may play a role in interaction of VEGF with neuropilin-1, or for bringing VEGFR2 together with neuropilin-1 in a proper orientation for full activation of the signalling pathway; other residues may also play a role in these interactions, such as D161 and R159.

The present results also show that a single mutation (K162E) or two mutations (K162E and P163G) may be sufficient to convert VEGF-A_{165} into an antagonist. This is in contrast with the natural splice variant (VEGF-A_{165}b) which has 6 mutations at the C-terminus, including a C160S mutation. The present is a more systematic approach to conferring antagonist activity, leading to a better and more stable antagonist. Furthermore, as the disulfide bridge between cysteines at positions 146 and 160 is conserved, the VEGF antagonist of the present invention may retain a wild-type like structure at its C-terminus.

The present invention also encompasses nucleic acid sequences encoding the VEGF antagonists as described above. The nucleic acid sequence may be codon-optimized. The present invention also encompasses vectors comprising the nucleic acids as just described. Furthermore, the invention encompasses cells comprising the nucleic acid and/or vector as described.

The present invention also provides a method of providing the VEGF antagonist of the present invention to a person in need thereof. For example, and without wishing to be limiting, a nucleic acid encoding the VEGF antagonist of the present invention may be administered by injection or through the use of viral delivery systems such as adenovirus, adeno-associated virus, retrovirus; alternatively, the purified VEGF antagonist could be delivered by injection into the person in need thereof. In yet another non-limiting example,

local implantation of encapsulated cells stably expressing the VEGF antagonist of the present invention can be performed.

The present invention will be further illustrated in the following examples. However, it is to be understood that these examples are for illustrative purposes only and should not be used to limit the scope of the present invention in any manner.

Example 1: Construction of VEGF-A_{165} mutants

Codon-optimized (human codon basis) VEGF-A_{165} cDNA was synthesized by GeneArt and cloned into the pTT5 vector (Shi et al, 2005). All mutants were created by cloning annealed oligonucleotides sequences comprising the mutated sequence between the SbfI and BamHI sites (underlined) of the VEGF-A_{165} cDNA in pTT5 vector as shown below (VEGF-A_{165} stop codon is shown in bold):

GAGGACCTGCAGGTGCGACGAGCCCAGGAGG**TGA**GGATCC (SEQ ID NO:12)

$VEGF_{165}C$:
sense oligo: 5'-GGTGCGACGAGCCCAGGAGGTGAG-3' (SEQ ID NO:13)
Antisense oligo: 5'-TGCAGGTGCGACGAGCCCAGGAGGTGAGGATC-3' (SEQ ID NO:14)

$VEGF_{165}D$:
sense oligo: 5'- GGTGCGGAGCCCCCAGGAGGTGAG-3' (SEQ ID NO:15)
Antisense oligo: 5'- TGCAGGTGCGGAGCCCCCAGGAGGTGAGGATC-3' (SEQ ID NO:16)

$VEGF_{165}E$:
sense oligo: 5'- GGTGCAAGAAGCCCAGGAGGTGAG-3' (SEQ ID NO:17)
Antisense oligo: 5'- TGCAGGTGCAAGAAGCCCAGGAGGTGAGGATC-3' (SEQ ID NO:18)

$VEGF_{165}F$:
sense oligo: 5'- GGTGCGACGCCCCCAGGAGGTGAG-3' (SEQ ID NO:19)
Antisense oligo: 5'- TGCAGGTGCGACGCCCCCAGGAGGTGAGGATC-3' (SEQ ID NO:20)

The $VEGF_{165}g$ mutant is cloned using an annealed oligonucleotide sequence comprising the mutated sequence between the SbfI and BamHI sites of the VEGF-A_{165} cDNA in pTT5 vector as shown below:

$VEGF_{165}G$:
sense oligo: 5'- TCTGCGACGAGCCCAGGAGGTGAG-3' (SEQ ID NO:21)
Antisense oligo: 5'- TGCATCTGCGACGAGCCCAGGAGGTGAGGATC-3' (SEQ ID NO:22)

Example 2: Expression and Purification of VEGF proteins

The VEGF mutants constructs described in Example 1 were expressed and purified for further experimentation. Human recombinant VEGF (isoform 165b) was from R&D Systems. Human recombinant VEGF (isoform 165) was produced and purified as described (Labrecque et al., 2004).

The VEGF mutants (shown in Figure 1) were expressed using methods known in the art. Briefly, 293-6E cells were cultured in Erlenmeyer flasks using 25% of their nominal volume at 110 rpm under standard humidified conditions (37°C and 5% CO_2) in F17 medium (Invitrogen, Grand Island NY) supplemented with 4 mM glutamine, 25 µg/ml Geneticin 418, and 0.1% Pluronic F-68. Cells were seeded two days prior to transfection at $2.5x10^5$ cells/ml in fresh medium at 90% of the final volume. Cells were transfected with the pTT5 vectors of Example 1 at a density of 0.8 to 1.2 $x10^6$ cells/ml. The transfection mixture was prepared in 10% of the final volume using F17 medium, 1 mg/L plasmid and 2 mg/L 25 kDa linear polyethylenimine (Polysciences, Warrington PA) as the transfection vehicle (Durocher et al, 2002; Tom et al, 2007). Cells were fed with 20% w/v TN1 peptone in F17 medium 24 hours after transfection, to give 0.5% w/v TN1 in the culture (Pham et al, 2005). VEGF was produced in 500 mL culture and harvested 5 days post-transfection. At harvest, cells were eliminated by centrifugation followed by 0.45 µm filtration. Clarified supernatant was either processed immediately, or stored frozen at -80°C until processing.

VEGF mutants were purified by chromatographic techniques. All chromatographic operations were performed using an AKTA Explorer 100 (GE Healthcare) controlled by UNICORN software version 5.01 (GE Healthcare). For purification by cation exchange, the clarified supernatant was acidified to pH 4.0 by dropwise addition of 12 N HCl. Acid precipitate was removed by centrifugation followed by filtration under vacuum using a 0.45 µm filter (Millipore). The supernatant was loaded onto Fractogel EMD SO3 650 (M) (EMD) packed in an XK16/20 column (GE Healthcare) equilibrated with wash buffer (100 mM sodium acetate / acetic acid, 80 mM NaCl, pH 4.0). The column was washed with 5 column volumes (CV) of wash buffer followed by 10 CV of 600 mM NaCl buffer to remove impurities, and the VEGF was eluted with 10 CV of 1.5 M NaCl and collected in 5 mL fractions. The VEGF fractions were pooled and loaded onto a HiPrep 26/10 Desalting column (GE Healthcare) equilibrated with PBS; the desalted VEGF fraction was then passed through Fractogel EMD DEAE 650 (M) (EMD) packed in a XK16/20 column (GE Healthcare) connected in series with the desalting column to remove impurities. The VEGF was sterile filtered at 0.22 µm and frozen in 1 ml aliquots.

The VEGF-A_{165} mutants were analyzed by SDS-PAGE electrophoresis (Figure 2). Approximately 7.5 µg of each purified mutant was resolved by reducing SDS-PAGE and the gel stained with Coomassie blue using methods well-known in the art.

Example 3: Effect of VEGF-A_{165} and VEGF-A_{165BRI} on VEGFR2 (Flk-1) Phosphorylation and ERK Activation in HUVEC

Human umbilical vein endothelial cells (HUVECs) were exposed to either VEGF-A_{165} or VEGF-A_{165BRI} to evaluate their effects on VEGFR2 phosphorylation and ERK activation.

HUVECs were purchased from Clonetics (San Diego, CA) and maintained in EC basal medium-2 (EBM-2) supplemented with EGM-2 Mv growth factor mixture (Clonetics). The cells were cultured at 37°C under a humidified 95%/5% (v/v) mixture of air and CO_2. For experimental purposes, cells were plated in 100 mm plastic dishes at 5000 cells/cm^2 and were grown to confluence before overnight serum starvation without supplements. Cells were treated with VEGF-A_{165} or VEGF-A_{165BRI}.

HUVEC cells were treated with VEGF-A_{165} (50 ng/ml) or 50-200 ng/ml of VEGF-A_{165BRI} using previously described methods (Gingras et al, 2000). Cells were then washed once with ice-cold PBS (pH 7.4) containing 1 mM Na_3VO_4 and were incubated in the same medium for 1 h at 4°C. The cells were solubilised on ice in lysis buffer (150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.5% (v/v) Nonidet P-40 and 1% (v/v) Triton X-100) containing 1 mM Na_3VO_4. Lysates were clarified by centrifugation at 10 000 g for 10 min, and the resulting supernatants were used for VEGFR-2 immunoprecipitation overnight with 1 mg/ml of anti-VEGFR-2 antibody (C-1158; Santa Cruz Biotechnologies, Santa Cruz, CA) at 4°C followed by the addition of rabbit primary antibody pre-bound to protein A-Sepharose beads at 4°C for 2 h, as described previously (Labrecque et al, 2004). Non-specifically bound proteins were removed by washing the beads three times with lysis buffer and once with PBS containing 1 mM Na_3VO_4. The proteins were extracted with 2-fold concentrated Laemmli sample buffer, boiled 4 min, and resolved by SDS–PAGE (7.5% gel). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, blocked overnight at 4°C in Tris-buffered saline/Tween-20 (147 mM NaCl, 20 mM Tris–HCl (pH 7.5) and 0.1% Tween-20) containing 2% (w/v) bovine serum albumin (BSA), and probed with primary antibodies (PY99 or VEGFR-2) for 2 h at room temperature. Immunoreactive bands were revealed after 1 h incubation with HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories (West Grove, PA)), and the signals were visualized by enhanced chemiluminescence; ECL reagents were obtained from PerkinElmer Life Sciences (Boston,

MA). Figure 3 shows the results of these experiments. As can be seen in the figure, VEGF-A_{165BRI} does not activate VEGFR2, but does activate ERK phosphorylation in HUVEC cells.

The immunoreactive phospho-VEGFR2 bands obtained as described above were quantified by scanning densitometry (Molecular Dynamics, Sunnyvale, CA). PTK (ACC Corp, San Diego CA) was used at 1 μM as a positive control to inhibit VEGFR2 activation. The results, shown in Figure 4, indicate that VEGF-A_{165BRI} has higher antagonistic activity (VEGFR2 activation) compared to that of VEGF-A_{165b}.

Example 4: Effect of VEGF-A_{165} and VEGF-A_{165BRI} on ERK Activation in HBEC

Human brain endothelial cells (HBECs) were exposed to either VEGF-A_{165} or VEGF-A_{165BRI} to evaluate their effects on ERK activation.

HBECs were obtained from small intracortical microvessels and capillaries (112-20 μm) harvested from temporal cortex surgically excised from patients treated for idiopathic epilepsy. Tissues were obtained with approval from the Institutional Research Ethics Committee. HBECs were separated from smooth muscle cells with cloning rings and grown at 37°C in HBEC media containing Earle's salts, 25 mM 4-(2-hydroxyethyl)-1-piperazineetahanesulfonic acid (HEPES), 4.35 g/L sodium bicarbonate, and 3 mM L-glutamine, 10% FBS, 5% human serum, 20% of media conditioned by murine melanoma cells (mouse melanoma, Cloudman S91, clone M-3, melanin-producing cells), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium and 10 μg/ml endothelial cell growth supplement. HBEC cultures were routinely characterized morphologically and biochemically. More than 95% of cells in culture stained immunopositive for the selective endothelial markers, angiotensin II-converting enzyme and Factor VIII-related antigen, incorporated fluorescently labeled Ac-LDL, and exhibited high activities of the blood-brain barrier-specific enzymes, γ-glutamyltranspeptidase and alkaline phosphatase.

HBECs were rinsed three time with DME before applying different concentrations (5-400 ng/ml) of either VEGF-A_{165BRI} or VEGF-A_{165b} for 10 min.

Cells were then rinsed with ice-cold HBSS and exposed to 250 μL ristocetin-induced platelet agglutination lysis buffer (1%NP-40 (IGEPAL, lysis buffer, Sigma-Aldrich), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate and protease inhibitor 100 μL/10 mL ristocetin-induced platelet agglutination in PBS), (Sigma-Aldrich) for 30 min at 4 °C. The cell lysates were centrifuged at 700 g for 30 min at 4°C, and the supernatants were collected. Protein concentration was measured in each of the cell lysates by Bicinchoninic Acid Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA), and 15 μg of protein was denatured

in protein loading buffer for 5 min at 100°C, and then separated on a 10% sodium dodecyl sulfate–polyacrylamide gel. Separated proteins were then transferred to polyvinylidene difluoride membranes. Blocking was performed by incubating membranes in Tris-buffered saline with Tween 20 (TBST) (20 mM Tris-buffer (pH 8.0) and 150 mM NaCl with 0.1% Tween 20) containing 5% BSA (Sigma-Aldrich) for 1 h at 21°C. Membranes were then incubated overnight at 4°C with primary antibodies against p44/42 MAPK (Cell Signaling Technology, Beverly, MA, USA) diluted 1/1000 in TBST containing 5% BSA. The membranes were washed with TBST and incubated for 1 h with anti-rabbit IgG (whole molecule)–peroxidase antibody (Sigma-Aldrich) diluted 1/5000 in TBST containing 5% skimmed milk. Immunoreactive proteins were visualized by Amersham enhanced chemiluminescence plus western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

The results are shown in Figure 5. Figure 5A (left) shows increasing concentrations of VEGF-A_{165BRI} (left panel), while Figure 5B (right) shows increasing concentrations of VEGF-A_{165b}; ERK phosphorylation was revealed by western lot as described above. These results show that VEGF-A_{165BRI} has less agonistic activity (ERK activation) compared to VEGF-A_{165b}.

Alternatively, VEGF-A_{165} (25 ng/ml) was added to the cells following their pre-incubation for 15 minutes in the absence or presence of 200 ng/ml of VEGF-A_{165BRI} or VEGF-A_{165b}. ERK phosphorylation was revealed by western blot, as described above, 10 minutes after VEGF-A_{165} addition. Results are shown in Figure 6. VEGF-A_{165BRI} appears more potent than VEGF-A_{165b} at blocking ERK activation by VEGF-A_{165}.

Example 5: Effect of VEGF-A_{165}, VEGF-A_{165b} and VEGF-A_{165} mutants on ERK Activation in Mouse Astrocytes

Mouse astrocytes were exposed to VEGF-A_{165}, VEGF-A_{165}b, or one of the mutants described in Example 2 to evaluate their effects on ERK activation.

Astrocytes were isolated from 8-day-old C57BL6/J (B6) mice, bred at the National Research Council of Canada (NRCC) Institute for Biological Sciences Animal Facility (Ottawa, ON, Canada). Mice were deeply anesthetized with 4% halothane B. P. (MTC Pharmaceuticals, Cambridge, ON, Canada) under the oxygen flow rate of 2 L/min and then killed by guillotine. All procedures used in these studies were approved by the NRCC Animal Care Committee. Brains were removed and placed into ice-cold Dulbecco's modified Eagle's medium (DMEM) with antibiotic-antimycotic solution (Sigma-Aldrich, Oakville, ON, Canada). The cerebellum, and the olfactory bulbs were removed and brain cortices (four brains per isolation) were homogenized by passing through sequentially smaller gauge needles (18G 11/2, 23G 3/4,

and 25G 5/8). Dispase (12 mg/mL, Sigma-Aldrich) in DMEM was added to the homogenate, and incubated at 37°C for 15 min. After incubation, the homogenate was filtered through a 53 mm Nitex filter (Sefar Canada Inc., Scarborough, ON, Canada) and then centrifuged at 4°C for 10 min at 700 g. The pellet was resuspended in astrocyte media containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 1X antibiotic-antimycotic, and DMEM, and the cells were plated on poly-L-lysine (Sigma-Aldrich) coated 75 cm^2 culture flasks. After 3 days, cells were rinsed 3X with warm Hank's buffered salt solution (HBSS) (Sigma-Aldrich) and fed every 3–4 days thereafter. Cells were kept in culture in an atmosphere of 5% CO_2/95% air at 37°C. Experiments were performed using astrocytes from the first passage only. The purity of astrocyte cultures (> 99%) was confirmed by positive immunostaining with a rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (Dako, Hamburg, Germany).

Cells were incubated for 10 minutes in the presence of 200 ng/ml of VEGF-A_{165}, VEGF-A_{165}c, VEGF-A_{165}d, VEGF-A_{165}e, or VEGF-A_{165}f; other cells were pre-incubated with VEGF-A_{165}b, VEGF-A_{165}c, VEGF-A_{165}d, VEGF-A_{165}e, or VEGF-A_{165}f, then challenged for 10 minutes with 25 ng/ml of VEGF-A_{165}. ERK phosphorylation was revealed by western blot as described in Example 4. This experiment was done in duplicate, and results are show in Figure 7 (upper and lower panels). Results indicate that mutants VEGF-A_{165}c and VEGF-A_{165}d have the least agonistic activity.

Example 6: Analysis of VEGF-A_{165} and VEGF-A_{165}b

The degree of aggregation of the purified VEGF-A_{165} and VEGF-A_{165b} proteins was analysed by gel filtration chromatography.

Briefly, gel filtration was performed on a HiLoad Superdex 75 16/60 prep grade column (GE Healthcare) equilibrated with 50 mM MES, 150 mM NaCl pH 6.0. A 2 ml sample loop was filled with sample and injected into the column. Elution was performed with 1 CV of running buffer. Molecular weights were determined using Bio-Rad gel filtration standards. Elution profiles are shown in Figure 8. $VEGF_{165b}$ shows significantly higher amounts of oligomers migrating with an apparent Mr of ~113 kDa (Figure 8B) compared to $VEGF_{165}$ (Figure 8A).

VEGF-A_{165} and VEGF-A_{165b} were further analyzed by SDS-PAGE. Samples of VEGF-A_{165} (lanes 1) and VEGF-A_{165b} (lanes 2) were resolved on 4-14% Bis-Tris gels under reducing (Figure 9A) or non-reducing (Figure 9B) conditions and stained with Coomassie blue R-250 using known methods; 10 µg of protein was loaded in each lane. The presence of oligomers is represented by the brace. VEGF-A_{165b} shows significantly higher amounts of SDS-

resistant oligomers migrating with an apparent Mr of 67-200 kDa (Figure 9B, lane 2) compared to VEGF-A_{165} (Figure 9B, lane 1). These oligomers appear to be DTT-sensitive.

Materials

Cell culture media were obtained from Life Technologies (Burlington, ON, Canada) and serum was purchased from Hyclone Laboratories (Logan, UT). Protein A-Sepharose was obtained from Amersham Pharmacia Biotech (Baie d'Urfé, Qc, Canada). Anti-VEGFR-2 (C-1158) and anti-ERK-1/2 (K-23) polyclonal antibodies and the anti-phosphotyrosine (PY99) monoclonal antibody were from Santa Cruz Biotechnologies (Santa Cruz, CA). Antiphospho-p44/42 MAPK (Thr 202/Tyr 204) polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences (Boston, MA). Human recombinant VEGF-A (isoform 165b) was from R&D Systems. All other reagents were from Sigma-Aldrich (Oakville, ON, Canada).

The embodiments and examples described herein are illustrative and are not meant to limit the scope of the invention as claimed. Variations of the foregoing embodiments will be readily apparent to those of skill in the art in light of the teachings of this invention, and are intended by the inventor to be encompassed by the claims. Furthermore, the discussed combination of features might not be necessary for the inventive solution.

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All patents, patent applications and publications referred to herein are hereby incorporated by reference.

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Jia, H., Bagherzadeh, A., Hartzoulakis, B., Jarvis, A., Lohr, M., Shaikh, S., Aqil, R., Cheng, L., Tickner, M., Esposito., D., Harris., R., Driscoll, P.C., Selwood, D.L., and Zachary, I.C. (2006) J. Biol. Chem. 281, 13493-13502.

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Tom, R., L. Bisson, and Y. Durocher, Transient expression in HEK293-EBNA1 cells, in Methods Express: Expression Systems, M.R. Dyson and Y. Durocher, Editors. 2007, Scion Publishing Limited: Bloxham, Oxfordshire, UK. p. 203-223.

Claims:

1. A VEGF antagonist based on the sequence of VEGF-A_{165} (SEQ ID NO:3) and comprising a mutation at residue 159, 161, 162, 163, 164, 165, or a combination thereof, wherein the Cys at residue 160 is retained.

2. The antagonist according to claim 1 comprising SEQ ID NO: 23 or a conservative amino acid mutation thereof in other than the six C-terminal amino acids, and wherein:

a) Xaa at residue 159 is an acidic or neutral amino acid;

b) Xaa at residue 161 is a basic or neutral amino acid;

c) Xaa at residue 162 is an acidic or neutral amino acid;

d) Xaa at residue 163 is a hydrophobic amino acid;

e) Xaa at residue 164 is an acidic or neutral amino acid;

f) Xaa at residue 165 is an acidic or neutral amino acid; or

g) a combination of a) to f).

3. The antagonist according to claim 1, comprising SEQ ID NO: 23 or a conservative amino acid mutation thereof in other than the six C-terminal amino acids, and wherein:

a) Xaa at residue 159 is isoleucine, glutamate, aspartate, alanine or glycine;

b) Xaa at residue 161 is glycine, alanine, lysine or arginine;

c) Xaa at residue 162 is glutamate, aspartate or alanine;

d) Xaa at residue 163 is glycine or alanine;

e) Xaa at residue 164 is glutamate, aspartate, alanine or glycine; and,

f) Xaa at residue 165 is glutamate, aspartate, alanine or glycine.

4. The antagonist according to claim 1 comprising the amino acid sequence as set forth in SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10, or a conservative amino acid mutation thereof in other than the six C-terminal amino acids.

5. The antagonist according to claim 1 comprising the amino acid sequence as set forth in SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10.

6. The antagonist according to any one of claims 1 to 5, further comprising an additional N- or C-terminal sequence to target protein expression or provide ease of purification.

7. The antagonist according to claim 6, wherein the additional sequence comprises a VEGF signal peptide at the N-terminus or a His tag.

8. The antagonist according to claim 7, wherein the VEGF signal peptide comprises SEQ ID NO:11.

9. A nucleic acid encoding the VEGF antagonist as defined in any one of claims 1 to 8.

10. A vector comprising the nucleic acid of claim 9.

```
VEGFA165     1   apmaegggqnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkps
VEGFA165b    1   apmaegggqnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkps
VEGFA165BRI  1   apmaegggqnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkps
VEGFA165C    1   apmaegggqnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkps
VEGFA165D    1   apmaegggqnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkps
VEGFA165E    1   apmaegggqnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkps
VEGFA165F    1   apmaegggqnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkps
VEGFA165G    1   apmaegggqnhhevvkfmdvyqrsychpietlvdifqeypdeieyifkps

VEGFA165     51  cvplmrcggccndeglecvpteesnitmqimrikphqgqhigemsflqhn
VEGFA165b    51  cvplmrcggccndeglecvpteesnitmqimrikphqgqhigemsflqhn
VEGFA165BRI  51  cvplmrcggccndeglecvpteesnitmqimrikphqgqhigemsflqhn
VEGFA165C    51  cvplmrcggccndeglecvpteesnitmqimrikphqgqhigemsflqhn
VEGFA165D    51  cvplmrcggccndeglecvpteesnitmqimrikphqgqhigemsflqhn
VEGFA165E    51  cvplmrcggccndeglecvpteesnitmqimrikphqgqhigemsflqhn
VEGFA165F    51  cvplmrcggccndeglecvpteesnitmqimrikphqgqhigemsflqhn
VEGFA165G    51  cvplmrcggccndeglecvpteesnitmqimrikphqgqhigemsflqhn

VEGFA165     101 kcecrpkkdrarqenpcgpcserrkhlfvqdpqtckcsckntdsrckarq
VEGFA165b    101 kcecrpkkdrarqenpcgpcserrkhlfvqdpqtckcsckntdsrckarq
VEGFA165BRI  101 kcecrpkkdrarqenpcgpcserrkhlfvqdpqtckcsckntdsrckarq
VEGFA165C    101 kcecrpkkdrarqenpcgpcserrkhlfvqdpqtckcsckntdsrckarq
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VEGFA165F    101 kcecrpkkdrarqenpcgpcserrkhlfvqdpqtckcsckntdsrckarq
VEGFA165G    101 kcecrpkkdrarqenpcgpcserrkhlfvqdpqtckcsckntdsrckarq

VEGFA165     151 lelnertcrcdkprr (SEQ ID NO:3)
VEGFA165b    151 lelnertcrsltrkd (SEQ ID NO:4)
VEGFA165BRI  151 lelnertcrcdegrr (SEQ ID NO:5)
VEGFA165C    151 lelnertcrcdeprr (SEQ ID NO:6)
VEGFA165D    151 lelnertcrcgaprr (SEQ ID NO:7)
VEGFA165E    151 lelnertcrckkprr (SEQ ID NO:8)
VEGFA165F    151 lelnertcrcdaprr (SEQ ID NO:9)
VEGFA165G    151 lelnertcicdeprr (SEQ ID NO:10)
```

FIG. 1

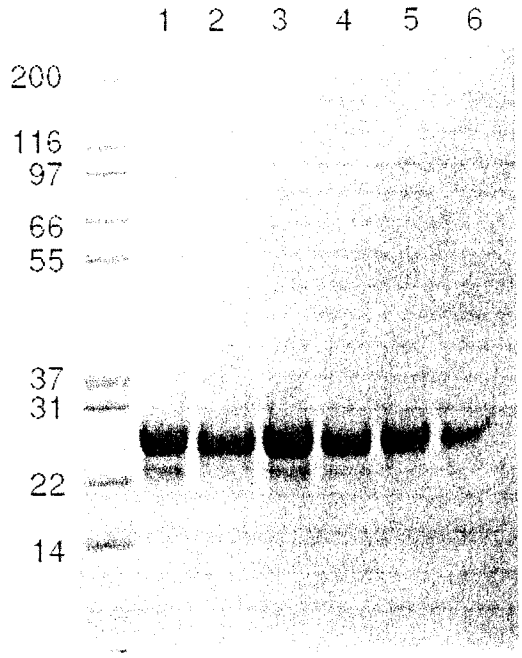

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200
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97
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55
37
31
22
14

FIG. 2

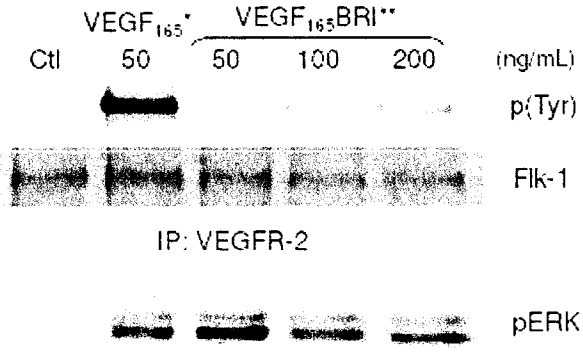

VEGF165*
VEGF165BRI**
Ctl
50
50
100
200
(ng/mL)
p(Tyr)
Flk-1
IP: VEGFR-2
pERK

FIG. 3

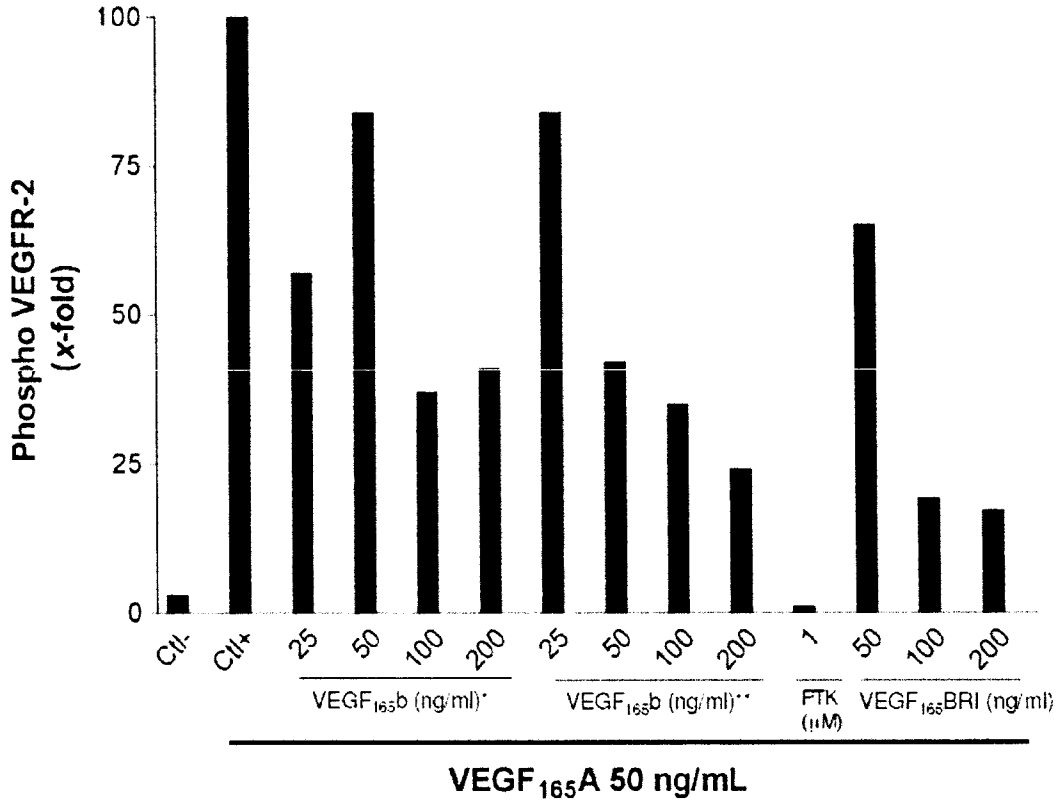


FIG. 4

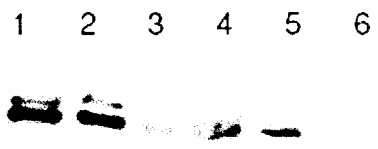


FIG. 5A

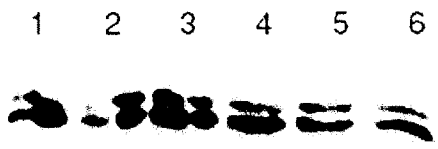


FIG. 5B

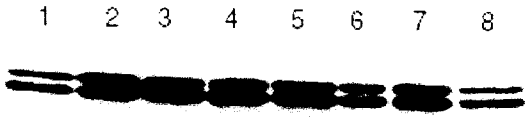

1 2 3 4 5 6 7 8

FIG. 6

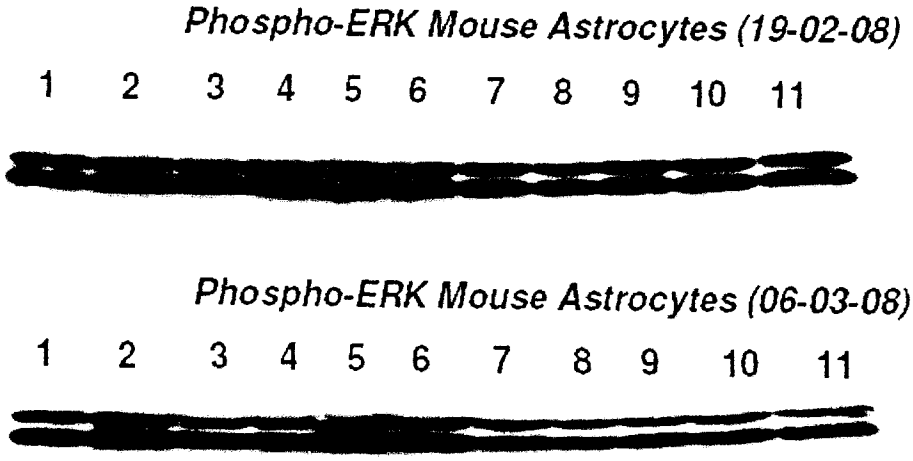

Phospho-ERK Mouse Astrocytes (19-02-08)
1 2 3 4 5 6 7 8 9 10 11
Phospho-ERK Mouse Astrocytes (06-03-08)
1 2 3 4 5 6 7 8 9 10 11

FIG. 7

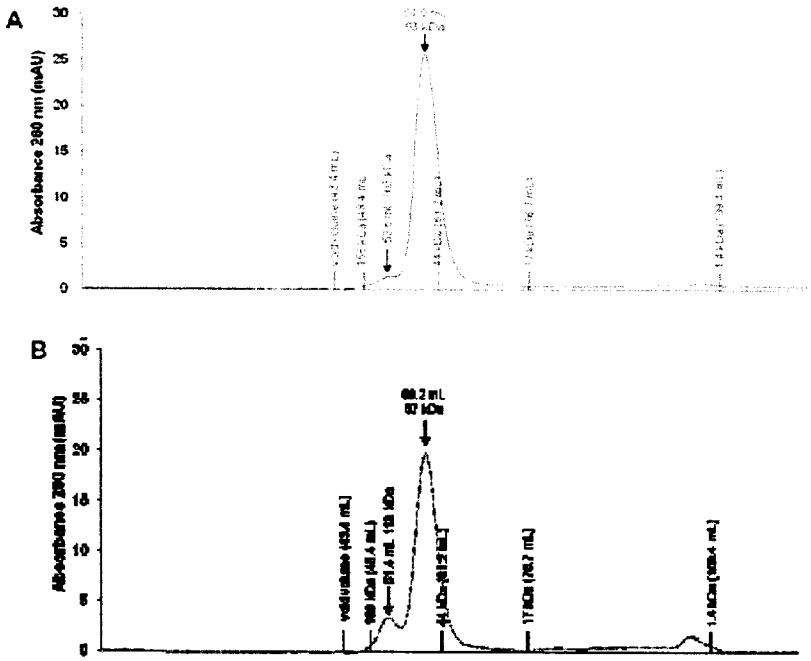

A
Absorbance 280 nm (mAU)
30
25
20
15
10
5
0
B
Absorbance 280 nm (mAU)
30
25
20
15
10
5
0
68.2 mL
17 kDa (68.7 mL)

FIG. 8

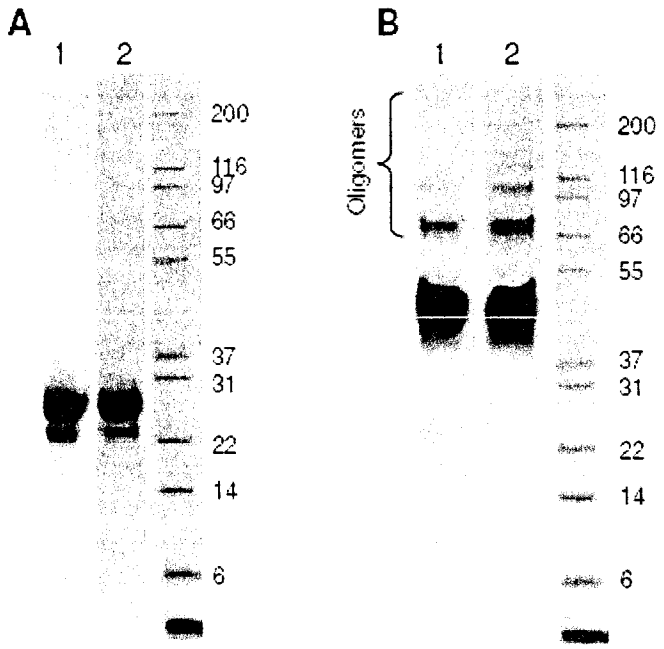

A
1
2
200
116
97
66
55
37
31
22
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6
B
1
2
Oligomers
200
116
97
66
55
37
31
22
14
6

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2010/000275

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07K 14/515*** (2006.01), ***A61K 38/18*** (2006.01), ***C07K 14/475*** (2006.01), ***C12N 15/18*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K, A61K, C12N

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Databases: PubMed, Delphion, Canadian Patent Database, GenomeQuest; Keywords: vascular endothelial growth factor, VEGF, VEGF-165, variant(s), antagonist(s), C-terminal, C-terminus, substitution, mutation, deletion, Exon 8 (in various combinations).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2007/044534 A2 (SZKUDLINSKI, M.W. et al) 19 April 2007 (19-04-2007); the whole document, particularly paragraph [0079].	1-3, 6-10 4, 5
X --- Y	US 7414027 B2 (KLAGSBRUN, M. et al) 19 August 2008 (19-08-2008); the whole document, particularly Figures 5 to 7 and SEQ ID NO:1.	1-3, 6-10 4, 5
X	WO 96/20403 A1 (STACKER, A. et al) 4 July 1996 (04-07-1996); Page 28, Figure 4, and SEQ ID NOS: 6, 7, 8, and 10.	1-3, 6-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :
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"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
31 May 2010 (31-05-2010)	8 June 2010 (08-06-2010)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Riad Qanbar (819) 934-7937

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BATES, D.O. et al. VEGF165b, an Inhibitory Splice Variant of Vascular Endothelial Growth Factor, Is Down-regulated in Renal Cell Carcinoma. Cancer Res. 2002, Vol.62, No.14, pp4123-4131, ISSN: 0008-5472; the whole document, particularly Figures 5C, 7, 8, and 9.	1-3, 6-10 4, 5
Y	JIA, H. et al, Characterization of a Bicyclic Peptide Neuropilin-1 (NP-1) Antagonist (EG3287) Reveals Importance of Vascular Endothelial Growth Factor Exon 8 for NP-1 Binding and Role of NP-1 in KDR Signaling. J. Biol. Chem. 2006, Vol.281, No.19, pp13493-13502, ISSN: 0021-9258; the whole document, particularly first paragraph on page 13498 and Table 2.	4, 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2010/000275

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

      [X] on paper

      [X] in electronic form

   b. (time)

      [X] in the international application as filed

      [ ] together with the international application in electronic form

      [ ] subsequently to this Authority for the purposes of search

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

3. Additional comments :

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2010/000275

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

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

1. [ ] Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

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

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

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

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

The claims are directed to a plurality of inventive concepts as follows:

Group A - Claims 1-10 (all partially) are directed to a VEGF antagonist of SEQ ID NO:5 and nucleic acids encoding said antagonist;

Continued in Supplemental Box.

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

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

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

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

1-10 insofar as they relate to a VEGF antagonist of SEQ ID NO:5.

Remark on Protest

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

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

[ ] No protest accompanied the payment of additional search fees.

Contiuation of Box No. III

Group B - Claims 1-10 (all partially) are directed to a VEGF antagonist of SEQ ID NO:6 and nucleic acids encoding said antagonist;

Group C - Claims 1-10 (all partially) are directed to a VEGF antagonist of SEQ ID NO:7 and nucleic acids encoding said antagonist;

Group D - Claims 1-10 (all partially) are directed to a VEGF antagonist of SEQ ID NO:8 and nucleic acids encoding said antagonist;

Group E - Claims 1-10 (all partially) are directed to a VEGF antagonist of SEQ ID NO:9 and nucleic acids encoding said antagonist; and

Group F - Claims 1-10 (all partially) are directed to a VEGF antagonist of SEQ ID NO:10 and nucleic acids encoding said antagonist.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.

The claims relate to antagonists of vascular endothelial growth factor (VEGF) obtained by mutating one or more of the last seven C-terminal amino acids of VEGF-165, excluding the cysteine at position 160. An *a posteriori* analysis revealed that VEGF antagonists that are obtained by amino acid mutations or deletions of VEGF-165 were known in the art on the relevant date. The prior art includes single amino acid mutations at various positions (WO 2007/044534 A2, SZKUDLINSKI, M.W. et al, 19 April 2007), mutations of the last six C-terminal amino acids (Bates, D.O. et al, 2002, Cancer Res, Vol.62, pp4123-4131; cited in application), and deletions of the last C-terminal five amino acids wherein the cysteine residue at position 160 is retained (US 7414027 B2, KLAGSBRUN, M. et al, 19 August 2008). As the prior art discloses VEGF antagonists in which each of the amino acids at positions 159, 161, 162, 163, 164, and 165 was altered by mutation or deletion, grouping of antagonists that share a mutation at a specific amino acid position (e.g., position 161, which is mutated in the VEGF antagonists of SEQ ID NO: 7 and SEQ ID NO: 8) is not possible. In view of the above, each antagonist is considered as a separate alleged invention.

A search and substantive examination were limited to the subject matter of group A, namely, to claims 1 to 10 insofar as they relate to a VEGF antagonist of SEQ ID NO:5.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2010/000275

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