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(54) **Title**: TRUNCATED CYSTINE-KNOT PROTEINS

(57) **Abstract**: The invention relates to the fields of protein chemistry, biology and medicine. More specifically, it relates to the design and preparation of proteinmimics of members of the cystine-knot growth factor superfamily. Further the invention relates to the use of these proteinmimics as a medicament or prophylactic agent. The invention provides proteinmimics of members of the cystine-knot growth factor superfamily, preferably for use in immunogenic and/or therapeutic compositions.

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Title: Truncated cystine-knot proteins

The invention relates to the fields of protein chemistry, biology and medicine. More specifically, it relates to the design and preparation of proteinmimics of members of the cystine-knot growth factor superfamily. Further the invention relates to the use of these proteinmimics as a medicament or prophylactic agent.

The cystine-knot three-dimensional structure is found in many extracellular molecules and is conserved among divergent species(ref 4). The cystine-knot structure is formed by the arrangement of six cysteines which, through their disulfide bonds form a knot. A typical consensus motif for a cystine-knot structure is: X0-C1-X1-C2-X2-C3-X3-C4-X4-C5-X5-C6-X6, wherein cysteines 2, 3, 5 and 6 form a ring that includes X2 and X3, by disulfide bonding of cysteines 2 and 5, and cysteines 3 and 6. The third disulfide bond between cysteines 1 and 4 penetrates the ring, thus forming a knot(ref 2,3). Figure 11 represents a schematic representation of a protein comprising a cystine-knot structure. This cystine-knot folding leads to the formation of three distinct domains, with two distorted beta-hairpin (beta-1 and beta-3) loops protruding from one side of the knot, and a single (beta-2) hairpin loop protruding from the other side of the knot. The beta-1 hairpin loop is formed by the stretch of amino acids between C1 and C2 and is designated “X1” in the above mentioned consensus motif; the beta-2 (“X3”) and beta-3 (“X4”) hairpin loops are formed by the amino acid stretch between C3 and C4, and between C4 and C5, respectively.

Growth factors represent a large group of polypeptides that share the property of inducing cell multiplication both *in vivo* and *in vitro*. Although the level of sequence similarity between growth factors is low, they can be classified into subfamilies based on their structural and functional similarities. For instance, the following growth factor subfamilies all show the cystine-knot conformation described above: glycoprotein hormone-beta (GLHB) subfamily, the platelet derived growth factor (PDGF) subfamily, the transforming growth factor beta (TGF-beta) subfamily, the nerve growth factor (NGF) subfamily, the glycoprotein hormone-alpha (GLHA) subfamily, CTCK subfamily, Noggin-like subfamily, Coagulin subfamily, Mucin-like subfamily, Mucin-like BMP-antagonist subfamily, Mucin-like hemolectin subfamily, Slit-like subfamily, and Jagged-like subfamily. However, the different sub-families have for instance different consensus lengths for X1, X2, X3, X4 and/or X5. Further, the different subfamilies have quite different functions and target organs. For instance, the GLHA and GLHB subfamilies are important for physiologic processes involved in reproduction, whereas members of the NGF subfamily exert their function mainly on nerve cells, and members of the PDGF subfamily mainly on endothelial cells.

Next to the cysteines involved in cystine-knot formation, other cysteines can be present in a cystine-knot protein, which are normally used to create further disulfide bonds within the cystine-knot, within the protruding domains, or between two proteins, for instance during dimerization.

There has been extensive research on cystine-knot growth factors in health and disease, and therapeutic examples, for instance, are the use of vascular endothelial growth factor (VEGF; a sub-subfamily of the PDGF subfamily) specific antibodies in the treatment of cancer, Bevacizumab (Avastin™), a monoclonal antibody developed by Genentech was approved in 2004 by the Food and Drug Administration (FDA) for the treatment of colorectal cancer, and the development of a follicle stimulating hormone (FSH; a member of the GLHA/B subfamily) vaccine as a contraceptive for men. Major drawbacks of the therapeutic VEGF specific monoclonal antibody Bevacizumab are the high production costs and relatively large amounts needed for treatment, sometimes low tumor penetration and its side effects. Furthermore, the antibody must be administered many times during a few months putting a high burden onto the patient.

A goal of the present invention is to provide proteinmimics of members of the cystine-knot growth factor superfamily, which are preferably capable of inducing an immune response against said members. Another goal of the present invention is to provide alternative means and methods for treatment and/or prophylaxis of cystine-knot protein-related conditions.

The invention provides proteinmimics of members of the cystine-knot growth factor superfamily, preferably for use in immunogenic and/or therapeutic compositions.

As said before, cystine-knot proteins have a complex conformation comprising a ring that is constituted of at least two amino acid stretches and two disulfide bonds connecting said amino acid stretches. A third disulfide bond penetrates the ring, forming a knot. All members of the cystine-knot growth factor superfamily further have in common that the amino acid stretches between the first and the second cysteine and the fourth and fifth cysteine form beta-hairpin loops that protrude in one direction, whereas another amino acid stretch, which is situated between cysteines three and four, protrudes from the opposite site of the molecule. (Figure 11). In a first embodiment, the invention provides a proteinmimic of a member of the cystine-knot growth factor superfamily, said proteinmimic having the motif X0-C1-X1-C2-X2-C3-X3-C4-X4-C5-X5-C6-X6, wherein C1 to C6 are cysteine residues which form a cystine-knot structure in which C1 is linked to C4, C2 is linked to C5 and C3 is linked to C6, and wherein X0 and X6 represent, independently from each other, an amino acid sequence with a length of 0 to 10 amino acids, preferably 0 to 5 amino acids, more preferably 0 to 3 amino acids, more preferably 0 to 2 amino acids, even more preferably 0 or 1 amino acids, most preferable 0 amino acids, X2 represents an amino acid sequence with a length of 2 to 24 amino acid residues with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to the amino acid sequence located between C2 and C3 of a member of the cystine-knot growth factor superfamily, X5 represents an amino acid sequence with a length of 1 amino acid residue, X1 represents an amino acid sequence with a length of 15 to 50 amino acids with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to the amino acid sequence located between C1 and C2 of a member of the cystine-knot growth factor superfamily, X3 represents an amino acid sequence with a length of 3 to 36 amino acids with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to the amino acid sequence located between C3 and C4 of a member of the cystine-knot growth factor superfamily, and X4 represents an amino acid sequence with a length of 15 to 50 amino acids with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to the amino acid sequence located between C4 and C5 of a member of the cystine-knot growth factor superfamily. Preferably, C2, C3, C5 and C6 form a ring by a bond between C2 and C5, and between C3 and C6, wherein the third bond between C1 and C4 penetrates the ring, thus forming a cystine-knot. In a preferred embodiment, a peptidomimetic according to the invention is provided, for which the total number of amino acids equals 130 or less, preferably 110 or less, more preferably 100 or less, even more preferably 90 or less, most preferably 80 or less.

In a preferred embodiment, a proteinmimic according to the invention is provided, wherein X1, X2, X3 and X4 each represent an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to an amino acid sequence of the same member of the cystine-knot growth factor superfamily. This thus means that the invention provides a proteinmimic of a member of the cystine-knot growth factor superfamily, said proteinmimic having the motif X0-C1-X1-C2-X2-C3-X3-C4-X4-C5-X5-C6-X6, wherein C1 to C6 are cysteine residues which form a cystine-knot structure in which C1 is linked to C4, C2 is linked to C5 and C3 is linked to C6, and wherein X0 and X6 represent, independently from each other, an amino acid sequence with a length of 0 to 10 amino acids, preferably 0 to 5 amino acids, more preferably 0 to 3 amino acids, more preferably 0 to 2 amino acids, more preferably 0 or 1 amino acids, most preferably 0 amino acids, X2 represents an amino acid sequence with a length of 2 to 24 amino acid residues with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to the amino acid sequence located between C2 and C3 of said member of the cystine-knot growth factor superfamily, X5 represents an amino acid sequence with a length of 1 amino acid residue, X1 represents an amino acid sequence with a length of 15 to 50 amino acids with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to the amino acid sequence located between C1 and C2 of said member of the cystine-knot growth factor superfamily, X3 represents an amino acid sequence with a length of 3 to 36 amino acids with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to the amino acid sequence located between C3 and C4 of said member of the cystine-knot growth factor superfamily, and X4 represents an amino acid sequence with a length of 15 to 50 amino acids with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to the amino acid sequence located between C4 and C5 of said member of the cystine-knot growth factor superfamily. Preferably, C2, C3, C5 and C6 form a ring by a bond between C2 and C5, and between C3 and C6, wherein the third bond between C1 and C4 penetrates the ring, thus forming a cystine-knot. In a preferred embodiment, a peptidomimetic according to the invention is provided, for which the total number of amino acids equals 130 or less, preferably 110 or less, more preferably 100 or less, even more preferably 90 or less, most preferably 80 or less.

A member of the cystine-knot growth factor superfamily is herein defined as any protein that forms a typical cystine-knot three-dimensional structure as described above, thus with at least six cysteines that form a cystine-knot and three hairpin loops protruding from the knot, wherein cysteines 2, 3, 5 and 6 form a ring by a bond between cysteines 2 and 5, as well as between cysteines 3 and 6, and wherein the third bond between cysteines 1 and 4 penetrates the ring, thus forming the knot. A person skilled in the art is able, for instance by a combination of pattern search and pair wise alignments, to identify structural motifs, present in members of the cystine-knot growth factor superfamily. A person skilled in the art may be guided in his search for instance by known cystine-knot proteins belonging to the cystine-knot growth factor superfamily, for instance by the non-limiting examples provided in Figure 10.

The inventors have provided the insight that so called "truncated cystine-knot proteins" according to the invention are especially useful for treating or preventing cystine-knot protein-related disorders. They have for instance shown that a truncated VEGF according to the invention shows negligible hormonal activity, whereas its immunological properties are excellent. One of the advantages of the negligible hormonal activity of truncated VEGF according to the invention is for instance, that a significant amount of truncated VEGF can be administered to an animal, without the hormonal side effects of the whole protein. Another advantage of truncated VEGF in comparison to the native protein or smaller fragments thereof, is that truncated VEGF is immunogenic per se. This is due to the fact that, in contrast to smaller fragments, truncated VEGF is large enough to be immunogenic without being coupled to a carrier protein and, in contrast to the native protein, is "non-native" enough to be seen as non-self by the immune system. With non-self is meant that the immune system does not consider the protein or parts of the protein as a self-protein and therefore mounts an immune response towards said protein. Without being bound to theory, the fact that a truncated protein according to the invention is seen as "non-self" is explained for instance by the concept of "cryptic peptides". Cryptic peptides are defined as peptides that are part of a (self-)protein, but under normal conditions are not presented to the immune system. The immune system is “ignorant” of these cryptic peptides. Proteins taken up by antigen presenting cells are processed, i.e. cut in small peptide fragments. Under normal conditions, these small peptide fragments of a given protein are more or less identical after each processing. These are so-called dominant peptides. Each time a given protein is processed it produces for instance peptides x, y and z in sufficient amounts to be effectively presented to the immune system. The immune system, constantly being exposed to peptides x, y and z of self proteins, ignores these dominant peptides of self proteins, whereas dominant peptides of non-self proteins, which are occasionally present, are reacted to. If, however, a self protein is for instance truncated according to the invention, the peptide fragments after processing in antigen presenting cells differ from those of the whole native protein. As a result so-called “cryptic peptides”, peptides that are not normally presented, are being generated and presented to the immune system in sufficient amounts. Instead of for instance the dominant self peptides x, y and z, peptides x, z and w are generated and presented to the immune system. As the immune system has not been exposed to cryptic peptide w previously, the immune system regards peptide w as non-self, and initiates an immune reaction. Without being bound to theory, this phenomenon may explain the enhanced immunogenicity of the truncated protein according to the invention as compared to the native protein.

The inventors have further shown that the cystine-knot structure is important for the immunological properties of the protein. This is especially true, if the native protein is to be immunologically mimicked. The inventors have for instance shown that a truncated VEGF protein in which the cysteines were blocked, disabling cystine-knot formation, is not recognized by the therapeutic VEGF monoclonal antibody Bevacizumab, whereas a truncated VEGF in which a cystine-knot is presented is recognized by said antibody. It is clear that what is said above for VEGF is equally well true for other members of the cystine-knot growth factor superfamily. If for instance a proteinmimic of FSH is used, it is preferred that the biological or hormonal activity is negligible, whereas the proteinmimic is preferably able to induce antibodies, preferably neutralizing antibodies that are capable of cross-reacting with the native protein. The same holds true for other members of the GLHA/GLHB subfamily, or members of other subfamilies.

A "truncated cystine-knot protein" is defined herein as a cystine-knot protein, in which at least part of the native amino acid sequence has been deleted, preferably N-terminal and/or C-terminal of the cystine-knot sequence. More preferably, the amino acid sequences N-terminal of C1 and C-terminal of C6 have been completely deleted. In a preferred embodiment, therefore, the invention provides a proteinmimic according to the invention, wherein said proteinmimic has the motif C1-X1-C2-X2-C3-X3-C4-X4-C5-X5-C6. Preferably, C2, C3, C5 and C6 form a ring by a bond between C2 and C5, and between C3 and C6, and a third bond between C1 and C4 penetrates the ring, thus forming a cystine-knot. In a more preferred embodiment, a peptidomimetic according to the invention is provided, for which the total number of amino acids equals 130 or less, preferably 110 or less, more preferably 100 or less, even more preferably 90 or less, most preferably 80 or less so that biological activity, e.g. hormonal side effects, are significantly reduced.

In a preferred embodiment, a proteinmimic according to the invention is provided, wherein X1 represents an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to an amino acid sequence of a member of the cystine knot-growth factor superfamily and wherein X2, X3 and/or X4 represent an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to an amino acid sequence of at least one other member of the cystine knot growth factor superfamily. This is called a chimeric proteinmimic because the proteinmimic contains amino acid sequences with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to sequences of at least two different members of the cystine-knot growth factor superfamily. Such a chimeric proteinmimic according to the invention preferably comprises loops, at least one of which representing a loop from another member of the cystine-knot growth factor superfamily than the other loops. In a preferred embodiment, each of said loops represents another member of the cystine-knot growth factor superfamily. In another preferred embodiment, the invention provides a proteinmimic according to the invention, wherein said proteinmimic comprises the motif C1-X1-C2-X2-C3-X3-C4-X4-C5-X5-C6, wherein each of said X1, X2, X3, X4 and X5 represents an amino acid sequence that has at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95% sequence identity with the corresponding part of a sequence selected from any of the sequences 1 to 145 of Figure 10. In a most preferred embodiment, each of said X1, X2, X3, X4 and X5 represents an amino acid sequence that is identical to the corresponding part of a sequence selected from sequences 1 to 145 of Figure 10.

It is especially useful to substitute at least one loop of one member of a cystine-knot growth factor superfamily with a loop of another member of a cystine-knot growth factor superfamily, wherein the latter loop is smaller, i.e. comprises lesser amino acids, than the loop which is substituted. One advantage of a substation with a smaller loop is that the proteinmimic is manufactured more easily. In a working example the invention for instance shows that the substitution of the b2 loop (represented by “X3”) of Transforming Growth Factor-B2 (TGFB2) consisting of 29 amino acids with the b2 loop of VEGF consisting of 6 amino acids provides a proteinmimic that is successfully used to induce antibodies that fully crossreact with the full-length TGFB2 protein. In a preferred embodiment therefore, the invention provides a proteinmimic according to the invention, wherein X3 represents an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to an amino acid sequence of a member of the cystine knot-growth factor superfamily and wherein X1, X2 and/or X4 represent an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to an amino acid sequence of at least one other member of the cystine knot growth factor superfamily, preferably wherein said at least one other member of the cystine knot growth factor superfamily is a member of the TGF-beta subfamily, more preferably TGFB2. Preferably X1, X2 and X4 each represent an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to an amino acid sequence of the corresponding part of the same cystine-knot growth factor superfamily, whereas X3 represents an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to an amino acid sequence of the corresponding part of another member of the cystine-knot growth factor superfamily. Preferably X1, X2 and X4 represent an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to an amino acid sequence of a member of the TGF-beta subfamily, more preferably to an amino acid sequence of TGFB2. In a more preferred embodiment, the chimeric proteinmimic consists of the amino acid sequence: **C1**ALRPLYIDFKRDLGWKWIHEPKGYNANF**C2**AGA**C3**NDEGLE**C4**VSQDLEPLTILYYIGKTPKIEQLSNMIVKS**C5**K**C6** **(TGFB2**$_{15\text{-}111/\Delta 49\text{-}77}$**-VEGF**$_{62\text{-}67}$), optionally comprising flanking sequences with a length of at most 5 amino acids. In a preferred embodiment, the flanking sequences have a length of at most 2 amino acids, preferably at most 1 amino acid. In a most preferred embodiment, the proteinmimic does not comprise flanking sequences.

TGF2B2 is a member of the TGF-beta subfamily. It is a secreted protein (cytokine) that performs many cellular functions and has a vital role during embryonic development. It is also known as Glioblastomaderived T-cell suppressor factor, G-TSF, BSC-1 cell growth inhibitor, Polyergin, and Cetermin. It is known to suppress the effects of interleukin dependent T-cell tumors.

In another preferred embodiment, the invention provides a proteinmimic according to the invention, wherein X0 represents acetyl and/or X6 represents amide. In a more preferred embodiment, X0 represents acetyl and X6 represents amide. Acetylation of the N-terminus and/or amidation of the C-terminus has several advantages, for instance the acetylated and amidated peptide ends are uncharged so they mimic natural peptides, stability toward digestions by aminopeptidases is enhanced and peptide ends are blocked against synthetase activities.

In another preferred embodiment, the invention provides a proteinmimic of a member of the cystine-knot growth factor superfamily, said proteinmimic having an identical sequence as said member, with the exception that the protein is truncated at position 0 to 10, preferably at position 0 to 5, more preferably at position 0 to 3, even more preferably at position 0 to 2, most preferably at position 0 or 1 N-terminal of C1 and at position 0 to 10, preferably at position 0 to 5, more preferably at position 0 to 3, even more preferably at position 0 to 2, more preferably at position 0 or 1, most preferably at position 0 C-terminal of C6.

Instead of the native sequence of a given member, consensus sequences of a subfamily can be used for designing a proteinmimic useful in the invention.

For the cystine-knot growth factor superfamily, several consensus sequences have been described(ref 1,3). For instance, for all but the Noggin-, Coagulin- and NGF-like cystine-knot proteins, X2 consists of 2 or 3 amino acids which can be defined as X2a-G-X2b, wherein X2a is any amino acid or none, G is glycine, and X2b is any amino acid. In a preferred embodiment, therefore, a proteinmimic according to the invention is provided, wherein X2 has the amino acid sequence X2a-G-X2b, wherein

X2a is any amino acid or none, G is glycine, and X2b is any amino acid. Other consensus sequences are known for instance for TGF-beta, GLHB, NGF, PDGF, GLHA, and CTCK. Known consensus sequences are depicted for the respective subfamilies in Fig. 10.In another preferred embodiment, a proteinmimic according to the invention is provided, which comprises at least one of the following consensus sequences:

- [GSRE]C3[KRL]G[LIVT][DE]XXX[YW]XSXC4;
- P[PSR]CVXXXRC2[GSTA]GCC3;
- [LIVM]XXPXX[FY]XXXXC2XGXC3;
- C2[STAGM]G[HFYL]C3X[ST];
- [PA]VAXXC5XC6XXCXXXX[STDAI][DEY]C;
- C2XGCC3[FY]S[RQS]A[FY]PTP; or
- CC4(X)13C(X)2[GN](X)12C5XC6(X)2,4C;

wherein

C2 to C6 are cysteine residues which are part of a cystine-knot structure;

X means any amino acid;

[GSRE] means G or S or R or E ; [KRL] means K or R or L;

[LIVT] means L or I or V or T; [DE] means D or E ; [YW] means Y or W;

[PSR] means P or S or R; [GSTA] means G or S or T or A;

[LIVM] means L or I or V or M ; [FY] means F or Y ;

[STAGM] means S or T or A or G or M; [HFYL] means H or F or Y or L;

[ST] means S or T; [PA] means P or A; [STDAI] means S or T or D or A or I;

[DEY] means D or E or Y; [GN] means G or N ; [RQS] means R or Q or S;

(X)13 means a sequence of 13 amino acids; (X)2 means a sequence of 2 amino acids; (X)12 means a sequence of 13 amino acids and (X)2,4 means a sequence of 2, 3 or 4 amino acids.

It is preferred to use a proteinmimic, which shows a considerable % sequence identity with a native amino acid sequence of said cystine-knot protein, in order to produce antibodies and/or T cells that are capable of cross-reacting towards the native protein. With considerably % sequence identity is meant: at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity with the native amino acid sequence of said cystine-knot protein. This is especially true if the proteinmimic is used as a vaccine to induce an immune response that is cross-reactive with a native cystine-knot protein, but also if the proteinmimic is used to induce T-cells and/or antibodies to be used as a medicament. The T-cells and/or antibodies that are raised against the proteinmimic are especially useful if they are able to cross-react with a native cystine-knot protein. However, in another embodiment, it can be especially useful to not generate antibodies against the native protein, for instance if the proteinmimic is to be used as an antagonist of a cystine-knot protein. In such a case, a proteinmimic according to the invention with a lower sequence identity with the native protein is designed, preferably between 70% and 90%, more preferably between 70% and 80%, most preferably between 70% and 75% sequence identity with the native amino acid sequence of said cystine-knot protein. Administration of such a proteinmimic with antagonistic properties to an individual preferably does not induce a T-cell and/or antibody response in said individual. In order to act as an antagonist, said proteinmimic preferably does not convey protein function to a receptor.

"% sequence identity" is defined herein as the percentage of residues in a candidate amino acid sequence that is identical with the residues in a reference sequence after aligning the two sequences and introducing gaps, if necessary, to achieve the maximum percent identity. Methods and computer programs for the alignment are well known in the art. One computer program which may be used or adapted for purposes of determining whether a candidate sequence falls within this definition is "Align 2", authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, D.C. 20559, on Dec. 10, 1991.

In a preferred embodiment, a proteinmimic according to the invention is provided, wherein the proteinmimic has an amino acid sequence with at least 70% sequence identity, preferably at least 80%, more preferably at least 85%, most preferably at least 90% sequence identity to the corresponding native amino acid sequence of said member of the cystine-knot growth factor superfamily. In another preferred embodiment, the invention provides a proteinmimic according to the invention, wherein said member of the cystine-knot growth factor superfamily is a member selected from the group consisting of the GLHB subfamily, the PDGF subfamily, the TGF-beta subfamily, the NGF subfamily, the GLHA subfamily, the CTCK subfamily, the Noggin-like subfamily, the Mucin-like subfamily, the Mucin-like BMP antagonist subfamily, the Mucin-like hemolectin subfamily, the Slit-like subfamily, and the Jagged-like subfamily.

In another preferred embodiment, a proteinmimic according to the invention is provided, wherein the proteinmimic has an amino acid sequence with between 70% and 90%, more preferably between 70% and 80%, most preferably between 70% and 75% sequence identity to the corresponding native amino acid sequence of said member of the cystine-knot growth factor superfamily. In another preferred embodiment, the invention provides a proteinmimic according to the invention, wherein said member of the cystine-knot growth factor superfamily is a member selected from the group consisting of the GLHB subfamily, the PDGF subfamily, the TGF-beta subfamily, the NGF subfamily, the GLHA subfamily, the CTCK subfamily, the Noggin-like subfamily, the Mucin-like subfamily, the Mucin-like BMP antagonist subfamily, Mucin-like hemolectin subfamily, the Slit-like subfamily, and the Jagged-like subfamily.

It is also useful to design a proteinmimic according to the invention with at least 70% sequence identity, preferably at least 80%, more preferably at least 85%, most preferably at least 90% sequence identity to the corresponding native amino acid sequence of said member of the cystine-knot growth factor superfamily, wherein at least one of the amino acid sequences represented by X1, X3, or X4 is at least partly deleted and/or modified. This is for instance especially useful if said amino acid sequence comprises an immunodominant peptide, or if said amino acid sequence has no function, for instance if said sequence it is not part of the immunogenic determinant of said member. Deletion of such an amino acid sequence can for instance significantly facilitate the manufacturing process, reduce manufacturing costs or improve solubility of the proteinmimic according to the invention. In a preferred embodiment therefore, the invention provides a proteinmimic according to the invention, wherein at least one of the amino acid sequences represented by X1, X3, or X4 is at least partly deleted and/or modified.

For instance PDGF plays a role in embryonic development, cell proliferation, cell migration, and angiogenesis. PDGF has also been linked to several diseases such as atherosclerosis, fibrosis and malignant diseases. Especially the VEGF family, a sub-subfamily of the PDGF subfamily has been linked to angiogenesis related to tumor growth and metastasis. Accordingly, in a preferred embodiment, the invention provides a proteinmimic according to the invention, wherein said member is a member of the PDGF subfamily, and wherein X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 29 to 32 amino acids, X3 represents an amino acid sequence with a length of 6 to 12 amino acids, and X4 represents an amino acid sequence with a length of 32 to 41 amino acids.

In a more preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Vascular Endothelial Growth Factor (hVEGF), and wherein X0 comprises amino acid sequence KFMDVYQRSY, X1 comprises amino acid sequence HPIETLVDIFQEYDPEIEYIFKPSAVPLMR, X2 comprises GGA, X3 comprises NDEGLE, X4 comprises VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNK, X5 comprises E, and X6 comprises RPKKDRARQE. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to X0-X6 of hVEGF, wherein X0-X6 are the respective hVEGF amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of hVEGF. In yet another more preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Vascular Endothelial Growth Factor (hVEGF), and wherein said proteinmimic consists of the amino acid sequence C1HPIETLVDIFQEYDPEIEYIFKPSAVPLMRC2GGAC3NDEGLEC4VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKC5EC6, optionally comprising flanking sequences with a length of at most 5 amino acids. In a preferred embodiment, the flanking sequences have a length of at most 2 amino acids, preferably at most 1 amino acid. In a most preferred embodiment, the proteinmimic does not comprise flanking sequences.

Placental growth factor (PLGF) is a member of the PDGF subfamily (subfamily 4) and a key molecule in angiogenesis and vasculogenesis, in particular during embryogenesis. The main source of PLGF during pregnancy is the placental trophoblast. PLGF is also expressed in many other tissues, including the villous trophoblast. PLGF expression within human atherosclerotic lesions is associated with plaque inflammation and neovascular growth.

Serum levels of PLGF and sFlt-1 (soluble fms-like tyrosine kinase-1, also known as soluble VEGF receptor-1) are altered in women with preeclampsia. Studies show that in both early and late onset preeclampsia, maternal serum levels of sFlt-1 are higher and PLGF lower in women presenting with preeclampsia. In addition, placental sFlt-1 levels were significantly increased and PLGF decreased in women with preeclampsia as compared to those with uncomplicated pregnancies. This suggests that placental concentrations of sFlt-1 and PLGF mirror the maternal serum changes. This is consistent with the view that the placenta is the main source of sFlt-1 and PLGF during pregnancy.

In yet another preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Placental Growth Factor (hPLGF), and wherein X0 comprises amino acid sequence PFQEVWGRSY, X1 comprises amino acid sequence RALERLVDVVSEYPSEVEHMFSPSAVSLLR, X2 comprises TGA, X3 comprises GDENLH, X4 comprises VPVETANVTMQLLKIRSGDRPSYVELTFSQHVR, X5 comprises E, and X6 comprises RHSPGRQSPD. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to X0-X6 of PLGF, wherein X0-X6 are the respective PLGF amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of PLGF. In yet another preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Placental Growth Factor (hPLGF), and wherein said proteinmimic consists of the amino acid sequence C1RALERLVDVVSEYPSEVEHMFSPSAVSLLRC2TGAC3GDENLHC4VPVETANVTMQLLKIRSGDRPSYVELTFSQHVRC5EC6 (hPLGF$_{34\text{-}112}$), optionally comprising flanking sequences with a length of at most 5 amino acids. In a preferred embodiment, the flanking sequences have a length of at most 2 amino acids, preferably at most 1 amino acid. In a most preferred embodiment, the proteinmimic does not comprise flanking sequences.

In yet another preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Platelet Derived Growth Factor A (hPDGF-A), and wherein X0 comprises amino acid sequence SIEEAVPAV, X1 comprises amino acid sequence KTRTVIYEIPRSQVDPTSANFLIWPPCVEVKR, X2 comprises TGC, X3 comprises NTSSVK, X4 comprises QPSRVHHRSVKVAKVEYVRKKPKLKEVQVRLEEHLE, X5 comprises A, and X6 comprises ATSLNPDYRE. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to

X0-X6 of hPDGF-A, wherein X0-X6 are the respective hPDGF-A amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of hPDGF-A.

In yet another preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Platelet Derived Growth Factor A (hPDGF-C), and wherein X0 comprises amino acid sequence LLTEEVRLYS, X1 comprises amino acid sequence TPRNFSVSIREELKRTDTIFWPGCLLVKR, X2 comprises GGN, X3 comprises ACCLHNCNECQ, X4 comprises VPSKVTKKYHEVLQLRPKTGVRGLHKSLTDVALEHHEE, X5 comprises D, and X6 comprises VCRGSTGG. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to X0-X6 of hPDGF-C, wherein X0-X6 are the respective hPDGF-C amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of hPDGF-C.

In yet another preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Vascular Endothelial Growth Factor C (hVEGF-C), and wherein X0 comprises amino acid sequence SIDNEWRKTQ, X1 comprises amino acid sequence MPREVAIDVGKEFGVATNTFFKPPCVSVYR, X2 comprises GGC, X3 comprises PDDGLE, X4 comprises VPTGQHQVRMQILMIRYPSSQLGEMSLEEHSQ, X5 comprises E, and X6 comprises RPKKKDSAVK. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to X0-X6 of hVEGF-C, wherein X0-X6 are the respective hVEGF-C amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of hVEGF-C.

Other subfamilies of the cystine-knot growth factor superfamily include the GLHA and GLHB subfamily. Members of said subfamilies comprise the glycoprotein hormone-alpha and glycoprotein hormone-beta subunits, respectively, that after dimerization form luteinizing hormone, (LH), thyroid stimulating hormone (TSH), chorionic gonadotropin (CG) and follicle stimulating hormone (FSH). These hormones all play a role in reproduction in mammals. For instance FSH stimulates testicular and ovarian functions through binding to a G-protein-coupled receptor on either Sertoli (male) or granulose (female) cells. Amongst other things, LH stimulates ovulation and sustains the corpus luteum during menstrual cycle, whereas CG for instance sustains the corpus luteum during pregnancy. TSH is important for Sertoli cell maturation and ovulatory function. The present invention also provides proteinmimics of this GLHB subfamily.

Thus, in another preferred embodiment, said member of the cystine-knot growth factor superfamily is a member of the GLHB subfamily, X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 23 to 28 amino acids, X3 represents an amino acid sequence with a length of 18 to 20 amino acids, and X4 represents an amino acid sequence with a length of 30 to 33 amino acids.

In a more preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Follicle Stimulating Hormone (hFSH), and wherein X0 comprises amino acid sequence NS, X1 comprises amino acid sequence ELTNITIAIEKEECRFCISINTTW, X2 comprises AGY, X3 comprises YTRDLVYKDPARPKIQKT, X4 comprises TFKELVYETVRVPGCAHHADSLYTYPVATQ, X5 comprises H, and X6 comprises KCDSDSTDCT. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to X0-X6 of FSH, wherein X0-X6 are the respective FSH amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of FSH.

In yet another more preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Choriogonadotropin (hCG), and wherein X0 comprises amino acid sequence SKEPLRPR, X1 comprises amino acid sequence RPINATLAVEKEGCPVCITVNTTI, X2 comprises AGY, X3 comprises PTMTRVLQGVLPALPQVV, X4 comprises NYRDVRFESIRLPGCPRGVNPVVSYAVALS, X5 comprises Q, and X6 comprises ALCRRSTTDC. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to X0-X6 of hCG, wherein X0-X6 are the respective hCG amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of hCG.

In yet another preferred embodiment, the invention provides a proteinmimic according to the invention, wherein said member of the cystine-knot growth factor superfamily is a member of the glycoprotein hormone-alpha (GLHA) subfamily, and wherein X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 13 to 17 amino acids, X3 represents an amino acid sequence with a length of 27 amino acids, and X4 represents an amino acid sequence with a length of 20 to 21 amino acids.

In yet another preferred embodiment, a proteinmimic according to the invention is provided, wherein said member of the cystine-knot growth factor superfamily is a member of the nerve growth factor (NGF) subfamily, and wherein X2 represents an amino acid sequence with a length of 9 to 24 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 41 to 44 amino acids, X3 represents an amino acid sequence with a length of 11 amino acids, and X4 represents an amino acid sequence with a length of 27 or 28 amino acids. In a more preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Nerve Growth Factor (hNGF), and wherein X0 comprises amino acid sequence PIFHRGEFSV, X1 comprises amino acid sequence DSVSVWVGDKTTATDIKGKEVMVLGEVNINNSVFKQYFFETK, X2 comprises RDPNPVDSG, X3 comprises RGIDSKHWNSY, X4 comprises TTTHTFVKALTMDGKQAAWRFIRIDTA, X5 comprises V, and X6 comprises VLSRKAVRRA. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to X0-X6 of hNGF, wherein X0-X6 are the respective hNGF amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of hNGF.

Members of the NGF subfamily play a role in survival and maintenance of sympathetic and sensory neurons and have been associated with Alzheimer disease. NGF plays a role in the repair, regeneration, and protection of neurons, and a proteinmimic of a member of the NGF subfamily according to the invention is thus especially useful for treating or preventing a neurodegenerative disorder.

Yet another subfamily of the cystine-knot growth factor superfamily is the TGF-beta subfamily. TGF-beta controls proliferation, cellular differentiation, and other functions in most cells. It plays a role in immunity, cancer, heart disease and in Marfan syndrome, a genetic disorder of the connective tissue.

In another preferred embodiment therefore, the invention provides a proteinmimic according to the invention, wherein said member of the cystine-knot growth factor superfamily is a member of the transforming growth factor beta (TGF-beta) subfamily, and wherein X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 23 to 41 amino acids, X3 represents an amino acid sequence with a length of 18 to 36 amino acids, and X4 represents an amino acid sequence with a length of 27 to 34 amino acids.

In a more preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is human Transforming Growth Factor beta2 (hTGF-beta2), and wherein X0 comprises amino acid sequence AYCFRNVQDN, X1 comprises amino acid sequence CLRPLYIDFKRDLGWKWIHEPKGYNANF, X2 comprises AGA, X3 comprises PYLWSSDTQHSRVLSLYNTINPEASASPC, X4 comprises VSQDLEPLTILYYIGKTPKIEQLSNMIVKS, X5 comprises K, and X6 comprises S. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to X0-X6 of hTGF-beta2, wherein X0-X6 are the respective hTGF-beta2 amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of hTGF-beta2.

Functional diverse modular proteins share a conserved domain of about 90 amino acids in their C-terminal cysteine-rich region, that has been proposed to be structurally related to the cystine-knot family and which is therefore called C-terminal cystine-knot (CTCK). Members of the C-terminal cystine knot family are, amongst others, von Willebrand factor (vWF), a multifunctional protein which is involved in maintaining homeostasis, mucins, CCN family members (cef-10/cyr61/CTFG/fisp-12/nov protein family)(ref 5), Drosophila slit protein, which is essential for development of midline glia and commissural axon pathways, Norrie disease protein (NDP), which may be involved in neuroectodermal cell- cell interaction and in a pathway that regulates neural cell differentiation and proliferation, and Silk moth hemocytin, an humoral lectin which is involved in a self-defence mechanism. The teaching of the present invention also encompasses this CTCK family.

In another preferred embodiment therefore, the invention provides a proteinmimic according to the invention, wherein said member of the cystine-knot growth factor superfamily is a member of the CTCK subfamily, and wherein X2 represents an amino acid sequence with a length of 2 to 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 22 to 35 amino acids, X3 represents an amino acid sequence with a length of 4 to 28 amino acids, and X4 represents an amino acid sequence with a length of 29 to 41 amino acids.

Sclerostin (or SOST) is a also a member of the CTCK-subfamily of the cystine-knot growth factor super family. Sclerostin, the product of the SOST gene, was originally believed to be a non-classical Bone morphogenetic protein (BMP) antagonist. More recently, Sclerostin has been identified as binding to LRP5/6 receptors and inhibiting the Wnt-signalling pathway. Wnt-activation under these circumstances is antagonistic to bone formation. More recently, it has been revealed that the antagonism of BMP-induced bone formation by sclerostin is mediated by Wnt signalling, but not BMP signalling pathways. The successful synthesis of **$\mathbf{SOST}_{67\text{-}144}$** in one of the examples serves to demonstrate that truncated cystine-knot proteins/peptides with an additional SS-bridge between C_{71} (loop-1; **X1**) and C_{125} (loop-3; **X4**) perfectly form the correctly folded cystine-knot structure in presence of the additional disulfide bond.

In a more preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is sclerostin, and wherein X0 comprises amino acid sequence FETKDVSEYS, wherein X1 comprises amino acid sequence RELHFTRYVTDGPCRSAKPVTELV, X2 comprises SGQ, X3 comprises GPARLLPNAIGRGKWWRPSGPDFR, X4 comprises IPDRYRAQRVQLLCPGGEAPRARKVRLVAS, X5 comprises K, and X6 comprises KRLTRFHNQS. In another more preferred embodiment, a proteinmimic is provided which has at least 70% sequence identity to X0-

X6 of sclerostin, wherein X0-X6 are the respective sclerostin amino acid sequences depicted in Figure 10. Preferably said proteinmimic has at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity to X0-X6 of sclerostin. In yet another more preferred embodiment, a proteinmimic according to the invention is provided, wherein said member is sclerostin, and wherein said proteinmimic consists of the amino acid sequence GGGC1RELHFTRYVTDGPCRSAKPVTELVC2SGQC3GPARLLPNAIGRGKWWRPSGPDFRC4IPDRYRAQRVQLLCPGGEAPRARKVRLVASC5KC6, optionally comprising flanking sequences with a length of at most 5 amino acids. In a preferred embodiment, the flanking sequences have a length of at most 2 amino acids, preferably at most 1 amino acid. In a most preferred embodiment, the proteinmimic does not comprise flanking sequences.

Members of the Noggin-like subfamily are for instance known to inhibit TGF-beta signal transduction by binding to TGF-beta family ligands and preventing them from binding to their corresponding receptors. Noggin plays a key role in neural induction by inhibiting BMP4. A proteinmimic of a member of the Noggin-like subfamily is thus especially useful for regulating TGF-beta and/or BMP4 activity.

In another preferred embodiment therefore, the invention provides a proteinmimic according to the invention,, wherein said member of the cystine-knot growth factor superfamily is a member of the Noggin-like subfamily, and wherein X2 represents an amino acid sequence with a length of 4 to 6 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 22 amino acids, X3 represents an amino acid sequence with a length of 7 to 9 amino acids, and X4 represents an amino acid sequence with a length of 35 to 98 amino acids.

A proteinmimic of a member of the Coagulin-like subfamily is for instance especially useful for treating coagulation disorders. Clinical trials have been started for instance with gene-therapy based coagulin B supplementiation for hemophilia B. However, a proteinmimic of a member of the coagulin-like subfamily as provided herewith is suitable for inhibiting coagulin B, for instance to reduce blood-clothing, thereby preventing thrombosis.

In another preferred embodiment therefore, the invention provides a proteinmimic according to the invention, wherein said member of the cystine-knot growth factor superfamily is a member of the Coagulin-like subfamily, and wherein X2 represents an amino acid sequence with a length of 7 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 38 amino acids, X3 represents an amino acid sequence with a length of 5 amino acids, and X4 represents an amino acid sequence with a length of 29 amino acids.

Members of the jagged-like subfamily are for instance ligands of the Notch family of receptors. The Notch signaling pathway plays a crucial role during embryonic pattern formation, controls many conserved cell determination events and defines a fundamental mechanism controlling cell fate. It is involved in lineage cell decisions in a variety of tissues. It plays a role in hematopoiesis, vascular development and angiogenesis, myogenesis, neurogenesis, somitogenesis, in kidney, eye, ear, and tooth development etc. Proteinmimics based on jagged-like members are especially useful for controlling the before mentioned biological processes.

In another preferred embodiment therefore, the invention provides a proteinmimic according to the invention, wherein said member of the cystine-knot growth factor superfamily is a member of the Jagged-like subfamily, and wherein X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 32 amino acids, X3 represents an amino acid sequence with a length of 25 amino acids, and X4 represents an amino acid sequence with a length of 26 amino acids.

As said before, Figure 10 depicts non-limiting examples of truncated proteins belonging to several cystine-knot growth factor subfamilies. It is especially useful to introduce small mutations, for instance exchange at least one cysteine, not being one of the conserved cysteines 1 to 6 which are necessary for cystine-knot formation, in order to prevent for instance dimer-formation. In a preferred embodiment therefore, a proteinmimic according to the invention is provided, wherein said X1 represents an amino acid sequence with at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95% sequence identity with any one of the sequences identified as a X1 in Figure 10, and/or wherein said X3 represents an amino acid sequence with at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95% sequence identity with any one of the sequences identified as X3 in Figure 10, and/or wherein said X4 represents an amino acid sequence with at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95% sequence identity with any one of the sequences identified as X4 in Figure 10, wherein X1, X3 and X4 are taken from a single amino acid sequence of Figure 10. In a more preferred embodiment, at least one cysteine in any of the sequences represented by X1, X2, X3, X4, and X6, is replaced by another amino acid, preferably alanine. In another preferred embodiment, said X1 represents an amino acid sequence which is identical with any one of the sequences identified as X1 in Figure 10, and/or X3 represents an amino acid sequence which is identical with any one of the sequences identified as X3 in Figure 10, and/or X4 represents an amino acid sequence which is identical with any one of the sequences identified as X4 in Figure 10, wherein X1, X3 and X4 are taken from a single amino acid sequence of Figure 10.

In another preferred embodiment, a proteinmimic according to the invention is provided, wherein said X2 represents an amino acid sequence with at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95% sequence identity with any of the sequences identified as X2 in Figure 10, and/or wherein X5 represents an amino acid sequence which is identical to any of the sequences identified as X5 in Figure 10, wherein X2 and X5 are taken from a single amino acid sequence of Figure 10. In a more preferred embodiment, at least one cysteine in any of the sequences represented by X1, X2, X3, X4, and X6, is replaced by another amino acid, preferably alanine. In another more preferred embodiment, said X2 represents an amino acid sequence which is identical with a sequence identified as X2 in Figure 10, wherein X2 and X5 are taken from a single amino acid sequence of Figure 10.

In another preferred embodiment, the invention provides a proteinmimic according to the invention, wherein said proteinmimic comprises the motif C1-X1-C2-X2-C3-X3-C4-X4-C5-X5-C6, wherein said sequence has at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95% sequence identity with a sequence selected from sequences 1 to 145 of Figure 10. In a most preferred embodiment, said proteinmimic sequence is identical to a sequence selected from sequences 1 to 145 of Figure 10. Such a proteinmimic is especially useful for induction of a cross-reactive, preferably a neutralizing antibody response, because the proteinmimic is identical to a part of the native protein.

In a preferred embodiment, a proteinmimic according to the invention is provided, wherein said C1 is linked to C4 through a disulfide bond and/or C2 is linked to C5 through a disulfide bond, and/or C3 is linked to C6 through a disulfide bond. In a more preferred embodiment, C1 is linked to C4 through a disulfide bond and C2 is linked to C5 through a disulfide bond, and C3 is linked to C6 through a disulfide bond.

Now that the invention provides proteinmimics of members of the cystine-knot growth factor superfamily, the invention also provides the insight that a proteinmimic according to the invention is especially useful for inducing an immune response, preferably said immune response is cross-reactive to a member of the cystine-knot growth factor superfamily.

With “cross-reactive” is meant that the antibody produced not only specifically binds the proteinmimic against which the antibody was raised, but also specifically binds to at least one of said members of the cystine-knot growth factor superfamily. In one embodiment therefore, an immunogenic composition is provided, comprising a proteinmimic according to the invention. Said immunogenic composition preferably further comprises a therapeutically acceptable carrier, adjuvant, diluent and/or excipient. "Immunogenic composition" is defined herein in its broad sense to refer to any type of biological agent in an administrable form capable of inducing and/or stimulating an immune response in an animal. In one preferred embodiment, an immunogenic composition according to the invention at least comprises a proteinmimic according to the invention and a pharmaceutically acceptable adjuvant.

In another preferred embodiment, an immunogenic composition according to the invention is provided, wherein said proteinmimic is coupled to an immunogenic carrier, preferably diphtheria toxin (DT) and/or keyhole limpet haemocyanin (KLH).

The invention further provides a pharmaceutical composition comprising a proteinmimic according to the invention and a pharmaceutically acceptable carrier, diluent and/or excipient. Suitable carriers, diluents, excipients and the like are commonly known in the art of pharmaceutical formulation and may be readily found and applied by the skilled artisan, references for instance Remmington’s Pharmaceutical Sciences, Mace Publishing Company, Philadelphia PA, 17th ed. 1985.

Members of the cystine-knot growth factor super-family are, as already mentioned before, associated with many diseases, including diseases of the nervous system, hematopoietic development, coagulation disorders, cancer, angiogenesis, etc. In one embodiment therefore, the invention provides a use of a proteinmimic according to the invention for the preparation of a medicament and/or prophylactic agent for the treatment and/or prevention of a disorder associated with a member of the cystine-knot growth factor superfamily.

The invention thus provides use of a proteinmimic in an immunogenic composition. Such immunogenic composition comprising a proteinmimic according to the invention is suitable for inducing an immune reaction in an animal, preferably a human. In a preferred embodiment, a proteinmimic of the invention is used to induce antibodies, which are preferably able to cross-react with the native protein. Even more preferably said antibodies are neutralizing antibodies, i.e. the function and/or activity of the native cystine-knot protein is diminished, inhibited, or at least reduced after binding of the native cystine-knot protein to said neutralizing antibody. It is possible to induce said antibodies in an individual in need thereof, for instance by administering a vaccine comprising a proteinmimic according to the invention to said individual. It is also possible to induce said antibodies in a non-human animal by administering an immunogenic composition of the invention to said animal and use antibodies obtained from said animal for the manufacture of a medicament. However, it is also possible to use a proteinmimic according to the invention to directly antagonize the function and/or activity of the native cystine-knot protein. This can for instance be achieved if the proteinmimic binds to the receptor but does not or does not fully activate the receptor signal pathway. In one embodiment, the invention provides a use of a proteinmimic according to the invention, or an immunogenic compound comprising a proteinmimic according to the invention, as a partial or full antagonist of a member of the cystine-knot growth factor superfamily.

Now that the invention provides the insight that a proteinmimic according to the invention is useful as an antagonist and/or agonist for a member of the cystine-knot growth factor superfamily or suitable for raising an immune response against a member of the cystine-knot growth factor superfamily, a method is provided for treating or preventing a disorder associated with a member of the cystine-knot growth factor superfamily, comprising administering a therapeutically effective amount of a proteinmimic according to the invention to a subject suffering from, or at risk of suffering from, said disorder.

One subfamily of the cystine-knot growth factor superfamily is the subfamily of vascular endothelial growth factors (VEGF) which is a subfamily of the PDGF subfamily. VEGFs act through a family of cognate receptor tyrosine kinases in endothelial cells to stimulate blood-vessel formation. Proteinmimics of, and/or antibodies specific for VEGF are thus especially useful for treating a disorder related to vascularization. One such disorder is age-related macula degeneration (AMD), which causes rapid and severe visual loss. This loss is due to development of choroidal neovascularisation under the macula. Inhibition of VEGF is therefore especially useful for the treatment and/or prevention of AMD. Another example of a disease that relates to vascularization is cancer. Tumors need neovascularization in order to grow. Fast growing tissue needs a continuous supply of oxygen and nutrients and therefore, the effective inhibition of neovascularization is thought to be one of the promising strategies for cancer therapy. This is for instance achieved by inhibiting for instance VEGF. As said before, Avastin™, a monoclonal antibody (Bevacizumab, Genentech) was approved in 2004 by the Food and Drug Administration (FDA) for the treatment of colorectal cancer when used with standard chemotherapy. In 2006, the FDA approved Bevacizumab for the treatment of lungcancer in combination with standard first line combination therapy.

The drawbacks of Bevacizumab, such as the high production costs and the relative large amounts needed for treatment, sometimes low tumor penetration and frequent administration are reduced when a proteinmimic or an immunogenic composition of the invention is used. For instance, an immunogenic composition comprising a proteinmimic of the invention is administered in a dose of a few mg, preferably 0.1 to 10 mg per subject in order to induce an immune response. Such an administration is generally repeated two or three times, in order to induce a proper protective response.

In one embodiment therefore, the invention provides use of a proteinmimic according to the invention for the preparation of a medicament and/or prophylactic agent for the treatment and/or prevention of a tumor related disease and/or age-related macular degeneration (AMD), wherein said member of the cystine-knot growth factor superfamily is a member of the VEGF subfamily or the TGF-beta subfamily.

Another cystine-knot growth factor subfamily, TGF-beta, is also related to cancer. In normal cells, TGF-beta, acting through its signaling pathway, stops the cell cycle at the G1 stage to stop proliferation, induce differentiation, or promote apoptosis. When a cell is transformed into a cancer cell, parts of the TGF-beta signaling pathway are mutated, and TGF-beta no longer controls the cell. These cancer cells proliferate. The surrounding stromal cells (fibroblasts) also proliferate. Both cells increase their production of TGF-beta. This TGF-beta acts on the surrounding stromal cells, immune cells, endothelial and smooth-muscle cells. It causes immunosuppression and angiogenesis, which makes the cancer more invasive. TGF-beta also converts effector T-cells, which normally attack cancer with an inflammatory (immune) reaction, into regulatory (suppressor) T-cells, which turn off the inflammatory reaction. Inhibiting TGF-beta for instance with an antogonistic proteinmimic according to the invention and/or an antibody of the invention or functional part and/or functional equivalent thereof of the invention, wherein said member belongs to the TGF-beta subfamily, is thus especially useful for the treatment of cancer.

In a preferred embodiment therefore, a method according to the invention is provided, wherein said disorder comprises a tumor-related disease and/or age-related macular degeneration (AMD), and wherein said member of the cystine-knot growth factor superfamily is a member of the VEGF subfamily or the TGF-beta subfamily. In a more preferred embodiment, said tumor-related disease is colorectal cancer or non small cell lung cancer (NSCLC).

In another preferred embodiment, a method according to the invention is provided, wherein said disorder comprises a connective tissue disorder, preferably Marfan syndrome. Marfan syndrome is carried by a gene called FBN1, which encodes a connective protein called fibrillin-1. People have a pair of FBN1 genes. Because it is dominant, people who have inherited one affected FBN1 gene from either parent will have Marfan's. In addition to being a connective protein that forms the structural support for tissues outside the cell, fibrillin-1 binds to another protein, TGF-beta. TGF-beta can cause inflammation. Researchers now believe that the inflammatory effects of TGF-beta, at the lungs, heart valves, and aorta, weaken the tissues and cause the features of Marfan syndrome. A proteinmimic of TGF-beta is thus especially useful for treatment of Marfan syndrome.

In contrast, neovascularization (vascular regeneration) is especially useful for the treatment of ischemic disease including but not limited to arteriosclerotic occlusion of the lower limbs, angina pectoris/myocardial infarction or cerebral infarction in order to rescue the ischemic tissue by developing collateral circulation. In another preferred embodiment therefore, said disorder comprises an ischemic disorder, preferably said ischemic disorder is taken from the group consisting of arteriosclerotic occlusion of the lower limbs, angina pectoris, myocardial infarction and cerebral infarction, wherein said member of the cystine-knot growth factor superfamily is a member of the VEGF subfamily.

As said before, members of the NGF subfamily are critical for the survival and maintenance of sympathetic and sensory neurons and have been associated with Alzheimer disease. As NGF plays a role in the repair, regeneration, and protection of neurons, a proteinmimic of a member for the NGF subfamily according to the invention is thus especially useful for treating a neurodegenerative disorder. Other possible applications are the use of a proteinmimic of a member of the NGF subfamily according to the invention, for instance through induction of NGF-specific antibodies, to diminish and/or treat chronic and/or neurodegenerative pain. Further, such NGF-specific antibodies are considered especially useful for the treatment of breast-tumors, as NGF is known to be a strong stimulator of breast cancer cell proliferation.

In another preferred embodiment therefore, a method according to the invention is provided, wherein said disorder comprises a disorder selected from the group consisting of a neurodegenerative disorder, preferably Alzheimer disease, a pain disorder, preferably a chronic and/or neuropathic pain disorder, and cancer, preferably breast cancer. In a more preferred embodiment, a method is provided, wherein said member belongs to the NGF subfamily.

Further provided is a method for producing antibodies against a member of the cystine-knot growth factor superfamily, comprising administering a proteinmimic according to the invention and or an immunogenic composition according to the invention to a non-human animal, and obtaining antibodies against a member of the cystine-knot growth factor superfamily, which antibodies are produced by said animal. Also provided is the use of a proteinmimic according to the invention in an *ex vivo* method for producing an antibody, or a functional part or functional equivalent of an antibody, which is specifically directed against a member of the cystine-knot growth factor superfamily. The skilled artisan is aware of the different methods for producing an antibody *ex vivo*, such as B-cell hybrodima techniques, antibody phage display technologies and the like.

A functional part of an antibody is defined herewith as a part which has at least one same property as said antibody in kind, not necessarily in amount. Said functional part is preferably capable of binding the same antigen as said antibody, albeit not necessarily to the same extent. A functional part of an antibody preferably comprises a single domain antibody, a single chain antibody, a Fab fragment or a $F(ab')_2$ fragment. A functional equivalent of an antibody is defined as an antibody which has been altered such that at least one property - preferably an antigen-binding property - of the resulting compound is essentially the same in kind, not necessarily in amount. An equivalent is provided in many ways, for instance through conservative amino acid substitution, whereby an amino acid residue is substituted by another residue with generally similar properties (size, hydrophobicity, etc), such that the overall functioning is likely not to be seriously affected.

The glycoprotein hormone subfamily (GLH), a subfamily of the cystine-knot superfamily of growth factors, comprises the hormones: luteinizing hormone, (LH), thyroid stimulating hormone (TSH) and chorionic gonadotropin (CG) and follicle stimulating hormone (FSH). These hormones all comprise an alpha and a beta subunit (GLHA and GLHB, respectively) and they play a role in reproduction in mammals. For instance FSH stimulates testicular and ovarian functions through binding to a G-protein-coupled receptor on either Sertoli (male) or granulose (female) cells. Amongst other things, LH stimulates ovulation and sustains the corpus luteum during menstrual cycle, whereas CG for instance sustains the corpus luteum during pregnancy. TSH is important for Sertoli cell maturation and ovulatory function.

In a preferred embodiment therefore, a method for treating or preventing a disorder associated with the presence of a member of the cystine-knot growth factor superfamily according to the invention is provided, wherein said disorder is a reproductive disorder. Apart from treating a reproductive disorder, a proteinmimic and/or an antibody or functional part or equivalent thereof according to the invention is also especially useful to prevent reproduction, i.e. prevent pregnancy. By inhibition of a GLH, for instance FSH, CG, LH or TSH, or inhibition of receptor binding and/or signaling of said GLH in a female or a male, ovulatory or testical function is disturbed and the chances of pregnancy are reduced. The invention thus provides a method for preventing pregnancy and/or reducing the chance of pregnancy in a female individual, comprising administering to said female or a sexual partner of said female an effective amount of a proteinmimic according to the invention, an immunogenic composition according to the invention, and/or an antibody obtainable by a method according to the invention or a functional part or functional equivalent of said antibody, wherein said member of the cystine-knot growth factor superfamily is a member of the GLHA or GLHB subfamily.

Further provided is a proteinmimic according to the invention, an immunogenic composition according to the invention, and/or an antibody obtainable by a method according to the invention, or a functional part or functional equivalent thereof, for use as a male and/or female contraceptive.

Further provided is a method for binding and/or neutralizing an antibody directed to a member of the cystine-knot growth factor superfamily, comprising administering a therapeutically effective amount of a proteinmimic according to any one of claims 1-17 to a subject comprising said antibody. Upon binding of the proteinmimic to said antibody, its activity is diminished. Antibodies that are specific for members of the cystine-knot protein are used in treatment protocols. One example thereof is Avastin™ specific for VEGF, which is used to treat metastatic cancer. Antibodies, once administered, have a half-life of several days, even up to several weeks. If, for instance such an antibody is over-dosed or if the action of such antibody is not desired anymore, a proteinmimic of the intvention is especially useful to counteract the action of said antibody by binding and/or neutralizing said antibody. A proteinmimic of the invention is especially useful because the proteinmimic as such is not or to a lesser extent bioactive and therefore does not interfere with a condition for which the antibody was initially administered. It is of course undesirable to treat a patient receiving for instance antibodies against VEGF with bio-active VEGF to neutralize the antibody. Bio-active VEGF administered in excess of the antibody present would exert its biological effect and would undermine the antibody treatment thus far received. An illustrative example, that does not limit the invention, is the use of a proteinmimic of VEGF, that can be used to bind and/or neutralize a monoclonal antibody against VEGF, preferably Avastin™. Avastin™ is a commercially available monoclonal antibody against VEGF, which is administered for instance to treat metastatic cancers. Treatment with Avastin™, however, can lead to slow or incomplete wound healing (for example, when a surgical incision has trouble healing or staying closed). In some cases, this event resulted in fatality. It is therefore not recommended to start Avastin™ therapy for at least 28 days after surgery and until the surgical wound is fully healed. Of course, during Avastin™ therapy, surgery should be avoided. However, it is sometimes necessary to perform surgery on a person that receives Avastin™ therapy. In such a case, a truncated VEGF, preferably $VEGF_{26-104}$ is preferably administered to neutralize the circulating anti-VEGF antibodies without inducing much biological effect resembling the action of VEGF itself. Shortly after administration of the truncated VEGF and neutralization of the anti-VEGF antibodies, the patient may undergo surgery without the above mentioned severe side effects which are normally observed after surgery during Avastin™ therapy.

In a preferred embodiment, therefore, a method for binding and/or neutralizing an antibody directed to a member of the cystine-knot growth factor superfamily comprising administering a therapeutically effective amount of a proteinmimic according to the invention to a subject comprising said antibody is provided, wherein said antibody is Avastin™ and said proteinmimic is $VEGF_{26-104}$.

Further provided is the use of a proteinmimic according to the invention for the manufacture of a medicament for neutralizing an antibody directed to a member of the cystine-knot growth factor superfamily. In a preferred embodiment said antibody is Avastin™ and said proteinmimic is $VEGF_{26-104}$ as explained before.

Another member of the cystine-knot growth factor superfamily, belonging to the TGF-beta subfamily, is sclerostin, the secreted protein product of the SOST gene, which is an osteocyte-derived inhibitor of cultured osteoblasts. Sclerostin deficiency leads to sclerosteosis and van Buchem disease, two closely related, rare sclerosing disorders characterized by substantial increase in bone mass of good quality which is due to increased bone formation. In contrast, osteoporosis, a disorder in which the density and quality of bone are reduced, leading to weakness of the skeleton and increased risk of fracture, particularly of the spine, wrist, hip, pelvis and upper arm, is possibly caused by an excess production of sclerostin, inhibiting bone formation. An agonistic or antagonistic proteinmimic of sclerostin and/or an antibody specific for sclerostin is thus especially useful for treatment of a bone disorder. In a preferred embodiment, therefore, a method according to the invention is provided, wherein said disorder comprises a disorder associated with disturbed bone-regulation. In a more preferred embodiment, said disorder comprises osteoporosis or sclerosteosis.

The invention is further explained in the following examples that do not limit the scope of the invention, but merely serve to clarify specific aspects of the invention.

Brief description of the figures

Figure 1. Electro Spray Ionization Mass Spectrum (ESI/MS) of humVEGF$_{25\text{-}107}$ (Boc) in A) fully reduced form (MW$_{calc}$ = 9569.1; MW$_{exp}$ = 9566.4), and B) after oxidative folding (MW$_{calc}$ = 9563.1; MW$_{exp}$ = 9560.7). Folding conditions as described above.

Figure 2. A) Inhibition of Avastin™-binding to surface-immobilized humVEGF$_{1\text{-}165}$ (1 µg/mL; GDA-coupling) in ELISA for varying concentrations (125 µM to 2 pM) of oxid-humVEGF$_{26\text{-}104}$ (■), humVEGF$_{1\text{-}165}$ (▲), and a backbone-cyclized peptide covering only the β5-turn-β6 loop of humVEGF (humVEGF$_{74\text{-}98}$) (x). B) Inhibition of Avastin™-binding to surface-immobilized humVEGF$_{1\text{-}165}$ (1 µg/mL; GDA-coupling) in ELISA for varying concentrations (5 µM to 12.8 pM) of humVEGF$_{1\text{-}165}$ (▲), oxid-humVEGF$_{26\text{-}104}$ synthesized via procedure-1 (grey square), or oxid-humVEGF$_{26\text{-}104}$ synthesized via procedure-2 (white square).

Figure 3. First neutralization data from BaF3/cell proliferation assay with non-purified rat anti-oxid-humVEGF$_{26\text{-}104}$ immune sera (I) 50.49 and 50.67 at 1/50 and 1/100 dilution. mAb Avastin™ (anti-humVEGF$_{1\text{-}165}$) was used as positive control, pre-immune (PI) sera (50.49 and 50.67) as negative control. Level of proliferation observed at humVEGF$_{1\text{-}165}$ = 0.6 ng/mL was set by default to 100%, sera proliferation levels were expressed as % of default. Pre-immune sera were taken just before first immunization. Immune sera were taken 6 weeks after first immunization. In grey: % of proliferation <50; in black: % of proliferation between 50 and 100.

Figure 4. Neutralization data from BaF3/cell proliferation assay with non-purified anti-oxid-humVEGF$_{26\text{-}104}$ rat sera A) 50.49 and B) 50.67 from 1/50 and 1/3200 dilution. For further details, see Figure 3.

Figure 5. Neutralization data from BaF3/cell proliferation assay with protG-purified anti-oxid-humVEGF$_{26\text{-}104}$ rat sera A) 50.49 and B) 50.67 from 1/50 and 1/3200 dilution. For further details, see Figure 3.

Figure 6. Neutralization data from BaF3/cell proliferation assay with non-purified mouse anti-oxid-humVEGF$_{26\text{-}104}$ immune sera (I) 59.01-59.05 (04 died). mAb Avastin™ (anti-humVEGF$_{1\text{-}165}$) and anti-oxid-humVEGF$_{26\text{-}104}$ rat-serum 50.67 were used as positive control; pre-immune (PI) sera as negative control. Level of proliferation observed at humVEGF$_{1\text{-}165}$=1.2 ng/mL was set by default to 100%, serum proliferation levels were expressed as % of default. PI: serum taken just before first immunzation; I: serum taken 6 weeks after first immunization.

Figure 7. Inhibition of Avastin™-binding to surface-immbolized **humVEGF$_{1\text{-}165}$** with non-purified rat immune sera 50.49 and 50.67 at 1/5 and 1/25 dilution. Peptide serum 31.1 (elicited against double-constrained **CLIPS**/SS-peptide derived from the β3-loop sequence hum**FSH**$_{56\text{-}79}$ of Follicle Stimulating Hormone; serum has high neutralizing activity for FSH in cell-based assay) and serum 45.09 (elicited against backbone-cyclized peptide derived from the β5-turn- β6 loop sequence 70-102 of VEGF; serum has neutralizing activity for humVEGF$_{1\text{-}165}$ in BaF3-cell proliferation assay) were used as negative controls. Minimal concentration of Avastin™ (~10 ng/mL) was

used (OD_{450nm}~0.4) in order to secure maximal sensitivity for the inhibition experiments.

Figure 8. Proliferation data from BaF3/cell assay with hum**VEGF$_{26\text{-}104}$** at various concentration (0.01-20 ng/mL), either in the absence and presence of hum**VEGF$_{1\text{-}165}$**. Level of proliferation observed at hum**VEGF$_{1\text{-}165}$**=1.2 ng/mL was set by default to 100%, other proliferation levels were expressed as % of default.

Figure 9. Schematic overview of the proliferation assay.

Figure 10. Full protein name, species from which the protein was isolated, and amino acid sequence for all proteins known to be part of the cystine-knot growth factor superfamily, subdivided in TGF-beta, GLH-beta, NGF, PDGF, GLHA, Noggin-like, Coagulin-like, and CTCK-like subfamilies. Defined consensus sequences per subfamily are projected on top of the listing of sequences for each member.

Figure 11. Schematic representation of the general structure of the various members of the cystine-knot growth factor superfamily.

Figure 12. A) Increase of average tumor volume (mm3) per mice in treatment group 1:PBS (●), 2:anti-oxid-humVEGF$_{26\text{-}104}$ (Δ), and 3:AVASTIN™ (V). In the PBS group 4/9 mice were euthanised (#) before the planned day, because the estimated volume of the tumors exceeded the (pre-set) maximum volume. B) Total average tumor weight (mgs) per mice in each different treatment group at the end of the experiment. C) Total tumor volume (mm3) of individual mice in each different treatment group at the end of the experiment (mouse 3 in PBS-group died before the start of the experiment).

Figure 13 HPLCs (A/C) and ElectroSpray Ionization Mass Spectra (B/D) of red-ratVEGF$_{26\text{-}104}$ (A/B) and oxid-ratVEGF$_{26\text{-}104}$ (C/D).

Figure 14. Plots of the binding in ELISA of anti-oxid-humVEGF$_{26\text{-}104}$ ratsera 1+2 (black ------ and - - - lines) and anti-oxid-ratVEGF$_{26\text{-}104}$ ratsera 3+4 (grey ------ and - - - lines) to both A) oxid-ratVEGF$_{26\text{-}104}$ and B) oxid-humVEGF$_{26\text{-}104}$.

Figure 15. HPLCs (A/C) and ElectroSpray Ionization Mass Spectra (B/D) of red-humPLGF$_{34\text{-}112}$ (A/B) and oxid-humPLGF$_{34\text{-}112}$ (C/D).

Figure 16. Three-fragment condensation of humSOST$_{57\text{-}144}$ from fragment humSOST-F1, humSOST-F2, and humSOST-F3 by Native Chemical Ligation. Step A: Ligation of the thiaproline-protected humSOST-F2 to humSOST-F3, generating protected humSOST-F2/3. Step B: Deprotection of humSOST-F2/3 with methoxyamine in at pH 4.0. Step C: Ligation of deprotected humSOST-F2/3 to humSOST-F1 generating humSOST$_{57\text{-}144}$ at pH 6.5.

Figure 17. Oxidative refolding of fully red-humSOST$_{57\text{-}144}$ after ion exchange chromatography. The peptide was folded in 0.4 M Arginine, 1.67mM Glutathione (red), 0.33 mM Glutathione (ox), 55 mM Tris-HCl, 21 mM sodium chloride, 0.88 mM potassium chloride, pH 8.0, yielding 10.2% of the desired product after 3.5 days at 4 °C.

Figure 18. HPLCs (A/C/E) and ElectroSpray Ionization Mass Spectra (B/D/F) of fully red-humSOST$_{57\text{-}144}$ (A/B), oxidatively refolded oxid-humSOST$_{57\text{-}144}$ (C/D), octa-acetamido derivatized humSOST$_{57\text{-}144}$ (E/F).

Figure 19. Binding data in ELISA for antibodies selected biotinylated oxid-humSOST$_{57\text{-}144}$ from a PDL-library. The positive binding to 1. Recombinant humSOST, 2. biotinylated oxid-humSOST$_{57\text{-}144}$ itself, and the absence of binding to 3. AA$_8$-SOST$_{57\text{-}144}$, 4. GST, 5) CD33, and finally 6. Bovine Serum Albumin (BSA) illustrate the high-specificity of the antibody binding.

Figure 20. HPLCs (A/C) and ElectroSpray Ionization Mass Spectra (B/D) of red-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$ (A/B) and oxid-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$ (C/D).

Figure 21. (A) Antibody titers in ELISA for 9wpv-ratsera (1 and 2 + pre-immune sera) that were elicited via immunization with **oxid-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$**. Titers were defined as the -10log[conc] at which the OD in ELISA is equal to 4x the background signal. (B) Antibody binding in ELISA of 9wpv-ratsera to surface-immobilized 1) **humTGFB2trunc-1** (with VEGF **b2-loop**), 2) **humTGFB2trunc-2** (with sequence PGGSPA replacing native humTGF-B2 **b2-loop**), and 3) hum**VEGFtrunc**.

humTGFB2trunc 1: acetyl-**C1**ALRPLYIDFKRDLGWKWIHEP KGYNANF**C2**AGA**C3**NDEGLE**C4**VSQDLEPLTILYYIGKTPKI EQLSNMIVKS**C5**K**C6**-amide

humTGFB2trunc 2: acetyl-**C1**ALRPLYIDFKRDLGWKWIHEP KGYNANF**C2**AGA**C3**PGGSPA**C4**VSQDLEPLTILYYIGKTPKI EQLSNMIVKS**C5**K**C6**-amide

VEGFtrunc: acetyl-**C1**HPIETLVDIFQEYPDEIEYIFKPSAVPL MR**C2**GGA**C3**NDEGLE**C4**VPTEESNITMQIMRIKPHQGQHIG EMSFLQHNK**C5**E**C6**-amide

Examples

EXAMPLE 1A: Synthesis of various forms of VEGF-truncated.

Three different forms of VEGF-truncated were synthesized:

humVEGF$_{26-104}$:

$_{26}$Ac-**C1**HPIETLVDIFQEYPDEIEYIFKPS**A**VPLMR**C1**GG**AC3**NDEGLE**C4**VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNK**C5**E**C6#**$_{104}$

humVEGF$_{25-107}$:

$_{25}$Ac-Y**C1**HPIETLVDIFQEYPDEIEYIFKPS**A**VPLMR**C2**GG**AC3**NDEGLE**C4**VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNK**C5**E**C6**RPK#$_{107}$

humVEGF$_{25-109}$:

$_{25}$Ac-Y**C1**HPIETLVDIFQEYPDEIEYIFKPS**A**VPLMR**C2**GG**SC3**NDEGLE**C4**VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNK**C5**E**C6**RPKKD#$_{109}$

Amino acids are indicated by the single-letter codes; "Ac" refers to N-terminal acetylation; "#" indicates C-terminal amidation; Cysteines (**C1-C6**) in **boldface** indicate cysteines involved in formation of the cystine-knot fold; alanines in **boldface** indicate native cysteines that were replaced by Ala.

Three different synthetic procedures were used:

I. Direct synthesis (Fmoc) of full-length peptide; only used for hum**VEGF**$_{26-104}$.
II. Peptide-thioester synthesis using Fmoc-chemistry. Subsequent Native Chemical Ligation (NCL) of peptide fragments hum**VEGF**$_{26-67}$(thioester) + hum**VEGF**$_{68-104}$(free N-terminal cysteine) for

hum**VEGF$_{\mathbf{26\text{-}104}}$**, hum**VEGF**$_{25\text{-}67}$(thioester) + hum**VEGF**$_{68\text{-}107}$(free N-terminal cysteine) for hum**VEGF$_{\mathbf{25\text{-}107}}$**, and hum**VEGF**$_{25\text{-}67}$(thioester) + hum**VEGF**$_{68\text{-}109}$(free N-terminal cysteine) for hum**VEGF$_{\mathbf{25\text{-}109}}$.**

III. Peptide-thioester synthesis using Boc-chemistry. Subsequent Native Chemical Ligation (NCL) of peptide fragments hum**VEGF**$_{25\text{-}67}$(thioester) + hum**VEGF**$_{68\text{-}107}$(free N-terminal cysteine) for hum**VEGF$_{\mathbf{25\text{-}107}}$** and hum**VEGF**$_{26\text{-}67}$(thioester) + hum**VEGF**$_{68\text{-}104}$(free N-terminal cysteine) or hum**VEGF**$_{26\text{-}60}$(thioester) + hum**VEGF**$_{61\text{-}104}$(free N-terminal cysteine) for **humVEGF$_{\mathbf{26\text{-}104}}$.**

Procedure I:

General procedure (A) for Fmoc-synthesis of peptides:

Peptides were synthesized on solid-phase using a 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (RinkAmide) resin (BACHEM, Germany) on a Symphony (Protein Technologies Inc., USA), Voyager (CEM GmbH, Germany), or SyroII (MultiSyntech, Germany) synthesizer. All Fmoc-amino acids were purchased from Biosolve (Netherlands) or Bachem GmbH (Germany) with side-chain functionalities protected as N-*t*-Boc (KW), O-*t*-Bu (DESTY), N-Trt (HNQ), S-Trt (C), or N-Pbf (R) groups. A coupling protocol using a 5-fold excess of HBTU/HOBt/amino acid/DIPEA (1:1:1:2) in NMP with a 20 minute activation time using double couplings was employed for every amino acid coupling step. Acetylation (Ac) of the peptide was performed by reacting the resin with NMP/Ac_2O/DIEA (10:1:0.1, v/v/v) for 30 min at room temperature. The acetylated peptide was cleaved from the resin by reaction with TFA (40 mL/mmol resin) containing 13.3% (w) phenol, 5% (v) thioanisole, 2.5% (v) 1,2-ethanedithiol, and 5% (v) milliQ-H_2O for 2 hrs at room temperature, unless indicated otherwise. Precipitation with ice-cold Et_2O + lyophilization of the precipitated material afforded the crude peptide.

humVEGF$_{26\text{-}104}$ was synthesized in one step following this procedure (resin-loading 0.88 mmol/g) on a Symphony synthesizer (Protein Technologies Inc., USA). In the first coupling step a 4:1 (w/w) mixture of Ac-Cys(Trt)-OH and Fmoc-Cys(Trt)-OH was used. The acylated peptide was cleaved from the resin by reaction with a slightly different mixture: TFA (40 mL/mmol resin) containing 5% (v) TES, 2.5% (v) 1,2-ethanedithiol, and 2.5% (v) milliQ-H_2O. Finally, the peptide was purified by HPLC and folded by oxidation following procedure G.
The fragment peptides hum**VEGF**$_{68\text{-}104}$, hum**VEGF**$_{68\text{-}107}$, and hum**VEGF**$_{68\text{-}109}$ (free N-terminal cysteine for NCL; see procedure II) were also synthesized following this procedure as described above for hum**VEGF$_{26\text{-}104}$** on a Rink-Made resin (loading 0.5 mmol/g) using a Liberty-synthesizer (CEM GmbH, Germany).

<u>Procedure II:</u>

<u>Fmoc-synthesis of peptide thioesters</u>:
The fragment peptides hum**VEGF**$_{25\text{-}67}$ and hum**VEGF**$_{26\text{-}67}$ (free C-terminus) were synthesized on a SASRIN-resin (loading 0.5 mmol/g; Bachem GmbH, Germany) following the general procedure for Fmoc-synthesis of peptides as described in procedure I. The peptide were cleaved from the resin by repetitive treatment (20 cycles) with 1% TFA (40 mL/mmol resin) in DCM. The combined fractions were neutralized with pyridine, whereafter DCM was removed by evaporation under reduced pressure. Finally, the peptides were precipitated by addition of excess of H_2O, followed by centrifugation and lyophilization. The crude lyophilized peptides were dissolved in DCM (2.0 mM), 12 equivalents of 4-acetamidothiophenol in DCM (0.334 mg/mL, 2.0 mM), 3 equivalents of PyBOP in DCM (1.040 mg/mL, 2.0 mM), and 2.6 equivalents of DIPEA in DCM (1 vol%) were subsequently added and the mixture was stirred at room temperature for 6 hours. Then, another 12 equivalents of 4-acetamidothiophenol in DCM (0.334 mg/mL, 2.0 mM) were added and the mixture was stirred overnight at room temperature. Finally, the mixture

was neutralized with ~2.6 equivalents of TFA and DCM was removed by evaporation under reduced pressure. The crude fragment peptide thioesters were then deprotected and purified by RP-HPLC following general procedures.

Native Chemical Ligation (NCL) of fragment peptides:

Condensation of fragment peptides hum**VEGF**$_{68\text{-}104}$, hum**VEGF**$_{68\text{-}107}$, or hum**VEGF**$_{68\text{-}109}$ (**A**) with either fragment peptide thioesters hum**VEGF**$_{25\text{-}67}$ or hum**VEGF**$_{26\text{-}67}$ (**B**) by native chemical ligation was performed by mixing almost equimolar (1:1.2) solutions of **A** (10 mg/mL; ~2.0 mM) and **B** (10 mg/mL; ~2.0 mM) in working buffer (6M guanHCl/20mM TCEP/200mM MPAA in 0.2M phosphate buffer pH 8.0;) and overnight stirring at room temperature. After mixing of the solutions (acidic!) the pH was adjusted to 6.5 by addition of 10M NaOH (μL of NaOH is roughly equal to mg of MPAA used). Excess of MPAA was removed by Amicon filtration using working buffer (without MPAA!!) in the washing steps. Finally, the crude hum**VEGF**$_{26\text{-}104}$, hum**VEGF**$_{25\text{-}107}$, or hum**VEGF**$_{25\text{-}109}$ in reduced form were purified by RP/HPLC following the standard procedure.

Oxidative Folding of red-hum**VEGF**$_{26\text{-}104}$, red-hum**VEGF**$_{25\text{-}107}$, and red-hum**VEGF**$_{25\text{-}109}$:

Fully reduced red-hum**VEGF**$_{26\text{-}104}$, red-hum**VEGF**$_{25\text{-}107}$, or red-hum**VEGF**$_{25\text{-}109}$ were dissolved in 0.1M Tris-buffer (pH 8.0), with or without 1M guanidine.HCl, containing 1.0 mM cystine (SS-form) and 8.0 mM cysteine (SH-form) in a final concentration of 0.1 mg/mL and stirred at room temperature. Immediately, a sharp peak appears at a lower retention time (more polar) in addition to some broad peaks that correspond to incomplete or incorrectly folded peptide. When HPLC-analysis showed no further change in peak intensities (usually after ~4 hours), the mixture was loaded onto a preparative RP/C_{18} column and purified following our standard procedure (see below).

Procedure III:

General procedure for tBoc-synthesis of peptides:

Fragment peptides were prepared by manual solid phase peptide synthesis (SPPS) typically on a 0.25 mmol scale using the in situ neutralization/ HBTU activation procedure for Boc chemistry as previously described. Each synthetic cycle consisted of Nα-Boc-removal by a 1-2 min treatment with neat TFA, a 1 min DMF-flow wash, a 10 to 20 min coupling time with 1.0 mmol preactivated Boc-amino acid in the presence of excess DIEA, followed by a second DMF-flow wash. Nα-Boc amino acids (1.1 mmol) were preactivated for 3 min with 1.0 mmol HBTU (0.5 M in DMF) in the presence of excess DIEA (3 mmol). After coupling of Gln residues, a DCM flow wash was used before and after deprotection using TFA, to prevent possible high temperature (TFA/DMF)-catalyzed pyrrolidonecarboxylic acid formation. Side-chain protected amino acids were: Boc-Arg (p-toluenesulfonyl)-OH, Boc-Asn(xanthyl)-OH, Boc-Asp(O-cyclohexyl)-OH, Boc-Cys(4-methylbenzyl)-OH, Boc-Glu(O-cyclohexyl)-OH, Boc-His(dinitrophenyl)-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Ser(benzyl)-OH, Boc-Thr(benzyl)-OH, and Boc-Tyr(2-Br-Z)-OH. Other amino acids were used without side-chain protection. Nα-acetylation of peptides was performed by treatment with acetic anhydride (0.1 M)/Pyridine (0.1 M) in DMF for 2 x 2 min). After chain assembly was completed, the peptides were deprotected and cleaved from the resin by treatment with anhydrous HF for 1 hr at 0°C with 4% *p*-cresol as a scavenger. In all cases, the imidazole side chain-dinitrophenyl (Dnp) protecting groups remained on His residues because the Dnp-removal procedure is incompatible with C-terminal thioester groups. However, Dnp is gradually removed by thiols during the ligation reaction yielding unprotected His. After cleavage, the peptide fragments were precipitated with ice-cold diethylether, dissolved in aqueous acetonitrile and lyophilized.

Preparation of thioester-generating (-COSR) resin:

1.1 mmol Nα-Boc Leu was activated with 1 mmol HBTU in the presence of 3 mmol DIEA and coupled for 10 min to 0.25 mmol MBHA resin. Next, 1.1

mmol S-trityl mercaptopropionic acid was activated with 1 mmol HBTU in the presence of 3 mmol DIEA and coupled for 30 min to Leu-MBHA resin. The resulting trityl-mercaptopropionic acid-leucine resin can be used as a starting resin for polypeptide chain assembly following removal of the trityl protecting group with 2 x 1 min treatments with 2.5% triisopropylsilane and 2.5% H_2O in TFA. The thioester bond was formed with the desired amino acid using standard peptide coupling protocols. Treatment of the final peptide with anhydrous HF yielded the C-terminal activated mercaptopropionic acid-leucine (MPAL) thioester (-COSR) peptides for participation in the native chemical ligation reaction.

Native Chemical Ligation (NCL) of fragment peptides:

The ligation of fully deprotected fragment peptide thioesters hum**VEGF**$_{26-60}$, hum**VEG**$_{26-67}$, and hum**VEGF**$_{25-67}$ with either the fragment peptides hum**VEGF**$_{61-104}$, hum**VEGF**$_{68-104}$, or hum**VEGF**$_{68-107}$ was performed as follows: peptide fragments were dissolved in a ~1:1 molar ratio at 10 mg/ml in 0.1 M tris buffer, pH 8.0, containing 6 M guanidine. Benzylmercaptan and thiophenol were added to 2% (v/v) resulting in a final peptide concentration of 1-3 mM at a pH ~ 7 (lowered due to addition of thiols and TFA from the lyophilized peptide). The ligation reaction was performed in a heating block at 37° and was vortexed periodically to equilibrate the thiol additives. The reaction was monitored by HPLC and ESI-MS until completion. Respective NCLs (hum**VEGF**$_{26-60}$ + hum**VEGF**$_{61-104}$; hum**VEGF**$_{26-67}$ + hum**VEGF**$_{68-104}$) yielded reduced **VEGF**$_{\mathbf{26-104}}$ with identical HPLC and ESI-MS specifications.

Oxidative Folding of red-hum**VEGF**$_{\mathbf{26-104}}$ and red-hum**VEGF**$_{\mathbf{25-107}}$:

Fully reduced red-hum**VEGF**$_{\mathbf{26-104}}$ and red-hum**VEGF**$_{\mathbf{25-107}}$ were dissolved in 0.1 M Tris-buffer (pH 8.0), with or without 1M guanidin.HCl, containing 1.0 mM cystine (SS-form) and 8.0 mM cysteine (SH-form) in a final concentration of 0.1 mg/mL and stirred at room temperature. Immediately, a sharp peak appears at a lower retention time (more polar) corresponding to the correctly folded cysknot structure, in addition to some broad peaks that correspond to incomplete or incorrectly folded peptide.

When HPLC-analysis showed no further change in peak intensities (usually after ~4 hours), the mixture was loaded onto a preparative RP/C_{18} column and purified following our standard procedure (see below).

General Procedure for Purification by HPLC:

Crude peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC), either on a "DeltaPack" (25x100 or 40x210 mm inner diameter, 15 µm particle size, 100 Å pore size; Waters, USA) or on a "Atlantis" (10x100 mm inner diameter, 5 µm particle size (Waters, USA) RP-18 preparative C_{18} column with a linear AB gradient of 1-2% B/min. where solvent A was 0.05% TFA in water and solvent B was 0.05% TFA in ACN. Alternatively, analytical reversed-phase HPLC was performed on a Varian Prostar system using Vydac C-18 columns (5 µm, 0.46 x 15 cm) and preparative reversed-phase HPLC was performed on a Waters system using Vydac C-18 columns (10 µm, 1.0/2.5 x 25 cm). Linear gradients of acetonitrile in water/0.1% TFA were used to elute bound peptides. The flow rates used were 1 ml/min (analytical), and 5/10 ml/min (preparative).

Analysis by RP-HPLC/ESI-MS:

Analysis of the purified peptide was performed by reversed-phase high performance liquid chromatography (RP-HPLC) on a "Acquity UPLC (Waters, USA) using a RP-18 preparative "BEH" column (2.1x50 inner diameter, 1.7 mm particle size, Waters, USA) with a lineair AB gradient (5-55% B, 25% B/min), where solvent A was 0.05% TFA in water and solvent B was 0.05% TFA in ACN. The primary ion molecular weight of the peptides was determined by electron-spray ionization mass spectrometry.

Analysis by ESI-MS:

Electrospray ionization mass spectrometry (ESI-MS) of HPLC samples was performed on an API-150 single quadrupole mass spectrometer

(Applied Biosystems). Peptide masses were calculated from the experimental mass to charge (m/z) ratios from all the observed protonation states of a peptide using Analysis software.

For each peptide the following characteristics were determined:

Peptide	**Oxidation state (RED/OX)**	**Retention (%ACN)**	**MW calculated**	**MW experimental**
Red-humVEGF$_{26\text{-}104}$	RED	48.5	9065.6	9064.4
Oxid-humVEGF$_{26\text{-}104}$	OX	42.5	9059.6	9058.5
Red-humVEGF$_{25\text{-}107}$ (Boc)	RED	45.8	9569.1	9566.4
Oxid-humVEGF$_{25\text{-}107}$ (Boc)	OX	40.5	9563.1	9560.7
Red-humVEGF$_{25\text{-}107}$ (Fmoc)	RED	45.8	9569.1	9568.8
Oxid-humVEGF$_{25\text{-}107}$ (Fmoc)	OX	40.5	9563.1	9561.7
Red-humVEGF$_{25\text{-}109}$	RED	43.8	9869.5	9869.6
Oxid-humVEGF$_{25\text{-}109}$	OX	38.2	9863.5	9863.8

These data and Figure 1 show that the various forms of humVEGF$_{trunc}$ can be synthesized in various different ways with identical outcomes.

EXAMPLE 1B: Inhibitory activity of oxid-humVEGF$_{26\text{-}104}$ in Avastin™-binding to surface-immobilized oxid-humVEGF$_{1\text{-}165}$.

Binding ELISA: Binding of various mAbs (Avastin™, mAb 293, PDL-antibody) to oxid-humVEGF$_{26\text{-}104}$ and humVEGF$_{1\text{-}165}$ was determined in ELISA. Therefore, polystyrene 96-well plates (Greiner, Germany) were treated with 100 μL/well of 0.2% glutaric dialdehyde in phosphate-buffer (0.1 M, pH=5) for 4 hours at room temperature while shaking, following by washing (3x10min) with phosphate-buffer (0.1 M, pH=8). Then, the wells were coated with 100 µL/well of a 1 µg/mL solution of oxid-humVEGF$_{26-104}$/humVEGF$_{1-165}$ in phosphate-buffer (0.1 M, pH=8) for 3 hours at 37 ^{0}C, followed by overnight standing at room temperature. After washing with 1%Tween80 (3x), the plates were incubated with the antibody at various different dilutions in horse serum (4% in PBS/1%Tween80/3%NaCl), starting with 1/10 dilution in the first well and 3-fold dilution steps in subsequent wells. Incubation was performed for 1 hour at 37 ^{0}C, followed by washing with 1%Tween-80 (3x). Then, the plates were incubated with 100 µL/well of peroxidase-labeled Goat-anti-rat serum (1/1000 dilution in 4% horse serum, see above) for 1 hour at 25 ^{0}C, followed by washing with 1%Tween80 (4x). Finally, the plates were incubated with a 0.5 µg/mL solution of ABTS (2,2'-azine-di(ethylbenzthiazoline sulfonate)) containing 0.006% H_2O_2 in citric acid/phosphate-buffer (0.1 M each, pH=4). OD$_{405nm}$-values were measured after 45 min. standing at room temperature in the dark.

Competition ELISA: ELISA binding competition studies were carried out following largely the procedure as described for binding in ELISA (see above). Incubation with antibody was carried out at one fixed antibody-concentration (10 ng/mL of Avastin™; OD$_{405nm}$ between 1.0-1.5) in the presence of decreasing amounts of **oxid-humVEGF$_{26-104}$** (start at 5 µM; 1/5 dilution steps) and **humVEGF$_{1-165}$** (positive control; start at 500 nM; 1/5 dilution steps).

The data in Figure 2 show that ***oxid-humVEGF$_{26-104}$*** *binds with less than 5-fold difference in affinity (as compared to* ***humVEGF$_{1-165}$****) to Avastin™, while the (cyclic) peptide-mimic derived from the beta3-loop of humVEGF is >10 000-fold less active in binding to Avastin™. This illustrates the big step forward in reconstruction of the discontinuous Avastin™ binding site on humVEGF using this novel technology of the present invention.*

EXAMPLE 1C: Use of oxid-humVEGF$_{26\text{-}104}$ for generating VEGF-neutralizing antibodies and sera in rats and mice.

Immunization experiments using **oxid-humVEGF$_{26\text{-}104}$** (not-conjugated to a carrier protein!!) were carried out both in female Wistar rats and female Balb/C mice. The antisera were analyzed for:

A) binding to surface-immobilized humVEGF$_{1\text{-}165}$ (titer determination)

B) ability to inhibit the binding of Avastin™ to surface-immobilized humVEGF$_{1\text{-}165}$

C) neutralizing activity for humVEGF$_{1\text{-}165}$ in a BaF3-cell proliferation assay

The results of these studies are shown below and in Figures 3-6.

Immunization Protocols:

Wistar rats: Female Wistar rats were immunized with anti-**humVEGF$_{26\text{-}104}$** at day 0 with 400 μL (intramuscular + subcutaneous, 200 μL each) of a 375 μg/mL solution of **humVEGF$_{26\text{-}104}$** in PBS/CoVaccine 1:1 (v/v) (PBS = Phosphate-Buffered Saline), followed by a booster (same quantity and concentration) at 2 and 4 weeks. Subsequently, the rats were bleeded after 6 weeks and the antisera collected. Anti-VEGF titers were determined as described as below.

Balb/C mice: Immunization with oxid-**humVEGF$_{26\text{-}104}$** was performed in female Balb/C mice, using 2 different formulations, i.e. with a CFA/IFA adjuvant (group 1: 2 anmials), and with a CoVaccine adjuvant (group 2: 3 animals). The animals (2) in group 1 were immunized intraperitoneal (i.p.) at day 0 with 250 μL of a 1.0 mg/mL solution of **oxid-humVEGF$_{26\text{-}104}$** in PBS/CFA 2:3 (v/v) (PBS = Phosphate-Buffered Saline, CFA = Complete Freund's Adjuvance), followed by a booster (same quantity, method and concentration; Incomplete Freund's Adjuvance (IFA) instead of CFA) at 4 weeks. The animals (3) in group 2 were immunized at day 0 with 210 μL (intramuscular + subcutaneous, 105 μL each) of a 1.25 mg/mL solution of VEGF$_{26\text{-}104}$ in PBS/CoVaccin 1:1 (v/v) (PBS = Phosphate-Buffered Saline), followed by a booster (same quantity, method and concentration) at 2 and 4 weeks. Subsequently, all 5 mice were bleeded after 6 weeks and the antisera collected. Anti-VEGF titers were determined as described as below.

ELISA titer determination:

Titers were calculated by determining the serum dilution for which OD_{405nm} is equal to $4xOD_{405nm}$ that of a buffer solution (see "ELISA-binding studies, example 1B"). The titer defines the negative ^{10}log-value of the dilution factor (1/10=1, 1/100=2, 1/1000=3, 1/10000=4, etc.).

Animal	**humVEGF$_{1\text{-}165}$ Titer 0 wpv**	**humVEGF$_{1\text{-}165}$ Titer 6 wpv**
50.49 (Wistar rat 1; CoVaccine)	<<2	4.8
50.67 (Wistar rat 2; CoVaccine)	<<2	5.4
59.01 (Balb/C mouse 1, CFA/IFA)	<<2	5.3
59.02 (Balb/C mouse 2, CFA/IFA)	<<2	5.2
59.03 (Balb/C mouse 3, CoVaccine)	<<2	5.4
59.04 (Balb/C mouse 4, CoVaccine)	<<2	†
59.05 (Balb/C mouse 5, CoVaccine)	<<2	5.3
Control Abs		
Avastin™ (500 ng/mL start)	-	4.4
BioVision™ (5000 ng/mL)	-	4.2

ELISA competition studies of rat antisera with Avastin™:

ELISA binding competition studies were carried out following largely the procedure as described for binding in ELISA (see above). Incubation with antibody was carried out at a fixed Avastin™-concentration (10 ng/mL; OD_{405nm} between 1.0-1.5) in the presence of decreasing amounts of rat antisera (start at 1/5; further 1/3 dilution steps).

Neutralization in BaF3-cell proliferation assay:

The cells which are used in the assay are murine pre-B lymphocytes stable expressing human (h) humVEGF-Receptor 2 (Makinen et al., 2001). These recombinant cells survive/proliferate only in the presence of IL-3 (natural cytokine required for the survival of the parental cells) or humVEGF. For the experiment IL-3 has to be washed off the medium so that proliferation capability in dependence of humVEGF can be tested.

Ba/F3 R2 cells were grown in DMEM (Gibco #31885) containing 10 % foetal bovine serum (Perbio #CH30160.03), 2 mM L-glutamine (Sigma #G7513), 2 ng/ml mIL-3 (Calbiochem #407631) and 500 µg/ml Zeocin (Invitrogen #450430). Cells were grown at 37°C in a humidified incubator with an atmosphere of 5% CO2/95% air.
Differently concentrated humVEGF (+ humVEGF) or medium (-humVEGF) was either added directly to the cells (to test the proliferation efficiency) or pre-incubated for 1 hour with different concentrations of Avastin™ (positive control), different concentrations of rat or mouse sera and then added to the cells (in case of inhibition experiments). Two days later cell proliferation was measured by adding WST-1 (Roche #1644807). See Figure 9 for a graphical representation of the assay.

The WST-1 assay is based on the measurement of the mitochondrial succinate deshydrogenase activity. To function correctly this enzyme requires the integrity of this organelle and is a good indicator of the number of proliferating cells present in the culture. A tetrazolium salt (WST-1) is used as substrate since it generates a soluble dark metabolic (formazon) through the action of the enzyme, which then be quantified by measuring the absorbance (450 nm) in an ELISA reader. The higher is the absorbance measured in the assay, the stronger the proliferation. Absorbance is positively correlated with proliferation. Experiments were repeated three times in triplicate showing overall similar results.

*The data obtained proves that high levels of antibodies were successfully generated via immunization with oxid-hum**VEGF$_{26\text{-}104}$** (not-conjugated to a carrier protein!!), both in female Wistar rats and female Balb/C mice. The* *antisera generated in this way exhibit strong neutralizing activity for humVEGF$_{1\text{-}165}$ in a BaF3-cell proliferation assay (Figure 3-6), and the ability to inhibit binding of Avastin™ to humVEGF (Figure 7).*

EXAMPLE 1D: oxid-humVEGF$_{26\text{-}104}$ does not induce BaF3-cell proliferating by itself.

In order to check whether oxid-hum**VEGF**$_{26\text{-}104}$, the truncated form of hum**VEGF**$_{1\text{-}165}$, is also able to induce BaF3-cell proliferation, we measured cell proliferation in the presence of varying amounts of oxid-hum**VEGF**$_{26\text{-}104}$ (0.01-20 ng/mL). In order to check if oxid-hum**VEGF**$_{26\text{-}104}$ was able to enhance or inhibit the proliferative capacity of hum**VEGF**$_{1\text{-}165}$, itself, the experiments with varying amounts of oxid-hum**VEGF**$_{26\text{-}104}$ were also run in the presence of hum**VEGF**$_{1\text{-}165}$=1.2 ng/mL .

*The results shown in Figure 8 clearly demonstrate no activity for oxid-hum**VEGF**$_{26\text{-}104}$ in BaF3-cell proliferation nor any effect on the proliferating ability of hum**VEGF**$_{1\text{-}165}$.*

EXAMPLE 1E: Passive immunization study with anti-humVEGF$_{26\text{-}104}$ rat-antisera in Swiss nu/nu mice inoculated with human LS174T tumor cells: in vivo proof of principle of the tumor-reducing potential of anti-humVEGF$_{26\text{-}104}$ antisera.

In order to demonstrate the tumor-reducing potential of anti-**humVEGF**$_{26\text{-}104}$ antisera, the following immunization experiment was carried out in 30 male Swiss nu/nu mice (Charles river), 6 weeks of age at the study begin. The animals were divided in the following 3 treatment groups:

Group 1: **PBS** (n=10; negative control group): intraperitoneal (i.p.) PBS injections (500 μl) after tumor cell inoculation.

Group 2: oxid-**humVEGF**$_{26\text{-}104}$ (n=10): i.p. injections (500 μl) with IgG-purified anti-VEGF peptide rat-antiserum after tumor cell inoculation.

Group 3: AVASTIN™ (n=10; positive control group): i.p. injections (500 µl) with anti-humVEGF mAb AVASTIN™ following tumor cell inoculation.

On day 1 of the study, all 30 mice were injected subcutaneously (right flank) with 10 million human LS174T tumor cells suspended in a 100 µL solution. Tumor-take was ~100%. Subsequently, the mice were given on day 1, 8, and 15 i.p. injections (500 µl) with either A) PBS (group 1), B) anti-**oxid-humVEGF$_{26\text{-}104}$** rat-antiserum (5x conc. rat-serum; group 2), and C) AVASTIN™ (group 3). **Anti-oxid-humVEGF$_{26\text{-}104}$** ratserum was obtained by immunizing a total number of 20 male Whistar rats in a separate experiment 4x with 250 microgram doses of humVEGF$_{26\text{-}104}$ using CoVaccine adjuvant (inoculations at day 0, 14, 28, and 49; bleed on day 63). The resulting ratsera were purified by affinity chromatography (ProtG-column) and concentrated 5x. The 10 most potent antisera (based on in-vitro neutralization data in BaF3 assay; see previous Example) of these were pooled and used for inoculation of the 10 mice in treatment-group 2. Lengths and breadths of the tumors were measured every other day, starting on the first day after tumor cell inoculation. Tumor volumes were estimated using the formula *(breadth2 x length)/2* (ref 6). The data are shown in Figure 12.

The data presented above lead to the following conclusions:

1. **anti-oxid-humVEGF$_{26\text{-}104}$** antisera have the ability to strongly reduce tumor growth in mice.
2. in this experimental setting, the observed effect of treatment with **anti-oxid-humVEGF$_{26\text{-}104}$** antisera was visibly more pronounced than that for AVASTIN™.
3. treatment of nude mice with **anti-oxid-humVEGF$_{26\text{-}104}$** antibodies was received well by all animals and is thus not toxic!

EXAMPLE 1F: Immunogenicity of oxid-ratVEGF$_{26-104}$ in rats.

Peptide sequence oxid-**ratVEGF$_{26-104}$**:
Acetyl-**C1**RPIETLVDIFQEYPDEIEYIFKPSAVPLMR**C2**AGA**C3**NDEALE
C4VPTSESNVTMQIMRIKPHQSQHIGEMSFLQHSR**C5**E**C6**-amide

Solid-phase synthesis of ratVEGF$_{26-104}$. ratVEGF$_{26-104}$ was synthesized by normal solid-phase synthesis on a Rink-amide resin (downloaded to 0.1 mmol/g) following standard procedures as described for **humVEGF$_{26-104}$** (see Example 1). Subsequent oxidative refolding was carried out exactly as described for **humVEGF$_{26-104}$**. Purification of both **red-ratVEGF$_{26-104}$** and **oxid-ratVEGF$_{26-104}$** was carried out by preparative High Performance Liquid Chromatography (HPLC). Characterization of both peptides was carried out by analytical HPLC and ElectroSpray Ionization Mass Spectrometry (ESI-MS).
The successful refolding of red-ratVEGF$_{26-104}$ was evidenced by the characteristic shift to lower Rf-values (from 48.5% to 41.3% ACN, see Table below), normally observed when proteins or fragments thereof are oxidative refolded. The characteristic narrow shape of the new peak at lower R_f-value provides evidence that an intact cystine-knot structure is indeed formed upon oxidative refolding of **red-ratVEGF$_{26-104}$**.
Also the ESI-MS spectrum undergoes a significant change upon oxidative refolding. First of all, the overall mass goes down by 6 mass units (formation of 3 disulfide bonds releases a total of 6H). Moreover, there is a very characteristic shift of MS-signals to higher m/z-values. For example, the MS-spectrum for **red-ratVEGF$_{26-104}$** gives the most intense signals for the M^{9+} and M^{10+} charged species, whereas these signals disappear and a much weaker signal at M^{5+} remains (see Figure 13) that is much less intense. Also this shift is characteristic for folding of proteins into their oxidized native structure and shows that oxidative refolding of red-**ratVEGF$_{26-104}$** has been successful. The reason is that the protein or protein fragment adopts a more condensed structure that is no longer able

to pick up so many charges. In contrast to this, the flexible and extended structure of the reduced protein is able to accommodate many more charges.

Peptide	**Oxidation state (RED/OX)**	**Retention (%ACN)**	**MW calculated**	**MW experimental**
red-ratVEGF$_{26\text{-}104}$	RED $(SH)_6$	48.5	9087.5	9085.3
oxid-ratVEGF$_{26\text{-}104}$	OX $(SS)_3$	41.3	9081.5	9080.0

This example describes the results of an immunization study in male Whistar rats with both oxid-**hum-VEGF$_{26\text{-}104}$** and oxid-**ratVEGF$_{26\text{-}104}$** with an intact cystine-knot fold (oxid-form). The data unequivocally show that oxid-**ratVEGF$_{26\text{-}104}$** is equally immunogenic and potent as compared to oxid-**humVEGF$_{26\text{-}104}$** in generating antibodies in rats. The use of truncated VEGF as described in this patent can thus be used to bypass immune tolerance to “self proteins”, like for example the full-length homodimeric VEGF protein in this particular case.

A total of 4 Whistar rats (2x2) were immunized on day 0 with 250 microgram each of either **oxid-ratVEGF$_{26\text{-}104}$** (2 rats) or **oxid-humVEGF**$_{26\text{-}104}$ (2 rats) using CoVaccine as adjuvant, followed by booster inoculations at day 14, 28, and 42. The rats were finally bled at day 56, and the sera were analyzed for antibody titers against ratVEGF$_{1\text{-}165}$, **humVEGF$_{1\text{-}165}$**, **oxid-ratVEGF$_{26\text{-}104}$**, and **oxid-humVEGF$_{26\text{-}104}$**. (Part of) the antibody-binding data are shown in Table 1 and Figure 14.
The data in Table 1 and Figure 14 do not show any detectable difference in binding between antisera elicited with **oxid-ratVEGF$_{26\text{-}104}$** and those elicited with **oxid-humVEGF$_{26\text{-}104}$ in rats**, which strongly suggests that **oxid-ratVEGF$_{26\text{-}104}$** is equally immunogenic in rats (homologous species) as compared to **oxid-humVEGF$_{26\text{-}104}$** (heterologous species), and is able to elicit comparable amounts of antibodies that even show crossreactivity with the **homodimeric VEGF$_{1\text{-}165}$ protein** (Table 1C).

Furthermore, the experiment provides very strong basis for the fact that oxid-humVEGF$_{26\text{-}104}$ can be used to elicit anti-VEGF in humans, and that oxid-humVEGF$_{26\text{-}104}$ will not suffer from lack of immunogenicity as a result of immune tolerance to self proteins.

Table 1. List of the binding of rat-antisera in ELISA to A) **oxid-ratVEGF$_{26\text{-}104}$**, B) **oxid-humVEGF$_{26\text{-}104}$**, C) **humVEGF$_{1\text{-}165}$ homodimer** (recombinant full-length humanVEGF), and D) **ratVEGF$_{1\text{-}165}$ homodimer** (recombinant full-length ratVEGF). For comparison, the binding data to the humanized anti-humVEGF mAb AVASTIN^{TM} are included.

A ratVEGF26-104

titers	1/100	1/300	1/1000	1/3000	1/10000	1/30000	1/100000	1/300000	titer endblood
rat 1 (a-oxid-ratVEGF26-104)	3298	3263	3123	3028	2357	1214	514	225	**5.1**
rat 2 (a-oxid-ratVEGF26-104)	3597	3424	3262	3197	2516	1241	532	237	**5.1**
rat 3 (a-oxid-humVEGF26-104)	3376	3172	3209	3176	2910	1951	861	355	**5.3**
rat 4 (a-oxid-humVEGF26-104)	3200	3263	3465	3060	2895	1736	754	349	**5.3**

B humanVEGF26-104

titers	1/100	1/300	1/1000	1/3000	1/10000	1/30000	1/100000	1/300000	titer endblood
rat 1 (a-oxid-ratVEGF26-104)	3334	3148	3210	3174	2989	1929	811	366	**5.3**
rat 2 (a-oxid-ratVEGF26-104)	3297	3121	3564	3329	2801	1871	728	332	**5.2**
rat 3 (a-oxid-humVEGF26-104)	3263	3098	3385	3300	2908	2188	898	409	**5.3**
rat 4 (a-oxid-humVEGF26-104)	3229	3174	3289	3298	3051	2166	873	373	**5.3**
Avastin (a-humVEGF mAb)	4037	3033	1839	736	333	158	116	97	**15-25 ng/mL**

C humanVEGF1-165

titers	1/100	1/300	1/1000	1/3000	1/10000	1/30000	1/100000	1/300000	titer endblood
rat 1 (a-oxid-ratVEGF26-104)	3404	3320	3449	2681	1305	548	280	158	**4.6**
rat 2 (a-oxid-ratVEGF26-104)	3245	3216	3672	2955	1588	955	301	166	**4.7**
rat 3 (a-oxid-humVEGF26-104)	3456	3406	3334	3078	1776	739	351	176	**4.7**
rat 4 (a-oxid-humVEGF26-104)	3758	3282	3604	3313	2508	1374	510	235	**5.1**
Avastin (a-humVEGF mAb)	3261	3016	2493	1322	528	222	129	100	**5-10 ng/mL**

D ratVEGF1-165

titers	1/100	1/300	1/1000	1/3000	1/10000	1/30000	1/100000	1/300000	titer endblood
rat 1 (a-oxid-ratVEGF26-104)	2993	2519	1481	731	346	172	122	98	**3.8**
rat 2 (a-oxid-ratVEGF26-104)	3032	3055	2717	1568	753	315	179	122	**4.2**
Avastin (a-humVEGF mAb)	236	148	103	89	93	89	91	88	**<1000 ng/mL**

EXAMPLE 1G: Synthesis of humPLGF$_{34\text{-}112}$ (humPLGFtrunc).

Peptide sequence of **humPLGF$_{34\text{-}112}$**:

Acetyl-**C1**RALERLVDVVSEYPSEVEHMFSPSAVSLLR**C2**TGA**C3**GDENLHC4VPVETANVTMQLLKIRSGDRPSYVELTFSQHVR**C5**E**C6**-amide

X0 = acetyl
X1 = RALERLVDVVSEYPSEVEHMFSPSAVSLLR (**A**-mutation for native **C**)
X2 = TGA (**A**-mutation for native **C**)
X3 = GDENLH
X4 = VPVETANVTMQLLKIRSGDRPSYVELTFSQHVR
X5 = E
X6 = amide

Solid-phase synthesis of red-PLGF$_{34\text{-}112}$. Red-PLGF$_{34\text{-}112}$ was synthesized by normal solid-phase synthesis on a Rink-amide resin (downloaded to 0.1 mmol/g) following standard procedures as described for **red-humVEGF$_{26\text{-}104}$** (see Example 1E). Subsequent oxidative refolding was carried out exactly as described for **oxid-humVEGF$_{26\text{-}104}$**. Purification of both **red-humPLGF$_{34\text{-}112}$** and **oxid-humPLGF$_{34\text{-}112}$** was carried out by preparative High Performance Liquid Chromatography (HPLC). Characterization of both **red-humPLGF$_{34\text{-}112}$** and **oxid-humPLGF$_{34\text{-}112}$** was carried out by analytical HPLC and ElectroSpray Ionization Mass Spectrometry (ESI-MS).
The successful refolding of **red-humPLGF$_{34\text{-}112}$** was evidenced by the characteristic shift to lower Rf-values (from 49% to 38.3% ACN, see Table below) that is normally observed when proteins or fragments thereof are oxidative refolded. The characteristic narrow shape of the new peak at lower Rf-value provides evidence that an intact cystine-knot structure is indeed formed upon oxidative refolding of **red-humPLGF$_{34\text{-}112}$**.

Also the ESI-MS spectrum undergoes a significant change upon oxidative refolding. First of all, the overall mass goes down by 6 mass units (formation of 3 disulfide bonds releases a total of 6H). Moreover, there is a very characteristic shift of MS-signals to higher m/z-values. For example, the MS-spectrum for **red-humPLGF$_{34-112}$** gives clear signals for the M^{6+} to M^{10+} charged species, whereas these signals disappear and a much weaker signal at M^{5+} remains (see Figure 15) that is much less intense. Also this shift is characteristic for folding of proteins into their oxidized native structure and shows that refolding of **red-humPLGF$_{34-112}$** was successful. The reason is that the protein or protein fragment adopts a more condensed structure that is no longer able to pick up so many charges. In contrast to this, the flexible and extended structure of the reduced protein is able to accommodate many more charges.

Peptide	Oxidation state (RED/OX)	Retention (%ACN)	MW calculated	MW experimental
red-humPLGF$_{34-112}$	RED (SH)$_6$	48.5	8855.2	8855.3
oxid-humPLGF$_{34-112}$	OX (SS)$_3$	38.3	8849.2	8847.5

EXAMPLE 1H: Synthesis of humSOST$_{57-144}$ (humSOSTtrunc):

Peptide sequence for humSOST$_{57-144}$:

Biotine-GGG**C1**RELHFTRYVTDGP*C*RSAKPVTELV**C2**SGQ**C3**GPARLLPNAIGRGKWWRPSGPDFR**C4**IPDRYRAQRVQLL*C*PGGEAPRARKVRLVAS**C5**K**C6#**

X0 = biotine-GGG
X1 = RELHFTRYVTDGP*C*RSAKPVTELV
X2 = SGQ
X3 = GPARLLPNAIGRGKWWRPSGPDFR
X4 = IPDRYRAQRVQLL*C*PGGEAPRARKVRLVAS
X5 = K
X6 = amide

Synthesis of red-humSOST$_{57\text{-}144}$ could not be performed directly on solid-phase on a downloaded resin, as described for humVEGF$_{26\text{-}104}$,. Therefore, the shorter fragments humSOST-F1/3 were synthesized and subsequently ligated by Native Chemical Ligation (NCL) as described below. Also, the subsequent oxidative refolding of fully **red-humSOST$_{57\text{-}144}$** was carried out as described below. Solid-phase synthesis of the fragments **humSOST-F1/3** was carried out following standard procedures as described for **humVEGF$_{26\text{-}104}$**.

Fragment condensation of humSOST-F1/3 by NCL to give red-humSOST$_{57\text{-}144}$ (for a schematic overview see Figure 16)

First, hum**SOST-F2** and hum**SOST-F3** were dissolved (2 mg/ml) in NCL reaction mixture (6 M guanidine, 20 mM TCEP, 200 mM MPAA, 0.2 M disodium hydrogenphosphate, adjusted with 10 M sodium hydroxide to pH 6.5) in a 1.2:1 ratio, and reacted for 24 hours at room temperature. The thiaproline-protected hum**SOST-F2/3** was obtained in 66.5% yield after reversed phase HPLC purification. Subsequently, the thiaproline was deprotected with 0.02 M methoxyamine in NCL buffer at pH 4.0 for 60 h. Then, the pH was adjusted to 6.5 and 1.2 equivalents of hum**SOST-F1** was added and reacted for 1.5 day. The reaction was monitored by RPLC/MS and each day 40 mM TCEP was added to completely reduce all reagents. After completion of the reaction, crude **red-humSOST$_{57\text{-}144}$** was purified using ion exchange chromatography, and subsequently by reversed phase HPLC giving pure **red-humSOST$_{57\text{-}144}$** in 24.2% yield (overall 16.1%).

Structure of peptide fragments used for the fragment condensation of reduced SOST$_{67\text{-}144}$

Name	Peptide Sequence
humSOST$_{57\text{-}144}$	Biotine-GGG**C**RELHFTRYVTDGP*C*RSAKPVTELV**C**SGQ**C**GPARLLPNAIGRGKWWRPSGPDFR**C**IPDRYRAQRVQLL*C*PGGEAPRARKVRLVAS**C**K**C**-amide
humSOST-F1	Biotine-GGG**C**RELHFTRYVTDGP*C*RSAKPVTELV**C**SGQ-thioester
humSOST-F2	BocNH-**C(Thz)**GPARLLPNAIGRGKWWRPSGPDFR-thioester
humSOST-F3	Amine-**C**IPDRYRAQRVQLL*C*PGGEAPRARKVRLVAS**C**K**C**-amide

C = cysteines involved in cystine-knot formation; *C* = cysteines forming SS-bond between loop-1 and loop-3 of humSOST

Oxidate refolding of red-humSOST$_{57\text{-}144}$ to give oxid-humSOST$_{57\text{-}144}$.

Subsequently, **red-humSOST**$_{57\text{-}144}$ was natively refolded by dissolving the peptide (2 mg/ml) in a pH 8.0 buffer solution, containing 55 mM Tris-HCl, 21 mM sodium chloride, 0.88 mM potassium chloride, 0.48 L-arginine, 20 mM Glutathion-SH, and 4 mM Glutathion-SS. The peptide was oxidized over time and yielded 10.2% of **oxid-humSOST**$_{57\text{-}144}$ after 3.5 days at 4 °C (see Figure 17).

Purification of both **red-humSOST**$_{57\text{-}144}$ and **oxid-humSOST**$_{57\text{-}144}$ was carried out by preparative High Performance Liquid Chromatography (HPLC). Characterization of both compounds was carried out by analytical HPLC and ElectroSpray Ionization Mass Spectrometry (ESI-MS; see below).

Peptide	Oxidation state (RED/OX)	Retention (%ACN)	MW calculated	MW experimental
red-humSOST$_{57\text{-}144}$	RED $(SH)_8$	35.0	10237.2	10235.0
oxid-humSOST$_{57\text{-}144}$	OX $(SS)_4$	30.0	10229.2	10229.8
AA$_8$-humSOST$_{57\text{-}144}$	RED $(S\text{-}AcNH_2)_8$	33.0	10694.1	10692.5

The successful refolding of hum**SOST**$_{57\text{-}144}$ was evidenced by the characteristic shift to lower Rf-values (from 35% to 30% ACN, see Table below) that is normally observed when proteins or fragments thereof are oxidative refolded. The characteristic narrow shape of the new peak at lower Rf-value provides evidence that an intact cystine-knot structure is indeed formed upon oxidative refolding.
Also the ESI-MS spectrum undergoes a significant change upon oxidative refolding. First of all, the overall mass goes down by 8 mass units (formation of 4 disulfide bonds releases a total of 8H). Moreover, there is a very characteristic shift of MS-signals to higher m/z-values. For example, the MS-spectrum for the **red-humSOST$_{57\text{-}144}$** gives clear signals for the M^{8+} to M^{12+} charged species, whereas these signals disappear and a much weaker signal at M^{6+} and M^{7+} remains (see Figure 18D) that is much less intense. Also this shift is characteristic for folding of proteins into their oxidized native structure and shows that refolding of red-hum**SOST**$_{57\text{-}144}$ was successful. The reason is that the protein or protein fragment adopts a more condensed structure that is no longer able to pick up so many charges. In contrast to this, the flexible and extended structure of the reduced protein is able to accommodate many more charges.

In order to prove further that **oxid-humSOST$_{57\text{-}144}$** adopts a native cystine-knot fold, we present binding data of a series of 3 mAbs that were selected from phage-display libraries using **oxid-humSOST$_{57\text{-}144}$.** It was shown that all 3 anti-**oxid-humSOST$_{57\text{-}144}$** antibodies

- bind strongly to **oxid-humSOST$_{57-144}$** in ELISA.
- bind strongly to **recombinant full length humSOST/sclerostin** in ELISA.
- do not bind at all to **AA$_8$-humSOST$_{57-144}$** in ELISA.
- do not bind at all to three other, non-related proteins in ELISA.

Altogether, these data show that **oxid-humSOST$_{57-144}$** can be used instead of **full length humSOST/sclerostin** to select antibodies from phage-display libraries (PDLs), that show full selectivity and specificity to **full length humSOST/sclerostin** with respect to non-related proteins , and that **oxid-humSOST$_{57-144}$** can therefore be used as an "easy-available" protein mimic of **full length humSOST/sclerostin** for purposes of antibody generation and selection.

EXAMPLE 1I. Synthesis of humTGFB2$_{15-111/\Delta 49-77}$-humVEGF$_{62-67}$ (chimeric humTGFB2-humVEGFtrunc)

In this example, we demonstrate the synthesis of the truncated protein mimic of oxid-hum**TGFB2**$_{15-111}$, in which the beta2-loop (28 amino acids long; **X3** in general sequence) was replaced by the hum**VEGF beta2-loop** (aa 62-67). The successful synthesis and oxidative (cystine-knot) folding of this **TGFB2$_{15-111/\Delta 49-77}$-humVEGF$_{62-67}$** mainly serves as an example to demonstrate that interchange of beta2-loop sequences amongst different cystine-knot proteins in general leads to chimeric peptides that retain the ability to form an intact **cystine-knot fold**, just like that observed for the fully homologous trunc-peptides (see other examples).

Peptide sequence of humTGFB2$_{15-111/\Delta 49-77}$-humVEGF$_{62-67}$:
Acetyl-**C1**ALRPLYIDFKRDLGWKWIHEPKGYNANF**C2**AGA**C3**NDEGLE**C4**VSQDLEPLTILYYIGKTPKIEQLSNMIVKS**C5**K**C6**-amide

X0 = acetyl
X1 = ALRPLYIDFKRDLGWKWIHEPKGYNANF (A-mutation for native C)
X2 = AGA
X3 = NDEGLE (beta2-loop sequence of humVEGF-A; aa 62-67)
X4 = VSQDLEPLTILYYIGKTPKIEQLSNMIVKS
X5 = K
X6 = amide

Solid-phase synthesis of red-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$. **Red-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$** was synthesized by normal solid-phase synthesis on a Rink-amide resin (downloaded to 0.1 mmol/g) following standard procedures as described for **humVEGF$_{26\text{-}104}$** (see Example 1). Subsequent oxidative refolding was carried out exactly as described for **humVEGF$_{26\text{-}104}$**. Purification of both red- and **oxid-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$** was carried out by preparative High Performance Liquid Chromatography (HPLC). Characterization of both the **red-** and **oxid-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$** was carried out by analytical HPLC and ElectroSpray Ionization Mass Spectrometry (ESI-MS).

The successful refolding of **red-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$** was evidenced by the characteristic shift to lower Rf-values upon oxidative refolding (from 46.8% to 42.0% ACN, see Table below) (see other examples). The characteristic narrow shape of the new peak at lower Rf-value provides evidence that an intact cystine-knot structure is indeed formed. Also the ESI-MS spectrum undergoes a significant change upon oxidative refolding. First of all, the overall mass goes down by 6 mass units (formation of 3 disulfide bonds releases a total of 6H). Moreover, there is a very characteristic shift of MS-signals to higher m/z-values. For example, the MS-spectrum for the **red-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$** gives clear signals for the M^{6+} to M^{11+} charged species, whereas these signals completely disappear and a much weaker signal at

M^{5+} remains (see Figure 20) that is much less intense. Also this shift is characteristic for folding of proteins into their oxidized native structure and shows that refolding of hum**TGFB2$_{15-111/\Delta49-77}$-humVEGF$_{62-67}$** was successful. The reason is that the protein or protein fragment adopts a more condensed structure that is no longer able to pick up so many charges. In contrast to this, the flexible and extended structure of **red humTGFB2$_{15-111/\Delta49-77}$-humVEGF$_{62-67}$** is able to accommodate many more charges.

Peptide	Oxidation state (RED/OX)	Retention (%ACN)	MW calc.	MW exper.
red-humTGFB2$_{15-111/\Delta49-77}$-humVEGF$_{62-67}$	RED	46.8	8498.1	8500.2
oxid-humTGFB2$_{15-111/\Delta49-77}$-humVEGF$_{62-67}$	OX	42.0	8492.1	8490.5

In order to prove that **oxid-humTGFB2$_{15-111/\Delta49-77}$-humVEGF$_{62-67}$** can be used to generate anti-TGF-B2 antibodies via immunization, we carried out an immunization experiment in 2 rats. Each animal received 4 inoculations (0, 2, 4, and 7.5 wks) with 2x450 + 2x130 microgram of **oxid-humTGFB2$_{15-111/\Delta49-77}$-humVEGF$_{62-67}$**. Analysis of the 9 weeks post vaccination (wpv) antisera (Figure 21) showed strong binding in ELISA to full length TGF-B2 (titers 3.8 and 4.1) compared to those of the pre-immune sera ($\leq$2.1) indicating that antibodies specific for TGF-B2 were generated upon immunization. Moreover, it was observed that the majority of antibodies in the sera were directed towards the TGFB2-part of the peptide in **oxid-humTGFB2$_{15-111/\Delta49-77}$-humVEGF$_{62-67}$** rather that to the VEGF-part (humVEGF$_{62-67}$). This indicates the humVEGF$_{62-67}$ sequence is a good substitute for the much longer b2-loop of humTGFB2 (28 amino acids), but that it does not disturb the making of humTGF-B2 specific antibodies, nor the oxidative refolding of **red-humTGFB2$_{15-111/\Delta49-77}$-humVEGF$_{62-67}$** into **oxid-humTGFB2$_{15-111/\Delta49-77}$-humVEGF$_{62-67}$.**

These data prove that **oxid-humTGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-humVEGF$_{62\text{-}67}$** can be used as a substitute for TGF-B2 for eliciting **anti-humTGFB2 antibodies** that are fully crossreactive with the native protein humTGF-B2.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

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Claims

1. Protein mimic of a member of the cystine-knot growth factor superfamily, said protein mimic being capable of inducing an immune response against said member and having the motif X0-C1-X1-C2-X2-C3-X3-C4-X4-C5-X5-C6-X6, wherein C1 to C6 are cysteine residues which form a cystine-knot structure in which C1 is linked to C4, C2 is linked to C5 and C3 is linked to C6, and wherein X0 and X6 represent, independently from each other, an amino acid sequence with a length of 0 to 10 amino acids, X2 represents an amino acid sequence with a length of 2 to 24 amino acid residues with at least 70% sequence identity to the amino acid sequence located between C2 and C3 of a member of the cystine-knot growth factor superfamily, X5 represents an amino acid sequence with a length of 1 amino acid residue, X1 represents an amino acid sequence with a length of 15 to 50 amino acids with at least 70% sequence identity to the amino acid sequence located between C1 and C2 of a member of the cystine-knot growth factor superfamily, X3 represents an amino acid sequence with a length of 3 to 36 amino acids with at least 70% sequence identity to the amino acid sequence located between C3 and C4 of a member of the cystine-knot growth factor superfamily, and X4 represents an amino acid sequence with a length of 15 to 50 amino acids with at least 70% sequence identity to the amino acid sequence located between C4 and C5 of a member of the cystine-knot growth factor superfamily and wherein at least one cysteine in any of the sequences represented by X1, X2, X3, X4 and X6 is replaced by another amino acid residue.

2. A protein mimic according to claim 1, wherein X1, X2, X3 and X4 each represent an amino acid sequence with at least 70% sequence identity to an amino acid sequence of the same member of the cystine-knot growth factor superfamily.

3. A protein mimic according to claim 1, wherein X1 represents an amino acid sequence with at least 70% sequence identity to an amino acid sequence of a member of the cystine knot-growth factor superfamily and wherein X2, X3 and/or X4 represent an amino acid sequence with at least 70% sequence identity to an amino acid sequence of at least one other member of the cystine knot growth factor superfamily.

4. A protein mimic according to any one of claims 1-3, wherein X2 has the amino acid sequence X2a-G-X2b, wherein X2a is any amino acid or none, G is glycine, and X2b is any amino acid.

5. Protein mimic according to any one of claims 1-4, wherein said member of the cystine-knot growth factor superfamily is a member selected from the group consisting of the glycoprotein hormone-beta (GLHB) subfamily, the platelet derived growth factor (PDGF) subfamily, the transforming growth factor beta (TGF-beta) subfamily, the nerve growth factor (NGF) subfamily, the glycoprotein hormone-alpha (GLHA) subfamily, the CTCK subfamily, the Noggin-like subfamily, the Mucin-like subfamily, the Mucin-like BMP antagonist subfamily, the Mucin-like hemolectin subfamily, the Slit-like subfamily, and the Jagged-like subfamily.

6. Protein mimic according to any one of claims 1-5, which comprises at least one of the following consensus sequences:

- [GSRE]C3[KRL]G[LIVT][DE]XXX[YW]XSXC4;
- P[PSR]CVXXXRC2[GSTA]GCC3;
- [LIVM]XXPXX[FY]XXXXC2XGXC3;
- C2[STAGM]G[HFYL]C3X[ST];
- [PA]VAXXC5XC6XXCXXXX[STDAI][DEY]C;
- C2XGCC3[FY]S[RQS]A[FY]PTP; or
- $CC4(X)_{13}C(X)_{2}[GN](X)_{12}C5XC6(X)_{2,4}C$;

wherein

C2 to C6 are cysteine residues which are part of a cystine-knot structure;

X means any amino acid;

[GSRE] means G or S or R or E ; [KRL] means K or R or L;

[LIVT] means L or I or V or T; [DE] means D or E ; [YW] means Y or W;

[PSR] means P or S or R; [GSTA] means G or S or T or A;

[LIVM] means L or I or V or M ; [FY] means F or Y ;

[STAGM] means S or T or A or G or M; [HFYL] means H or F or Y or L;

[ST] means S or T; [PA] means P or A; [STDAI] means S or T or D or A or I;

[DEY] means D or E or Y; [GN] means G or N ; [RQS] means R or Q or S;

$(X)_{13}$ means a sequence of 13 amino acids; $(X)_2$ means a sequence of 2 amino acids; $(X)_{12}$ means a sequence of 12 amino acids and $(X)_{2,4}$ means a sequence of 2, 3 or 4 amino acids.

7. Protein mimic according to any one of claims 1-6, wherein said member of the cystine-knot growth factor superfamily is a member of platelet derived growth factor (PDGF) subfamily, and wherein X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 29 to 32 amino acids, X3 represents an amino acid sequence with a length of 6 to 12 amino acids, and X4 represents an amino acid sequence with a length of 32 to 41 amino acids.

8. Protein mimic according to claim 7, wherein said member of the cystine-knot growth factor superfamily is placental growth factor (PLGF), and wherein said protein mimic consists of the amino acid sequence C**1**RALERLVDVVSEYPSEVEHMFSPSAVSLLR**C2**TGA**C3**GDENLH**C4**VPVETANVTMQLLKIRSGDRPSYVELTFSQHVR**C5**E**C6** **(**PLGF$_{34\text{-}112}$**).**

9. Protein mimic according to any one of claims 1-7, wherein said member is human Vascular Endothelial Growth Factor (hVEGF), and wherein X0 comprises the amino acid sequence KFMDVYQRSY, X1 comprises the amino acid sequence HPIETLVDIFQEYPDEIEYIFKPSAVPLMR, X2 comprises the amino acid sequence GGA, X3 comprises the amino acid sequence NDEGLE, X4 comprises the amino acid sequence VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNK, X5 comprises the amino acid sequence E, and X6 comprises the amino acid sequence RPKKDRARQE, or wherein said protein mimic consists of the amino acid sequence **C1**HPIETLVDIFQEYPDEIEYIFKPSAVPLMR**C2**GGA**C3**NDEGLE**C4**VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNK**C5**E**C6** (VEGF$_{26\text{-}104}$).

10. Protein mimic according to any one of claims 1-6, wherein said member of the cystine-knot growth factor superfamily is:

- a member of the glycoprotein hormone-beta (GLHB) subfamily, and wherein X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 23 to 28 amino acids, X3 represents an amino acid sequence with a length of 18 to 20 amino acids, and X4 represents an amino acid sequence with a length of 30 to 33 amino acids, or
- a member of the glycoprotein hormone-alpha (GLHA) subfamily, and wherein X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 13 to 17 amino acids, X3 represents an amino acid sequence with a length of 27 amino acids, and X4 represents an amino acid sequence with a length of 20 to 21 amino acids, or
- a member of the nerve growth factor (NGF) subfamily, and wherein X2 represents an amino acid sequence with a length of 9 to 24 amino acids,

X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 41 to 44 amino acids, X3 represents an amino acid sequence with a length of 11 amino acids, and X4 represents an amino acid sequence with a length of 27 or 28 amino acids, or

- a member of the transforming growth factor beta (TGF-beta) subfamily, and wherein X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 23 to 41 amino acids, X3 represents an amino acid sequence with a length of 18 to 36 amino acids, and X4 represents an amino acid sequence with a length of 27 to 34 amino acids, or

-a member of the CTCK subfamily, and wherein X2 represents an amino acid sequence with a length of 2 to 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 22 to 35 amino acids, X3 represents an amino acid sequence with a length of 4 to 28 amino acids, and X4 represents an amino acid sequence with a length of 29 to 41 amino acids, or

- a member of the Noggin-like subfamily, and wherein X2 represents an amino acid sequence with a length of 4 to 6 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 22 amino acids, X3 represents an amino acid sequence with a length of 7 to 9 amino acids, and X4 represents an amino acid sequence with a length of 35 to 98 amino acids, or

- a member of the Coagulin-like subfamily, and wherein X2 represents an amino acid sequence with a length of 7 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 38 amino acids, X3 represents an amino acid sequence with a length of 5 amino acids, and X4 represents an amino acid sequence with a length of 29 amino acids, or

- a member of the Jagged-like subfamily, and wherein X2 represents an amino acid sequence with a length of 3 amino acids, X5 represents an amino acid sequence with a length of 1 amino acid, X1 represents an amino acid sequence with a length of 32 amino acids, X3 represents an amino acid sequence with a length of 25 amino acids, and X4 represents an amino acid sequence with a length of 26 amino acids.

11. Protein mimic according to claim 3, wherein said protein mimic consists of the amino acid sequence: **C1**ALRPLYIDFKRDLGWKWIHEPKGYNANF**C2**AGA**C3**NDEGLE**C4**VSQDLEPLTILYYIGKTPKIEQLSNMIVKS**C5**K**C6** (TGFB2$_{15\text{-}111/\Delta 49\text{-}77}$-VEGF$_{62\text{-}67}$).

12. Protein mimic according to any one of claims 1-11, wherein said protein mimic comprises the sequence C1-X1-C2-X2-C3-X3-C4-X4-C5-X5-C6, wherein said sequence has at least 80% sequence identity with a sequence selected from sequences 1 to 145 of Figure 10.

13. Pharmaceutical or immunogenic composition comprising a protein mimic according to any one of claims 1-12 and a pharmaceutically acceptable carrier, diluent and/or excipient.

14. Immunogenic composition according to claim 13, wherein said protein mimic is coupled to an immunogenic carrier, preferably dyphteria toxin (DT) and/or keyhole limpet haemocyanin (KLH).

15. Use of a protein mimic according to any one of claims 1-12 for the preparation of a medicament and/or prophylactic agent for the treatment and/or prevention of a disorder associated with a member of the cystine-knot growth factor superfamily.

16. Use of a protein mimic according to any one of claims 1-12 for the preparation of a medicament and/or prophylactic agent for the treatment and/or prevention of a tumor related disease and/or age-related macular degeneration (AMD), wherein said member of the cystine-knot growth factor superfamily is a member of the VEGF subfamily or the TGF-beta subfamily.

17. A method for treating or preventing a disorder associated with a member of the cystine-knot growth factor superfamily, comprising administering a therapeutically effective amount of a protein mimic according to any one of claims 1-12 to a subject suffering from, or at risk of suffering from said disorder.

18. A method according to claim 17, wherein said disorder comprises a tumor-related disease and/or age-related macular degeneration (AMD), and wherein said member of the cystine-knot growth factor superfamily is a member of the VEGF subfamily or the TGF-beta subfamily.

19. A method for producing antibodies against a member of the cystine-knot growth factor superfamily, comprising administering a protein mimic according to any one of claims 1-12 and/or an immunogenic composition according to claim 13 or 14 to a non-human animal, and obtaining antibodies against a member of the cystine-knot growth factor superfamily, which antibodies are produced by said animal.

20. A method for reducing the chance of pregnancy in a female individual, comprising administering to said female or to a sexual partner of said female an effective amount of a protein mimic according to any one of claims 1-12, an immunogenic composition according to claim 13 or 14, and/or an antibody obtainable by a method according to claim 19 or functional part or functional equivalent of said antibody, wherein said

member of the cystine-knot growth factor superfamily is a member of the GLHA or GLHB subfamily.

21. A method for binding and/or neutralizing an antibody directed to a member of the cystine-knot growth factor superfamily, comprising administering a therapeutically effective amount of a protein mimic according to any one of claims 1-12 to a subject comprising said antibody.

22. Use of a protein mimic according to any one of claims 1-12 for the manufacture of a medicament for neutralizing an antibody directed to a member of the cystine-knot growth factor superfamily.

23. The method of claim 21 or use of claim 22, wherein said antibody is bevacizumab (Avastin™) and said protein mimic is oxid-VEGF$_{26\text{-}104.}$

Figure 1

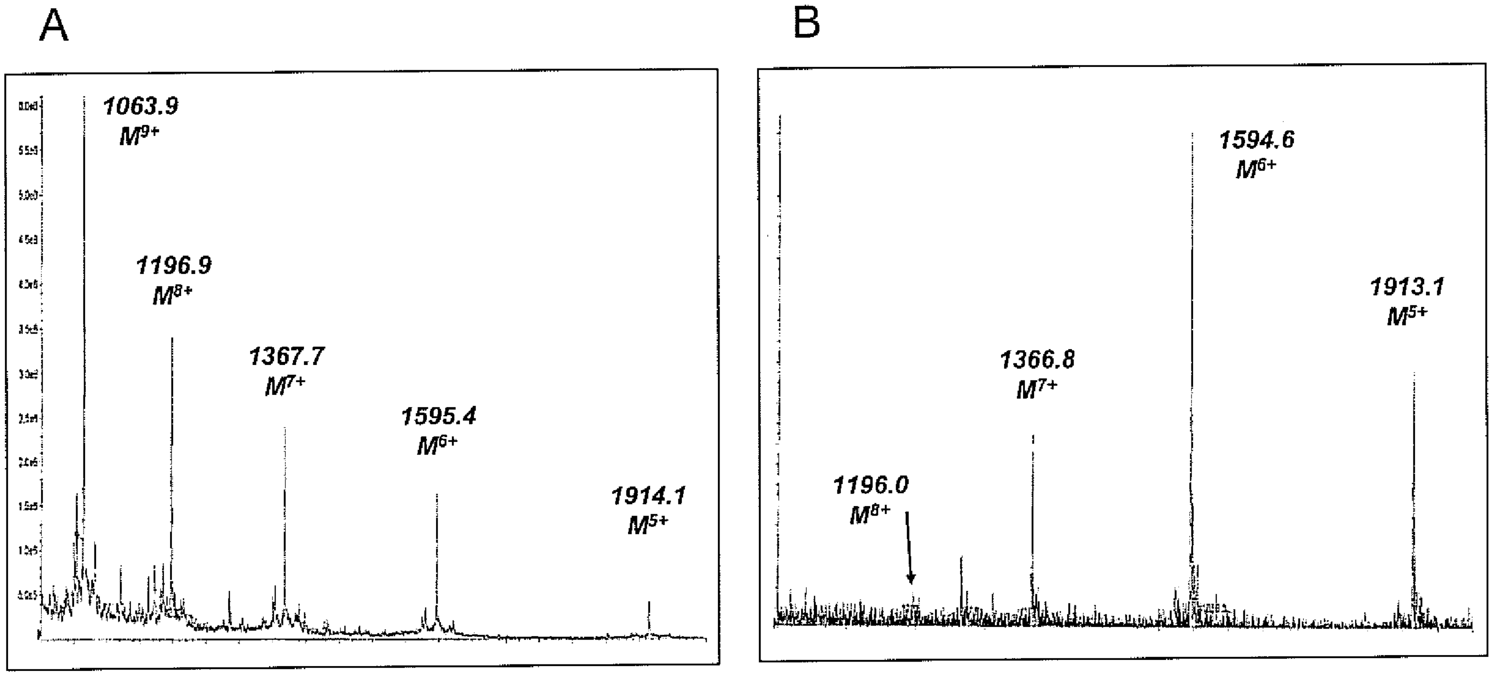

A
1063.9
M^{9+}
1196.9
M^{8+}
1367.7
M^{7+}
1595.4
M^{6+}
1914.1
M^{5+}
B
1196.0
M^{8+}
1366.8
M^{7+}
1594.6
M^{6+}
1913.1
M^{5+}

Figure 2

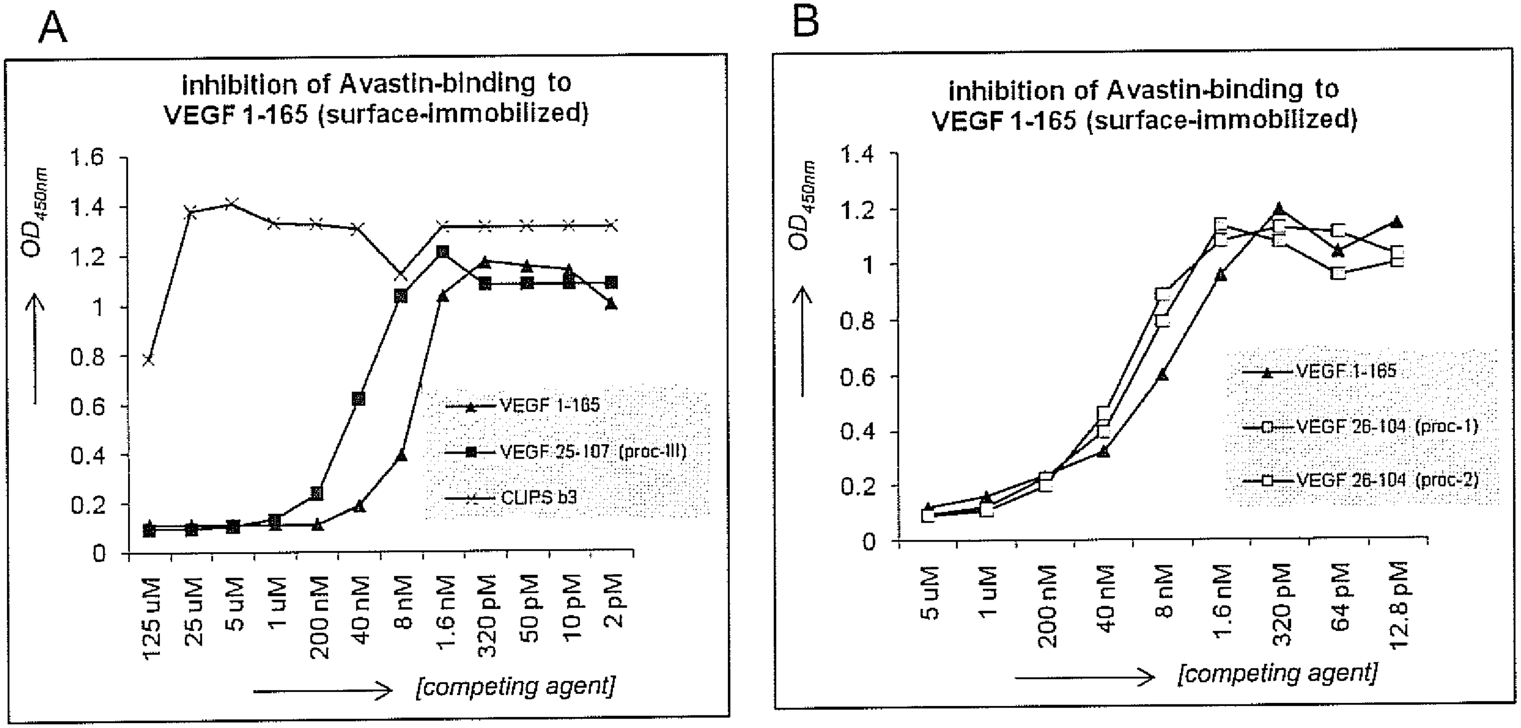

A
inhibition of Avastin-binding to VEGF 1-165 (surface-immobilized)
OD_{450nm}
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
125 uM
25 uM
5 uM
1 uM
200 nM
40 nM
8 nM
1.6 nM
320 pM
50 pM
10 pM
2 pM
[competing agent]
VEGF 1-165
VEGF 25-107 (proc-III)
CLIPS b3
B
inhibition of Avastin-binding to VEGF 1-165 (surface-immobilized)
OD_{450nm}
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5 uM
1 uM
200 nM
40 nM
8 nM
1.6 nM
320 pM
64 pM
12.8 pM
[competing agent]
VEGF 1-165
VEGF 26-104 (proc-1)
VEGF 26-104 (proc-2)

Figure 3

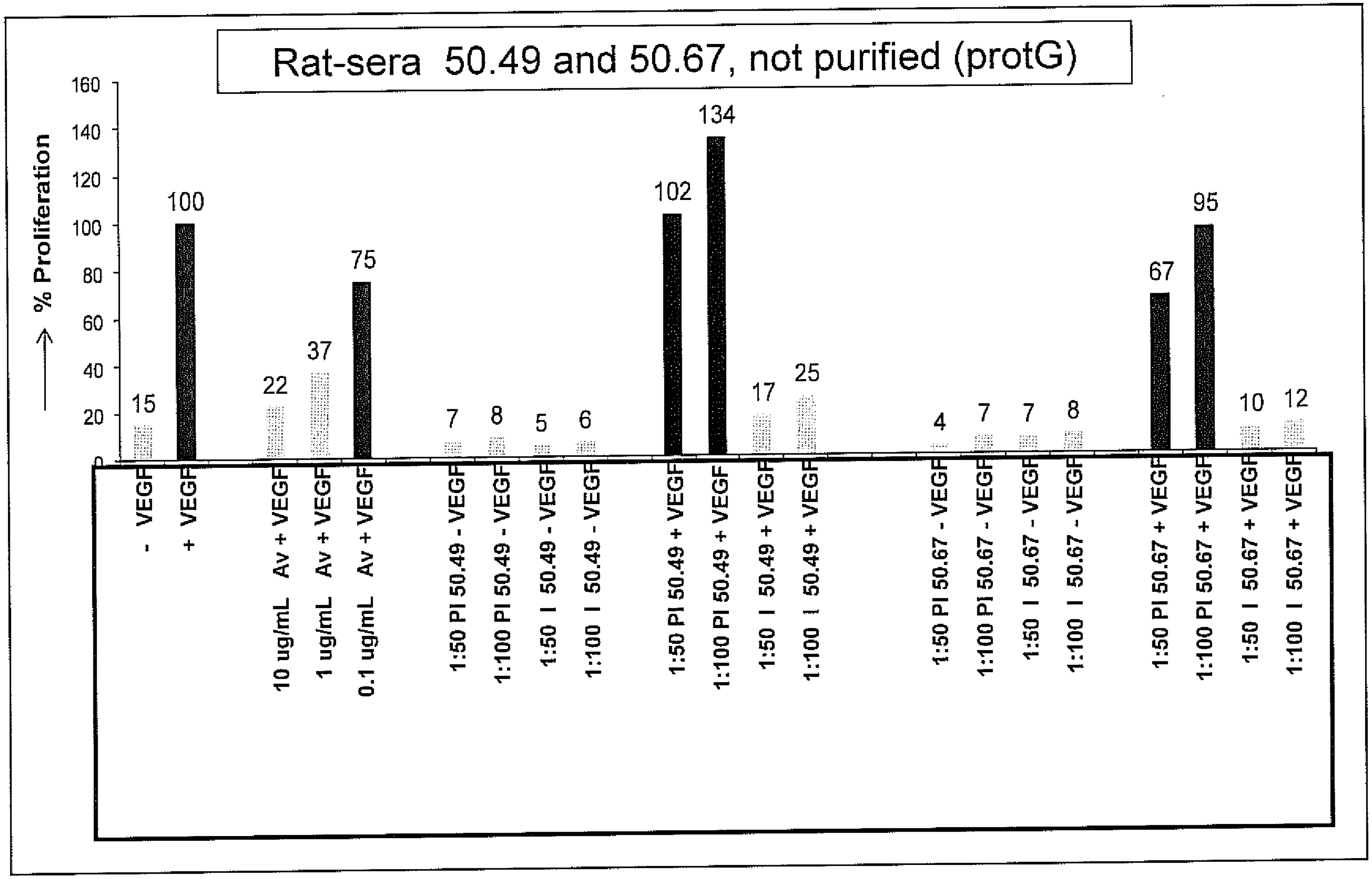

Rat-sera 50.49 and 50.67, not purified (protG)
% Proliferation
160
140
120
100
80
60
40
20
0
15
100
22
37
75
7
8
5
6
102
134
17
25
4
7
7
8
67
95
10
12
- VEGF
+ VEGF
10 ug/mL Av + VEGF
1 ug/mL Av + VEGF
0.1 ug/mL Av + VEGF
1:50 PI 50.49 - VEGF
1:100 PI 50.49 - VEGF
1:50 I 50.49 - VEGF
1:100 I 50.49 - VEGF
1:50 PI 50.49 + VEGF
1:100 PI 50.49 + VEGF
1:50 I 50.49 + VEGF
1:100 I 50.49 + VEGF
1:50 PI 50.67 - VEGF
1:100 PI 50.67 - VEGF
1:50 I 50.67 - VEGF
1:100 I 50.67 - VEGF
1:50 PI 50.67 + VEGF
1:100 PI 50.67 + VEGF
1:50 I 50.67 + VEGF
1:100 I 50.67 + VEGF

Figure 4

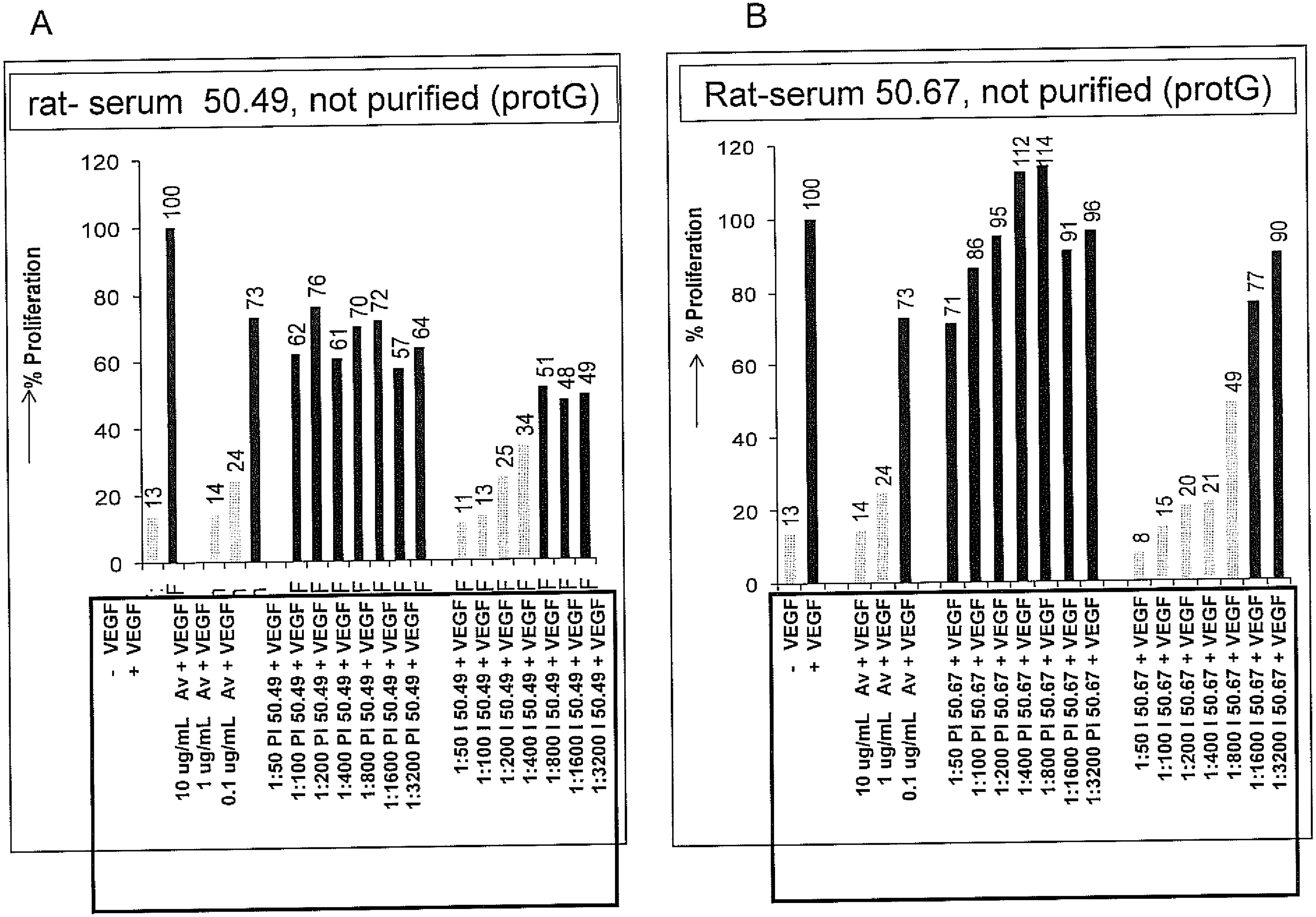

A
rat- serum 50.49, not purified (protG)
% Proliferation
0
20
40
60
80
100
120
13
100
14
24
73
62
76
61
70
72
57
64
11
13
25
34
51
48
49
- VEGF
+ VEGF
10 ug/mL Av + VEGF
1 ug/mL Av + VEGF
0.1 ug/mL Av + VEGF
1:50 PI 50.49 + VEGF
1:100 PI 50.49 + VEGF
1:200 PI 50.49 + VEGF
1:400 PI 50.49 + VEGF
1:800 PI 50.49 + VEGF
1:1600 PI 50.49 + VEGF
1:3200 PI 50.49 + VEGF
1:50 I 50.49 + VEGF
1:100 I 50.49 + VEGF
1:200 I 50.49 + VEGF
1:400 I 50.49 + VEGF
1:800 I 50.49 + VEGF
1:1600 I 50.49 + VEGF
1:3200 I 50.49 + VEGF
B
Rat-serum 50.67, not purified (protG)
% Proliferation
0
20
40
60
80
100
120
13
100
14
24
73
71
86
95
112
114
91
96
8
15
20
21
49
77
90
- VEGF
+ VEGF
10 ug/mL Av + VEGF
1 ug/mL Av + VEGF
0.1 ug/mL Av + VEGF
1:50 PI 50.67 + VEGF
1:100 PI 50.67 + VEGF
1:200 PI 50.67 + VEGF
1:400 PI 50.67 + VEGF
1:800 PI 50.67 + VEGF
1:1600 PI 50.67 + VEGF
1:3200 PI 50.67 + VEGF
1:50 I 50.67 + VEGF
1:100 I 50.67 + VEGF
1:200 I 50.67 + VEGF
1:400 I 50.67 + VEGF
1:800 I 50.67 + VEGF
1:1600 I 50.67 + VEGF
1:3200 I 50.67 + VEGF

Figure 5

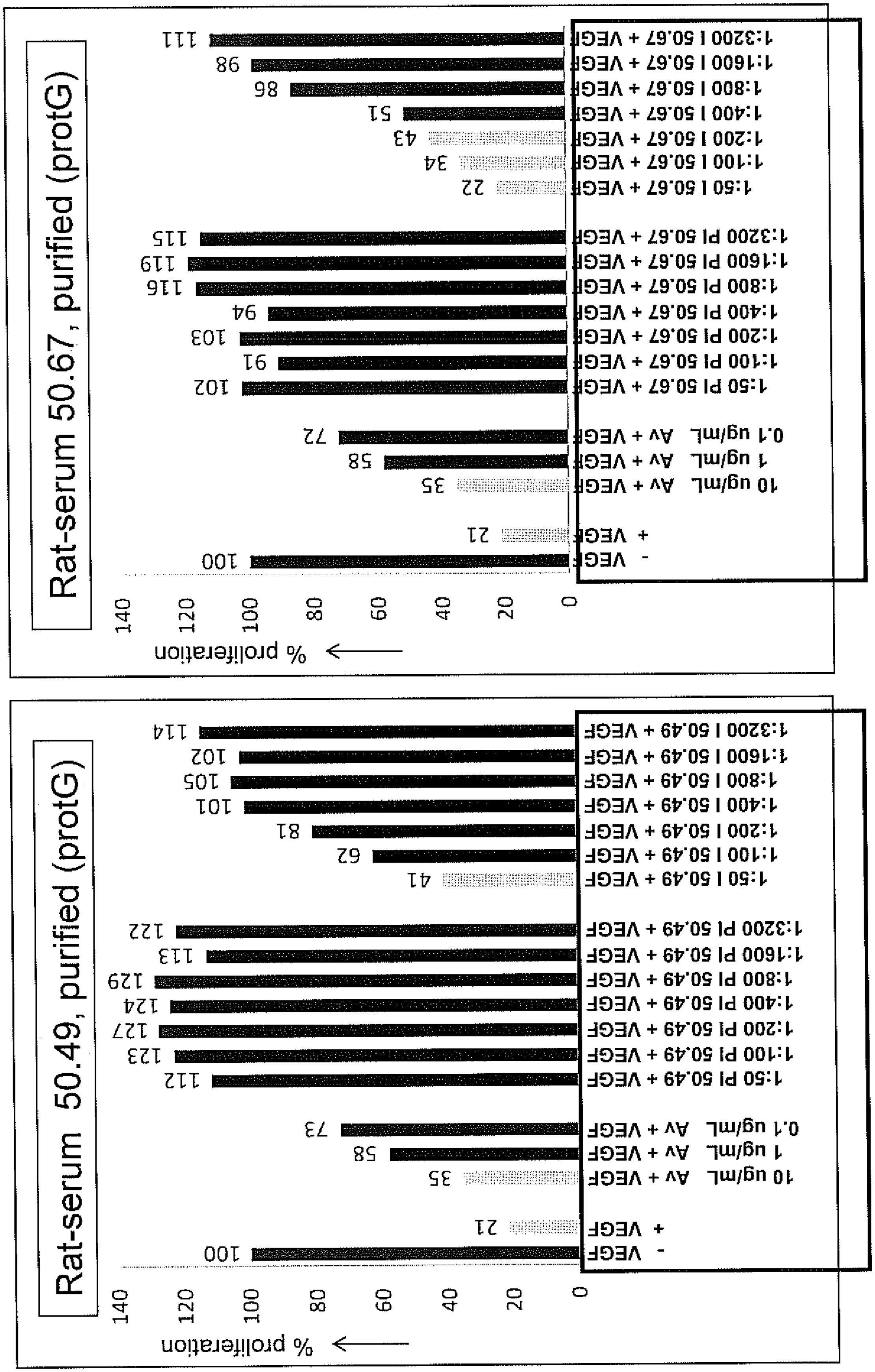

A
Rat-serum 50.49, purified (protG)
% proliferation
0
20
40
60
80
100
120
140
- VEGF
+ VEGF
10 ug/mL Av + VEGF
1 ug/mL Av + VEGF
0.1 ug/mL Av + VEGF
1:50 PI 50.49 + VEGF
1:100 PI 50.49 + VEGF
1:200 PI 50.49 + VEGF
1:400 PI 50.49 + VEGF
1:800 PI 50.49 + VEGF
1:1600 PI 50.49 + VEGF
1:3200 PI 50.49 + VEGF
1:50 I 50.49 + VEGF
1:100 I 50.49 + VEGF
1:200 I 50.49 + VEGF
1:400 I 50.49 + VEGF
1:800 I 50.49 + VEGF
1:1600 I 50.49 + VEGF
1:3200 I 50.49 + VEGF
100
21
35
58
73
112
123
127
124
129
113
122
41
62
81
101
105
102
114
B
Rat-serum 50.67, purified (protG)
% proliferation
0
20
40
60
80
100
120
140
- VEGF
+ VEGF
10 ug/mL Av + VEGF
1 ug/mL Av + VEGF
0.1 ug/mL Av + VEGF
1:50 PI 50.67 + VEGF
1:100 PI 50.67 + VEGF
1:200 PI 50.67 + VEGF
1:400 PI 50.67 + VEGF
1:800 PI 50.67 + VEGF
1:1600 PI 50.67 + VEGF
1:3200 PI 50.67 + VEGF
1:50 I 50.67 + VEGF
1:100 I 50.67 + VEGF
1:200 I 50.67 + VEGF
1:400 I 50.67 + VEGF
1:800 I 50.67 + VEGF
1:1600 I 50.67 + VEGF
1:3200 I 50.67 + VEGF
100
21
35
58
72
102
91
103
94
116
119
115
22
34
43
51
86
98
111

Figure 6

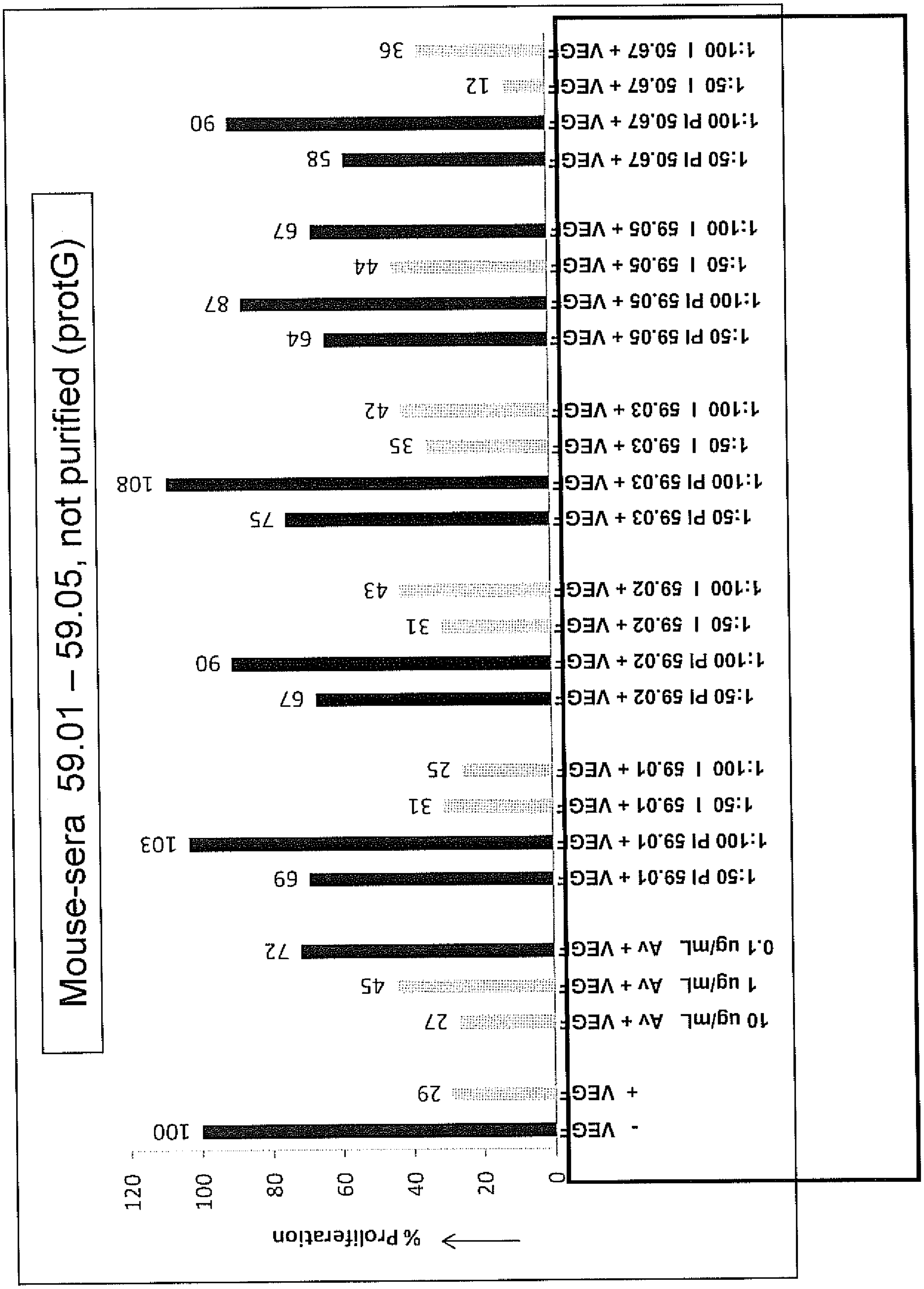
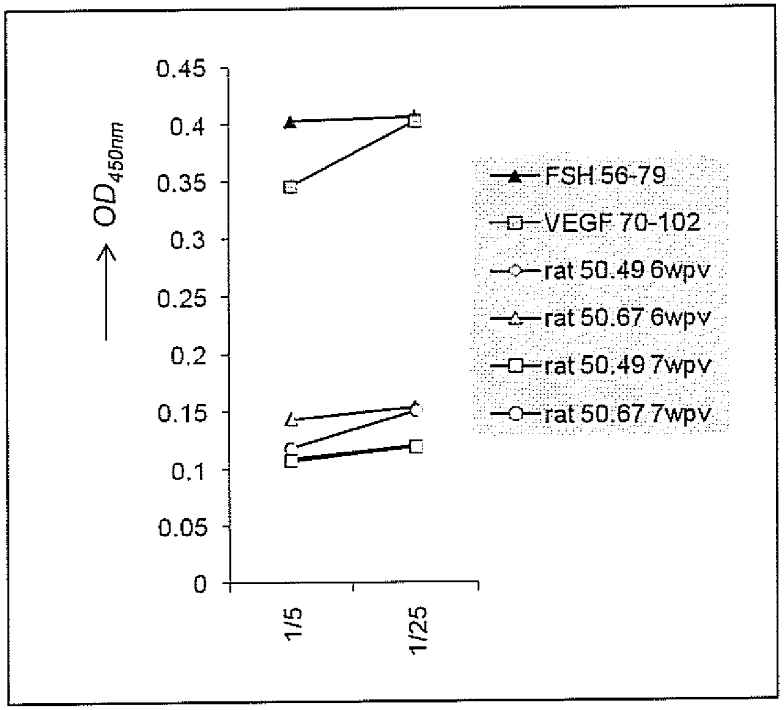

Mouse-sera 59.01 – 59.05, not purified (protG)
% Proliferation
0
20
40
60
80
100
120
- VEGF
100
+ VEGF
29
10 ug/mL Av + VEGF
27
1 ug/mL Av + VEGF
45
0.1 ug/mL Av + VEGF
72
1:50 PI 59.01 + VEGF
69
1:100 PI 59.01 + VEGF
103
1:50 I 59.01 + VEGF
31
1:100 I 59.01 + VEGF
25
1:50 PI 59.02 + VEGF
67
1:100 PI 59.02 + VEGF
90
1:50 I 59.02 + VEGF
31
1:100 I 59.02 + VEGF
43
1:50 PI 59.03 + VEGF
75
1:100 PI 59.03 + VEGF
108
1:50 I 59.03 + VEGF
35
1:100 I 59.03 + VEGF
42
1:50 PI 59.05 + VEGF
64
1:100 PI 59.05 + VEGF
87
1:50 I 59.05 + VEGF
44
1:100 I 59.05 + VEGF
67
1:50 PI 50.67 + VEGF
58
1:100 PI 50.67 + VEGF
90
1:50 I 50.67 + VEGF
12
1:100 I 50.67 + VEGF
36

Figure 7



◇ ○ □ △ antisera elicited with new protein mimic **VEGF**$_{26-104}$

▨ antiserum elicited with **CLIPS** peptide derived from VEGF β5-turn-β6-loop (70-102)

▲ antiserum elicited with **CLIPS** peptide derived from FSH β3-loop (56-79)

Figure 8

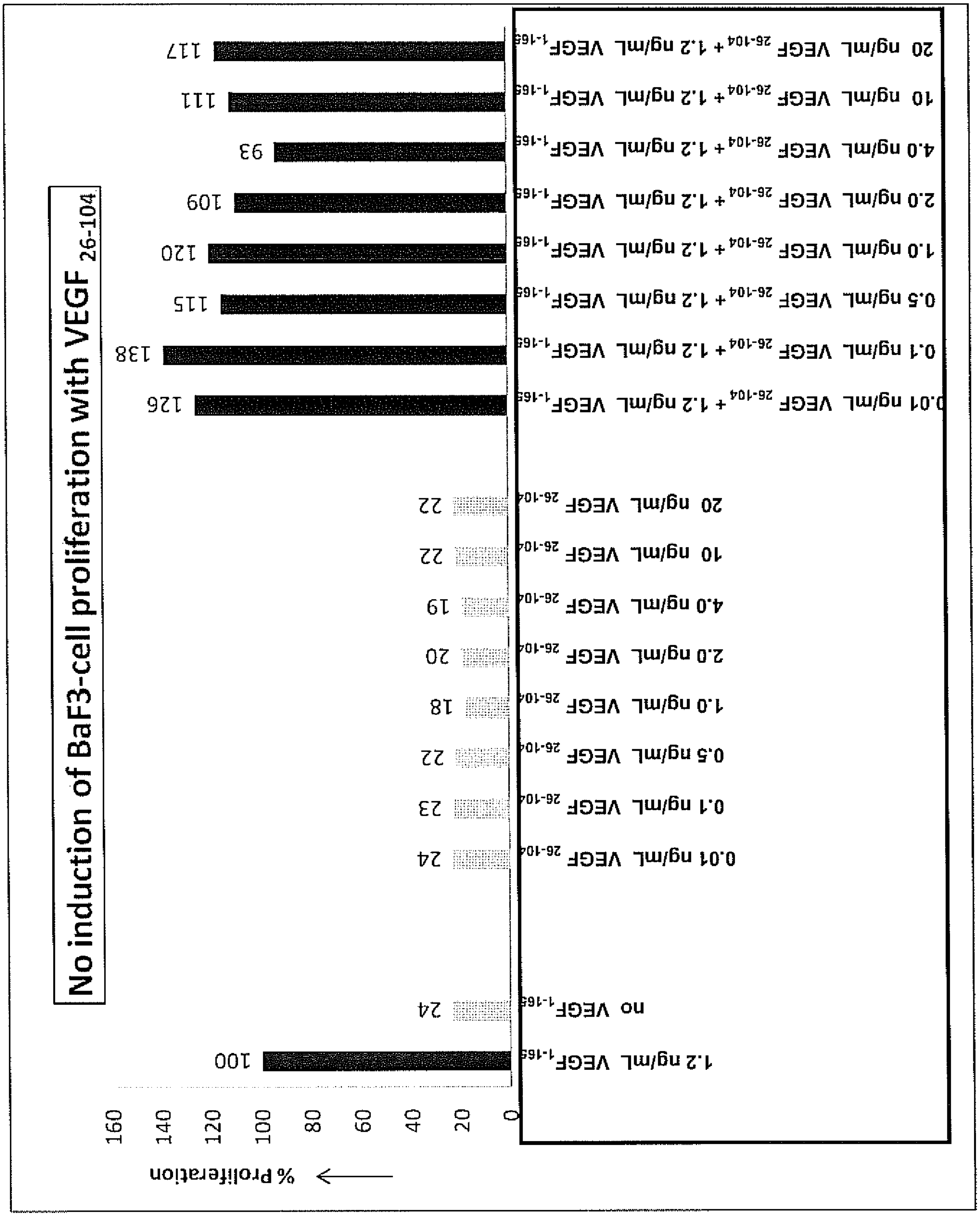

No induction of BaF3-cell proliferation with VEGF $_{26-104}$
% Proliferation
0
20
40
60
80
100
120
140
160
1.2 ng/mL VEGF$^{1-165}$
100
no VEGF$^{1-165}$
24
0.01 ng/mL VEGF $^{26-104}$
24
0.1 ng/mL VEGF $^{26-104}$
23
0.5 ng/mL VEGF $^{26-104}$
22
1.0 ng/mL VEGF $^{26-104}$
18
2.0 ng/mL VEGF $^{26-104}$
20
4.0 ng/mL VEGF $^{26-104}$
19
10 ng/mL VEGF $^{26-104}$
22
20 ng/mL VEGF $^{26-104}$
22
0.01 ng/mL VEGF $^{26-104}$ + 1.2 ng/mL VEGF$^{1-165}$
126
0.1 ng/mL VEGF $^{26-104}$ + 1.2 ng/mL VEGF$^{1-165}$
138
0.5 ng/mL VEGF $^{26-104}$ + 1.2 ng/mL VEGF$^{1-165}$
115
1.0 ng/mL VEGF $^{26-104}$ + 1.2 ng/mL VEGF$^{1-165}$
120
2.0 ng/mL VEGF $^{26-104}$ + 1.2 ng/mL VEGF$^{1-165}$
109
4.0 ng/mL VEGF $^{26-104}$ + 1.2 ng/mL VEGF$^{1-165}$
93
10 ng/mL VEGF $^{26-104}$ + 1.2 ng/mL VEGF$^{1-165}$
111
20 ng/mL VEGF $^{26-104}$ + 1.2 ng/mL VEGF$^{1-165}$
117

Figure 9

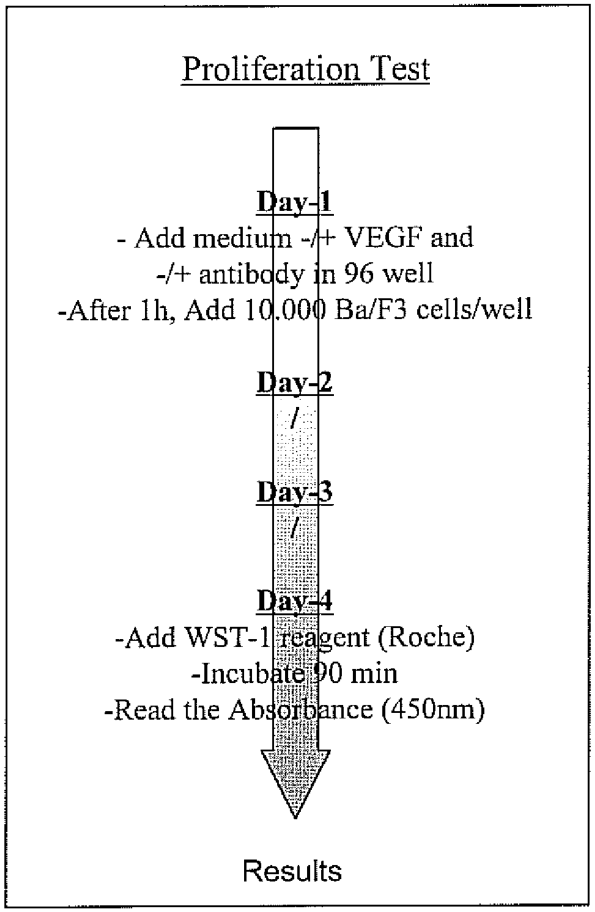

Proliferation Test
Day-1
- Add medium -/+ VEGF and
-/+ antibody in 96 well
-After 1h, Add 10.000 Ba/F3 cells/well
Day-2
/
Day-3
/
Day-4
-Add WST-1 reagent (Roche)
-Incubate 90 min
-Read the Absorbance (450nm)
Results

Figure 10 (Overview)

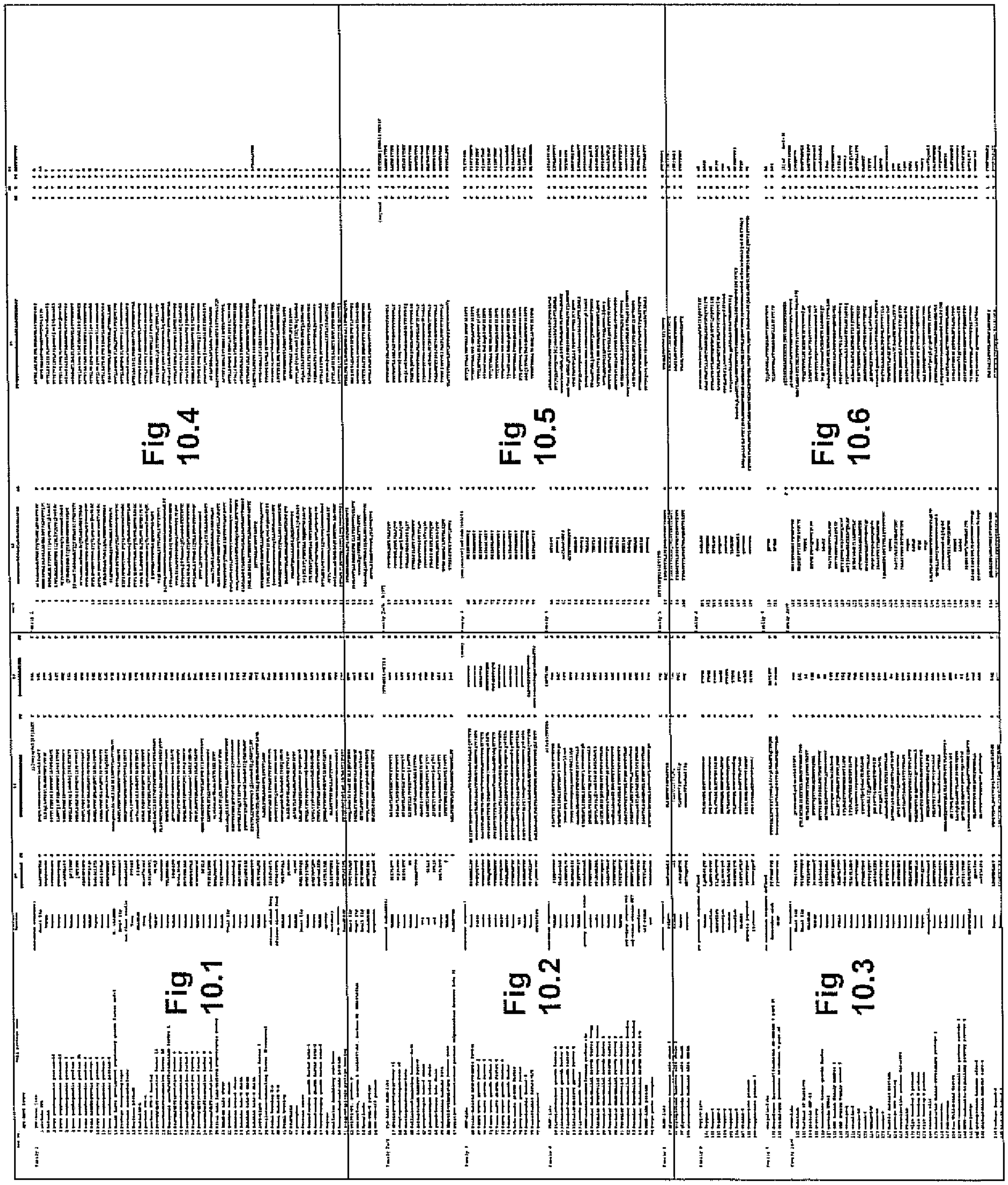


Figure 10.1

Seq Nr.	Full protein name	Species	X0	C1	X1	C2	X2	C3
	cys-knot trunc		XXXXXXXXXX	C1	XXXXXXXXXXXXXXXXXXXXXXXXX	C2	XXXXXXXXXXXXXX	C3
Family 1	TGF-beta like	consensus sequence:			[LIVH]XXFXX[FY]XXXX	C	XGX	C
1	protein 60A	fruit fly	LLEPMESTRS	C	QHQTLYIDFKDLGWHDWIIAPEGYGAFY	C	SGE	C
2	Artemin	human	SRARAAGARG	C	RLRSQLVPVRALGLGHRSDELVRFRF	C	SGS	C
3	bone morphogenetic protein-10	human	RIRRNAKGNY	C	KRTPLYIDFKEIGWDSWIIAPPGYEAYE	C	RGV	C
4	bone morphogenetic protein-15	human	KHSGPEENNQ	C	SLHPFQISFRQLGWDHWIIAPPFYTPNY	C	KGT	C
5	bone morphogenetic protein 2	human	HKQRKRLKSS	C	KRHPLYVDFSDVGWNDWIVAPPGYHAFY	C	HGE	C
6	bone morphogenetic protein 3	human	QWIEPRN	C	ARRYLKVDFADIGWSEWIISPKSFDAYY	C	SGA	C
7	bone morphogenetic protein 3b	human	QWDEPRV	C	SRRYLKVDFADIGWNEWIISPKSFDAYY	C	AGA	C
8	bone morphogenetic protein 4	human	SQRARKKNKN	C	RRHSLYVDFSDVGWNDWIVAPPGYQAFY	C	HGD	C
9	bone morphoganetic protein 5	human	DYNTSEQKQA	C	KKHELYVSFRDLGWQDWIIAPEGYAAFY	C	DGE	C
10	bone morphogenetic protein 6	human	DYNSSELKTA	C	RKHELYVSFQDLGWQDWIIAPKGYAANY	C	DGE	C
11	bone morphogenetic protein 7	human	AENSSSDRQA	C	KKHELYVSFRDLGWQDWIIAPEGYAAYY	C	EGE	C
12	bone morphogenetic protein-8	human	DVRGSHGRQV	C	RRHELYVSFQDLGWLDWVIAPQGYSAYY	C	EGE	C
13	Dauer larva development regulatory growth factor daf-7	C. elegans	SHAKPV	C	NAEAQSKGCCLYDLEIEFEKIGWDWIVAPRYNAYM	C	RGD	C
14	Protein decapentaplegic	fruit fly	PTRRKNHDDT	C	RRHSLYVDFSDVGWDDWIVAPLGYDAYY	C	HGK	C
15	Protein decapentaplegic	red flour beatle	RRHRKNLKDP	C	RRRQMYVDFGSVGWNDWIVAPLGYDAYY	C	GGE	C
16	Derriere protein	frog	PTPSNI	C	KKRRLYIDFKDVGWQNWVIAPRGYMANY	C	HGE	C
17	Dorsalin-1	chicken	SIGANH	C	RRTSLHVNFKEIGWDSWIIAPKDYEAFE	C	KGG	C
18	protein DVR-1	frog	SKLPFTASNI	C	KKRHLYVEFKDVGWQNWVIAPQGYMANY	C	YGE	C
19	protein DVR-1 homolog	urchin	SASSLNSDWQ	C	KRKHLFVNFEDLDWQEWIIAPLGYVAFY	C	QGE	C
20	Growth/differentation factor 11	human	NLGLD	C	DEHSSESRCCRYPLTVDFEAFGWDWIIAPKRYKANY	C	SGQ	C
21	Growth/differentation factor 15	human	ARARNGDH	C	PLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTM	C	IGA	C
22	Embryonic growth/differentation factor 1	human	PVLGGGPGGA	C	RARRLYVSFREVGWHRWVIAPRGFLANY	C	QGQ	C
23	Growth/differentation factor 2	human	SAGAGSH	C	QKTSLRVNFEDIGWDSWIIAPKEYEAYE	C	KGG	C
24	Growth/differentation factor 3	human	PVPKLSCKNL	C	HRHQLFINFRDLGWHKWIIAPKGFMANY	C	HGE	C
25	Growth/differentation factor 5	human	KRPSKNLKAR	C	SRKALHVNFKEMGWDDWIIAPLEYEAFH	C	EGL	C
26	Growth/differentation factor 6	human	KRHGKKSRLR	C	SKKPLHVNFKELGWDDWIIAPLEYEAYH	C	EGV	C
27	Growth/differentation factor 7	human	RGHGRRGRSR	C	SRKPLHVDFKELGWDDWIIAPLDYEAYH	C	EGL	C
28	Growth/differentation factor 8	human	DFGLD	C	DEHSTESRCCRYPLTVDFEAFGWDWIIAPKRYKANY	C	SGE	C
29	Growth/differentation factor 9	human	FRQFLLPQHE	C	ELHDFRLSFSQLKWDNWIVAPHRYNPRY	C	KGD	C
30	Glial cell line-derived neurotrophic factor	human	RGGQRGKNRG	C	VLTAIHLNVTDLGLGYETKEELIFRY	C	SGS	C
31	Inhibin alfa chain	human	PPEEPAAHAN	C	HRVALNISFQELGWERWIVYPPSFIFHY	C	HGG	C
32	Inhibin beta chain	fruit fly	TRRVRRRAVD	C	GGALNGQCCKESFYVSFKALGWDDWIIAPRGYFANY	C	RGD	C
33	Inhibin beta-A chain	human	PHRRRRRGLE	C	DGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANY	C	EGE	C
34	Inhibin beta-B chain	human	RHRIRKRGLE	C	DGRTNLCCRQQFFIDFRLIGWNDWIIAPTGYYGNY	C	EGS	C
35	Inhibin beta-C chain	human	KHQIHRRGID	C	QGGSRMCCRQEFFVDFREIGWHDWIIQPEGYAMNF	C	IGQ	C
36	Inhibin beta-E chain	human	AGRARRRTPT	C	EPATPLCCRRDHYVDFQELGWRDWILQPEGYQLNY	C	SGQ	C
37	left-right determination factor 2	human	DLRDYGAQGD	C	DPEAPMTEGTRCCRQEMYIDLQGMKWAKNWVLEPPGFLAYE	C	VGT	C
38	Muellerian-inhibiting factor [Precursor]	human	SAGATAADGP	C	ALRELSVDLRAERSVLIPETYQANN	C	QGV	C
39	Nodal homolog 2-A	African clawed frog	IPSRSVGKTL	C	RRVDMIVDFEKIEWGDRIVYPKRFNAYR	C	EGA	C
40	Nodal homolog 4-A	African clawed frog	VPPADSSRTL	C	RRVDFFVDFKQIGWDSWIIHPMKYNAYR	C	EGE	C
41	Nodal homolog	human	HHLPDRSQL	C	RKVKFQVDFNLIGWGSWIIYPKQYNAYR	C	EGE	C
42	neurturin	human	RLGARP	C	GLRELEVRVSELGLGYASDETVLFRY	C	AGA	C
43	persephin	human	ALSGP	C	QLWSLTLSVAELGLGYASEEKVIFRY	C	AGS	C
44	Protein screw	fruit fly	PVDLYRPPQS	C	ERLHFTVDFKELHMHNWVIAPKKFEAYF	C	GGG	C
45	transforming growth factor beta-1	human	HYCFSSTEKN	C	CVRQLYIDFRKDLGWKWIHEPKGYHANF	C	LGP	C
46	transforming growth factor beta-2	human	AYCFRNVQDN	C	CLRPLYIDFKRDLGWKWIHEPKGYNANF	C	AGA	C
47	transforming growth factor beta-3	human	HYCFRNLEEN	C	CVRPLYIDFRQDLGWKWVHEPKGYYANF	C	SGP	C
48	Univin	urchin	SFPTASLTHL	C	QRHRLFVSFRDVGWENWIIAPMGYQAYY	C	DGE	C
49	Mullerian inhibitory substance	hatcheri	GLDSPSGSNI	C	GLRSLTVSFEKLLLGPQTANINN	C	QGS	C
50	Decapentaplegic-like protein	sea anemone	GGAKRRRPQY	C	RRHPLYVDFTDVGWNDWIVAPPGYHAFY	C	TGV	C
51	decapentaplegic protein homolog	roundworm	HHNTEAESNL	C	RRTDFYVDFDDLNWQDWIMAPKGYDAYQ	C	QGS	C

Figure 10.2

	52 CG1901-PB, isoform B	fruit fly	TNNCYKLHQR	C	CRNQLDVAFKSIKGFEFILPQKVFDAGY	C	HGR	C
	53 CG16987-PA, isoform A (CG16987-PB, isoform B) (GH144433p)	fruit fly	SINCSSGMTE	C	CREHLYISFRDIGWSNWILKPEGYNAYF	C	RGS	C
	54 Myoglianin	fruit fly	DCTENDHDMR	C	CRYPLKVNFTSFGWHFVVAPTSFDAYF	C	SGD	C
	55 CBR-DBL-1 protein	roundworm	NHNTEAESNL	C	RRTDLYVDFDDLGWQDWIMAPKGYDAYQ	C	QGS	C
	56 CBR-UNC-129 protein	roundworm	RVVLLQNKNR	C	HKEGTLVSLKHFGWDKFVMEPRTIETSF	C	KGK	C
Family 2a/b	Cys-knot: GLHB-like	consensus sequences:				C	[STAGM]G[HFYL]	C
	57 choriogonadotropin-beta v1	human	SKEPLRPR	C	RPINATLAVEKEGCPVCITVNTTI	C	AGY	C
	58 choriogonadotropin-beta v2	human	SKEPLRPR	C	RPINATLAVEKEGCPVCITVNTTI	C	AGY	C
	59 choriogonadotropin-beta	human	SKEPLRPR	C	RPINATLAVEKEGCPVCITVNTTI	C	AGY	C
	60 follicle-stimulating hormone-beta	human	NS	C	ELTNITIAIEKEECRFCISINTTW	C	AGY	C
	61 glycoprotein hormone beta-5	human	SSGNLRTFVG	C	AVREFTFLAKKPGCRGLRITTDA	C	WGR	C
	62 gonadotropin beta-1 chain	eel		C	GLANISISVENEECGGCITFNTTA	C	AGL	C
	63 gonadotropin beta-2 chain	eel	SLLLP	C	EPINETISVEKDGCPKCLVFQTSI	C	SGH	C
	64 gonadotropin beta chain	eel	SVLQP	C	QPINETISVEKDGCPKCLVFQTSI	C	SGH	C
	65 luteinizing hormone beta	human	SREPLRPW	C	HPINAILAVEKEGCPVCITVNTTI	C	AGY	C
	66 thyroid-stimulating hormone beta	human	F	C	IPTEYTMHIERRECAYCLTINTTI	C	AGY	C
	67 putative uncharacterized protein (Glycoprotein hormone beta 5)	roundworm		C	HRLVPGFNPLRQVDANGKECRGNVELPF	C	KGY	C
Family 3	NGF-like	consensus sequence:					[GSRE]	C
	68 Brain-derived neurotrophic factor	human	DPARRGELSV	C	DSISEWVTAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETK	C	NPMGYTKEG	C
	69 Venom nerve growth factor 1	viper	PVHNQGEYSV	C	DSVSVWVANKTTATDIRGNLVTVMVDINLNNNVYKQYFFETK	C	RNPNPVPSG	C
	70 Venom nerve growth factor 2	snake	PVNDHGEYSV	C	DSVSVWVNKTTATDIKGKPVTVMVDVNLNNHVYKQYFFETK	C	RNPNPVPSG	C
	71 Venom nerve growth factor 3	snake	PVHNQGEHSV	C	DSVSDWVIKTTATDIRGNMVTVMVDINRDNEVYKQYFFETK	C	RNPNPNPVQSE	C
	72 Venom nerve growth factor 4	snake	PVHNQGEHSV	C	DSVSDWVIKTTATDIRGNVVTVMEDINLNNEVYKQYFFETK	C	RNPNPNPVQSE	C
	73 Venom nerve growth factor 5	snake	PVYNRGEHSV	C	DSVSVWVTNKTKATDIKGNMVTVMVDINLNNEVYKQYFFETK	C	RNPNPVPSG	C
	74 Venom nerve growth factor	snake	PVHNLGEHSV	C	DSISVWVTNKTKATDIKDNMVTVMVDINLNNEVYKQYFFETK	C	RNPNPVPSG	C
	75 Beta-nerve growth factor	human	PIFHRGEFSV	C	DSVSVWVGDKTTATDIKGKEVMVLGEVNINNSVFKQYFFETK	C	RDPNPVDSG	C
	76 Beta-nerve growth factor	rat	PVFHMGEFSV	C	DSVSVWVGDKTTATDIKGKEVTVLGEVNINNSVFKQYFFETK	C	RAPNPVESG	C
	77 Neurotrophic factor-3	human	HKSHRGEYSV	C	DSESLWVTDKSSAIDIRGHQVTVLGEIKTGNSPVKQYFYETR	C	KEARPVKNG	C
	78 Neurotrophic factor-4/5	human	PASRRGELAV	C	DAVSGWVTDRRTAVDLRGREVEVLGEVPAAGGSPLRQYFFETR	C	KADNAEEGGPGAGGGG	C
	79 Neurotrophin-7	zebrafish	DFLHRGEYSV	C	DSEEHWVGNLTHATDLGGNEVMVLPHFRINNVVKKQLFYETT	C	RVKKPIGAPKPGQGASGVKAGTSS	C
Family 4	PDGF-like	consensus sequence:			P[PSR]CVXXXR	C	[GSTA]GC	C
	80 Plateled-derived growth factor A	human	SIEEAVPAV	C	KTRTVIYEIPRSQVDPTSANFLIWPPCVEVKR	C	TGC	C
	81 Plateled-derived growth factor B	human	TIAEPAMIAE	C	KTRTEVFEISRRLIDRTNANFLVWPPCVEVQR	C	SGC	C
	82 Plateled-derived growth factor C	human	LLTEEVRLYS	C	TPRNFSVSIREELKRTDTIFWPGCLLVKR	C	GGN	C
	83 Plateled-derived growth factor D	human	RLNDDAKRYS	C	TPRNYSVNIREELKLANVVFFPRCLLVQR	C	GGN	C
	84 Placenta growth factor	human	PFQEVWGRSY	C	RALERLVDVVSEYPSEVEHMFSPSCVSLLR	C	TGC	C
	85 PDGF-related-transforming protein sis	monkey sarcoma virus	SVAEPAMIAE	C	KTRTEVFEISRRLIDRTNANFLVWPPCVEVQR	C	SGC	C
	86 Vascular Endothelial Growth Factor toxin	snake	PFMEVYRHSV	C	QPRETLVSILEEYPGEIAHIFRPSCVTALR	C	GGC	C
	87 Vascular Endothelial Growth Factor toxin	snake	PFLEVHERSA	C	QARETLVSILQEYPDEISDIFRPSCVAVLR	C	SGC	C
	88 Vascular Endothelial Growth Factor A	human	KFMDVYQRSY	C	HPIETLVDIFQEYPDEIEYIFKPSCVPLMR	C	GGA	C
	89 Vascular Endothelial Growth Factor B	human	SWIDVYTRAT	C	QPREVVVPLTVELMGTVAKQLVPSCVTVQR	C	GGC	C
	90 Vascular Endothelial Growth Factor C	human	SIDNEWRKTQ	C	MPREVAIDVGKEFGVATNTFFKPPCVSVYR	C	GGC	C
	91 Vascular Endothelial Growth Factor D	human	VIDEEWQRTQ	C	SPRETAVEVASELGKSTNTFFKPPCVNVFR	C	GGC	C
	92 Vascular Endothelial Growth Factor homolog	orf-virus strain NZ2	GNSEVLKGSE	C	KPRPIVVPVSETHPELTSQRFNPPCVTLMR	C	GGC	C
	93 Vascular Endothelial Growth Factor homolog	orf-virus strain NZ7	DWMRTLDKSG	C	KPRDTVVYLGEEYPESTNLQYNPRCVTVKR	C	SGC	C
	94 Vascular Endothelial Growth Factor A-A	zebrafish	PFMDVYKKSA	C	KTRELLVDIIQEYPDEIEHTYIPSCVVLMR	C	AGC	C
	95 VEGF-like protein	orf-virus	KWPEVLEGSK	C	KPRPTVLSVNGEHPELTSQRFNPPCVTLMR	C	GGC	C
	96 C-sis proto oncogene	cat	TVAEPAMIAE	C	KTRTEVFEVSRRLIDRTNANFLVWPPCVEVQR	C	SGC	C
Family 5	GLHA-like	consensus sequence:				C	XGC	C
	97 glycoprotein hormones alfa chain 1	salmon	SDHTNVGCEE	C	KLKENKVFSNPGAPVYQ	C	TGC	C

Figure 10.3

	98 glycoprotein hormones alfa chain 2	salmon	CEE	C	TLKPNTIFPNIMQ	C	TGC	C
	99 glycoprotein hormones alfa chain	human	APDVQDCPE	C	TLQENPFFSQPGAPILQ	C	MGC	C
	100 glycoprotein hormones alfa chain	macaque	GEFTMQDCPE	C	KPRENKFFSKPGAPIYQ	C	MGC	C
Family 6	Noggin-like	no consensus sequence defined						
	101 Noggin	human	LQMWLWSQTF	C	FVLYAWNDLGSRFWPRYVKVGS	C	FSKRS	C
	102 Noggin-1	zebrafish	LQMWLWSYSF	C	PVLYAWNDLGSRFWPRFVRAGS	C	YTKRS	C
	103 Noggin-2	zebrafish	FLQWLWMYTH	C	PVLYTWKDLGLRFWPRYIKEGN	C	FSERS	C
	104 Noggin-3	zebrafish	LQLWLWSYTF	C	PVVHTWQDLGNRFWPRYLKVGS	C	YNKRS	C
	105 Noggin-4	chicken	LRRWLVERAS	C	RLTSAWVDLGPVFWPRWVRHTA	C	RTGPPA	C
	106 Noggin-5	zebrafish	MRRWMWSYTR	C	PVLSMWKDLGVRFWPRYVKEGQ	C	STERS	C
	107 Noggin-I	sponge	AIRRTLNTLN	C	RVTYNWADAGVNFFPRYFSAGS	C	FERR	C
	108 Noggin-like protein	dugesia japonica	IRRWHVQQAA	C	KIDYLWKRLDDTHWPSFIKHGV	C	NSRES	C
	109 Noggin-like protein 1	flatworm	IRRWHVQQAT	C	KTDYIWKRLDETHWPSWIKHGI	C	SSTEP	C
Family 7	Coagulin-like	no consensus sequence defined						
	110 Coagulogen [precursor] (contains AB-chains + pept.C)	horseshoe crab	FFPFHKFPSE	C	PVSVSACEPTFGYTTSNELRIIVQAPKAGFRQCVWQHK	C	RAYGSNF	C
	111 Coagulogen (contains AB-chains + pept.C)	crab	FPPFVHFTSE	C	PVSTRDCEPVFGYTVAGEFRVIVQAPRAGFRQCVWQHK	C	R-YGSNN	C
Family 2c/8	CTCK-like	consensus sequence:						
	112 Bursicon	fruit fly	TDNDITHLGD	C	QVTEVIHVLQYPGCVPKPIPSFA	C	VGR	C
	113 Partner of bursicon	fruit fly	RYSQGTGDEN	C	ETLKSEIHLIKEEFDELGRMQRTCNADVIVNK	C	EGL	C
	114 protein CEF-10	chicken	SYASLKKGKK	C	TKTKKSPSPVRFTYAGCSSVKKYRPKY	C	GS	C
	115 Cerberus	human	IKSHEVHWET	C	RTVPFSQTITHEGCEKVVVQNNL	C	FGK	C
	116 Connective tissue growth factor	human	LEENIKKGKK	C	IRTPKISKPIKFELSGCTSMKTYRAKF	C	GV	C
	117 protein CYR61	human	VYSSLKKGKK	C	SKTKKSPEPVRFTYAGCLSVKKYRPKY	C	GS	C
	118 DAN domain family member 5	human	LNPQEVIQGM	C	KAVPFVQVFSRPGCSAIRLRNHL	C	FGH	C
	119 DAN domain family member 5	xenla	IGQDALKRSR	C	HALPFIQNVFRKNCFPVRLPNKF	C	FGQ	C
	120 Gremlin-1	human	TERKYLKRDW	C	KTQPLKQTIHEEGCNSRTIINRF	C	YGQ	C
	121 Gremlin-2	human	TERKYLKRDW	C	KTQPLRQTVSEEGCRSRTILNRF	C	YGQ	C
	122 mucin-19	human	KKCCYTCKNN	C	RSSLVNVTVIYSGCKKRVQMAK	C	TGE	C
	123 mucin-2	human	CTPRNETRVP	C	STVPVTTEVSYAGCTKTVLMNH	C	SGS	C
	124 mucin-5AC	human	PPPPYQNQST	C	AVYHRSLIIQQQGCSSSEPVRLAY	C	RGH	C
	125 mucin-5B	human	GCCYSCEEDS	C	QVRINTTILWHQGCETEVNITF	C	EGS	C
	126 mucin-6	human	GTPTPTSPGV	C	SVREQQEEITFKGCMANVTVTR	C	EGA	C
	127 Norrie disease protein	human	SFIMDSDPRR	C	MRHHYVDSISHPLYKCSSKMVLLAR	C	EGH	C
	128 protein NOV homolog	human	EQPTDKKGKK	C	LRTKKSLKAIHLQFKNCTSLHTYKPRF	C	GV	C
	129 Otogelin-like protein C12orf64	human	CKICKREERI	C	QKVIIKSVIRKQDCMSQSPINVAS	C	DGK	C
	130 Otogelin	human	CRTCKEDGRS	C	KKVTIRMTIRKNECRSSTPVNLVS	C	DGR	C
	131 Slit homolog 1 protein	human	SGELCEQESE	C	RGDPVRDFHQVQRGYAICQTTRPLSWVE	C	RGS	C
	132 Slit homolog 2 protein	human	TGDSCDREIS	C	RGERIRDYYQKQQGYAACQTTKKVSRLE	C	RGG	C
	133 Slit homolog 3 protein	human	SGEHCQQENP	C	LGQVVREVIRRQKGYASCATASKVPIME	C	RGG	C
	134 Protein slit	drosophila	EPPTVTAAST	C	RKEQVREYYTENDCRSRQPLKYAK	C	VGG	C
	135 sclerostin domain-containing protein 1	human	LDRNTRVQVG	C	RELRSTKYISDGQCTSISPLKELV	C	AGE	C
	136 sclerostin	human	FETKDVSEYS	C	RELHFTRYVTDGPCRSAKPVTELV	C	AGE	C
	137 SCO-spondin	human	VLEPGSCCPS	C	RREAPEEQSPSCQLLTELRNFTKGTCYLDQVEVSY	C	SGY	C
	138 von Willebrand factor	human	TCCDTCEEPE	C	NDITARLQYVKVGSCKSEVEVDIHY	C	QGK	C
	139 WNT1-inducible-signalling pathway protein 1	human	IHTLIKAGKK	C	LAVYQPEASMNFTLAGCISTRSYQPKY	C	GV	C
	140 WNT1-inducible-signalling pathway protein 3	human	LKTIKIPKGK	C	QPTFQLSKAEKFVFSGCSSTQSYKPTF	C	GI	C
	141 Hemolectin	drosophila	SEPLVEDKSS	C	LPVSLAESRTKEILKFPVQGHGTCVNADPIQGFTD	C	EGA	C
	142 glycoprotein hormone alfa-2	human	QEAVIPG	C	HLHPFNVTVRSDRQGTCQGSHVAQA	C	VGH	C
	143 glycoprotein hormone alfa-2	mouse	HSPETAIPG	C	HLHPFNVTVRSDRLGTCQGSHVAQA	C	VGH	C
	144 Protein jagged-1	human	LDANECEAKP	C	VNAKSCKNLIASYYCDCLPGWNGQNCDININD	C	LGQ	C
	145 Protein jagged-2	human	LDANECEGKP	C	LNAFSCKNLIGGYYCDCIPGWKGINCHINVND	C	RGQ	C

Figure 10.4

Family 1

Seq Nr.	X3	C4	X4	C5	X5	C6	X6
	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX		XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX		X		XXXXXXXXXX
1	NFPLNAHMNATNHAIVQTLVHLLEPKKVPKPC	C	APTRLGALPVLYHLNDENVHLKKYRNMIVKS	C	G	C	H
2	RRARSPHDLSLASLLGAGALRPPPGSRPVSQPC	C	RPTRYEAVSFMDVHSTWRTVDRLSATA	C	G	C	LG
3	NYPLAEHLTPTKHAIIQALVHLKNSQKASKAC	C	VPTKLEPISILYLDKGVVTYKFKYEGMAVSE	C	G	C	R
4	LRVLRDGLNSFNHAIIQNLINQLVDQSVPRPS	C	VPYKYVPISVLMIEANGSILYKEYEGMIAES	C	T	C	R
5	PFPLADHLNSTNHAIVQTLVNSVNSKIPKAC	C	VPTELSAISMLYLDENEKVVLKNYQDMVVEG	C	G	C	R
6	QFPMPKSNHATIQSIVRAVGVVPGIPEPC	C	VPEKMSSLSILFFDENKNVVLKVYPNMTVES	C	A	C	R
7	EFPMPKIVRPSNHATIQSIVRAVGIIPGIPEPC	C	VPDIGMNSLGVLFLDENRNVVLKVYPNMSVDT	C	A	C	R
8	PFPLADHLNSTNHAIVQTLVNSVNSSIPKAC	C	VPTELSAISMLYLDEYDKVVLKNYQEMVVEG	C	G	C	R
9	SFPLNAHMNATNHAIVQTLVHLMFPDHVPKPC	C	APTKLNAISVLYFDDSSNVILKKYRNMVVRS	C	G	C	H
10	SFPLNAHMNATNHAIVQTLVHLMNPEYVPKPC	C	APTKLNAISVLYFDDNSNVILKKYRNMVVRA	C	G	C	H
11	AFPLNSYMNATNHAIVQTLVHFINPETVPKPC	C	APTQLNAISVLYFDDSSNVILKKYRNMVVRA	C	G	C	H
12	SFPLDSCMNATHAILQSLVHSMKPNAVPKAC	C	APTKLSATSVLYYDSSNNVILRKHRNMVVKA	C	G	C	H
13	PSQFRAANMHAQIKTSLHRLKPDTVPAPC	C	VPASYNFMVLIQKTDTGVSLQTYDDLLAKD	C	H	C	I
14	PFPLADHFNSTNHAVVQTLVNMHNPGKVPKAC	C	VPTQLDSVAMLYLNDQSTVVLKNYQEMTVVG	C	G	C	R
15	EYPIPDHMNTTNHAIVQSLVNSHKPKEVPGPC	C	VPTQLGQMSMLYLGSDGSVILKNYKEMVVVG	C	G	C	R
16	PYPLTEMLRGTNHAVLQTLVHSVEPENTPLPC	C	APTKLSPISMLYYDNNDNVVLRHYEDMVVDE	C	G	C	K
17	FFPLTDHVTPTKHAIVQTLVHLQNPKKASKAC	C	VPTKLDAISILYKDDAGVPTLIYNYEGMKVAE	C	G	C	R
18	PYPLTEILNGSNHAILQTLVHSIEPEDIPLPC	C	VPTKMSPISHLFYDNNDNVVLRHYENMAVDE	C	G	C	R
19	AFPLNGHANATNHAIVQTLVHHMSPSHVPQPC	C	APTKLSPITVLYYDDSRNVVLKKYKNMVVRA	C	G	C	L
20	EYMFMQKYPHTHLVQQANPRGSAGPC	C	TPTKMSPINMLYFNDKQQIIYGKIPGMVVDR	C	G	C	S
21	PSQFRAANMHAQIKTSLHRLKPDTVPAPC	C	VPASYNFMVLIQKTDTGVSLQTYDDLLAKD	C	H	C	I
22	ALPVALSGSGGPPALNHAVLRALMHAAAPGAADLPC	C	VPARLSPISVLFFDNSDNVVLRQYEDMVVDE	C	G	C	R
23	FFPLADDVTPTKHAIVQTLVHLKFPTKVGKAC	C	VPTKLSPISVLYKDDMGVPTLKYHYEGMSVAE	C	G	C	R
24	PFSLTISLNSSNYAFMQALMHAVDPEIPQAV	C	IPTKLSPISMLYQDNNDNVILRHYEDMVVDE	C	G	C	G
25	EFPLRSHLEPTNHAVIQTLMNSMDPESTPPTC	C	VPTRLSPISILFIDSANNVVYKQYEDMVVES	C	G	C	R
26	DFPLRSHLEPTNHAIIQTLMNSMDPGSTPPSC	C	VPTKLTPISILYIDAGNNVVYKQYEDMVVES	C	G	C	R
27	DFPLRSHLEPTNHAIIQTLLNSMAPDAAPASC	C	VPARLSPISILYIDAANNVVYKQYEDMVVEA	C	G	C	R
28	EFVFLQKYPHTHLVHQANPRGSAGPC	C	TPTKHSPINMLYFNGKEQIIYGKIPAMVVDR	C	G	C	S
29	PRAVGHRYGSPVHTHVQNIIYEKLDSSVPRPS	C	VPAKYSPLSVLTIEPDGSIAYKEYEDMIATK	C	T	C	R
30	DAAETTYDKILKNLSRNRRLVSDKVGQAC	C	RPIAFDDDLSFLDDNLVYHILRKHSAKR	C	G	C	I
31	GLHIPPNLSLPVPGAPPTPAQPYSLLPGAQPC	C	AALPGTMRPLHVRTTSDGGYSFKYETVPNLLTQH	C	A	C	I
32	TGSFRTPDTFQTFHAHFIEEYRKMGLMNGHRPC	C	APIKFSSHSLIYYGDDGIIKRDLPKMVVDE	C	G	C	P
33	PSHIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSC	C	VPTKLRPMSMLYYDDGQNIIKKDIQNMIVEE	C	G	C	S
34	PAYLAGVPGSASSFHTAVVNQYRMRGLNPGTVNSC	C	IPTKLSTMSMLYFDDEYNIVKRDVPNMIVEE	C	G	C	A
35	PLHIAGMPGIAASFHTAVLNLLKANTAAGTTGGGSC	C	VPTARRPLSLLYYDRDSNIVKTDIPDHVVEA	C	G	C	S
36	PPHLAGSPGIAASFHSAVFSLLKANNPWPASTSC	C	VPTARRPLSLLYLDHNGNVVKTDVPDMVVEA	C	G	C	S
37	QQPPEALAFNWPFLGPRQ	C	IASETASLPMIVSIKEGGRTRPQVVSLPNMRVQK	C	S	C	ASDGALVPRR
38	GWPQSDRNPRYGNHVVLLLKHQVRGAALARPPC	C	VPTAYAGKLLISLSEERISAHHVPNHVATE	C	G	C	R
39	PIPLNETFKPTNHAYIKSLVKLYDQEKVECSS	C	VPVKMSPLSMLLYEDGEVVLKHHEDMIVDE	C	G	C	N
40	PSPVNESVKPNNHAYMQSLLNYVKGKAPEVC	C	VPIRMSSLSMVYYDHDDIAFQNHEGMIVEE	C	G	C	Q
41	PNPVGEEFHPTNHAYIQSLLKRYQPHRVPSTC	C	APVKTKPLSMLYVDNGRVLLDHHKDMIVEE	C	G	C	L
42	EAAARVYDLGLRRLRQRRRLRRERVRAQPC	C	RPTAYEDEVSFLDAHSRYHTVHELSARE	C	A	C	V
43	PRGARTQHGLALARLQGQERAHGGPC	C	RPTRYTDVAFLDDRHRWQRLPQLSAAA	C	G	C	G
44	NFPLGTKMNATNHAIVQTLMHLKQPHLPKPC	C	VPTVLGAITILRYLNEDIIDLTKYQKAVAKE	C	G	C	H
45	PYIWSLDTQYSKVLALYNQHNPGASAAPC	C	VPQALEPLPIVYYVGRKPKVEQLSNMIVRS	C	K	C	S
46	PYLWSSDTQHSRVLSLYNTINPEASASPC	C	VSQDLEPLTILYYIGKTPKIEQLSNMIVKS	C	K	C	S
47	PYLRSADTTHSTVLGLYNTLNPEASASPC	C	VPQDLEPLTILYYVGRTPKVEQLSNMVVKS	C	K	C	S
48	PFPLGERLNGTNHAIIQTLVNSIDNRAVPKVC	C	APTKLSGISMLYFDNNENVVLRQYEDMVVEA	C	G	C	R
49	AFPLTNGNNHAVLLNSHVESGNANERAPC	C	VPVAYDPLEVMDWNAEESFLSIKPDMIVKE	C	G	C	R
50	PYPIAKHLNATNHAIVQTIMNTVD-SNVPNAC	C	IPTTLNPISILSLNEFDKVVLKNYKDMVIEG	C	G	C	R
51	PNPHPAQLNATNHAIIQSLLHSLRPDEVPPPC	C	VPTETSPLSILYMDVDKVIVIREYADMRVES	C	G	C	R

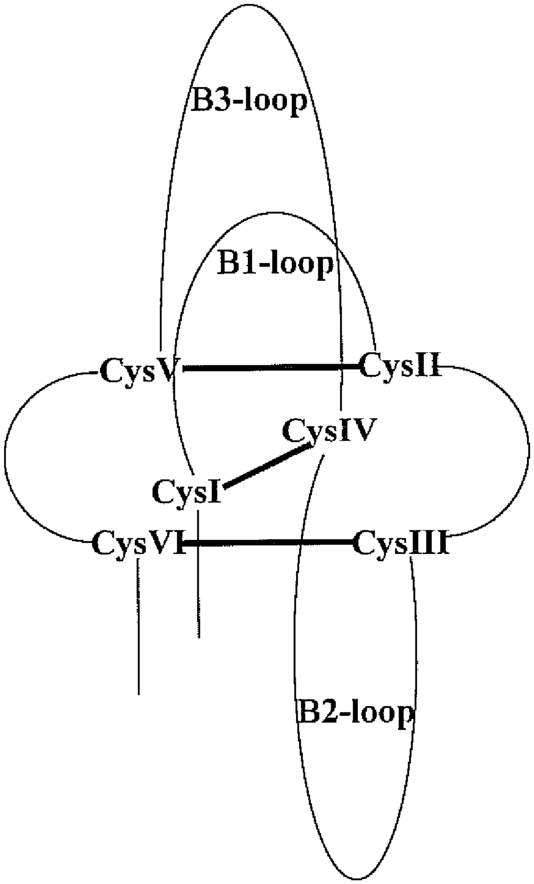
Figure 10.5

	52	PPRHNPAHHHALLQSLIWQEDHKRAPRPC	C	TPSKLEMLEILHVDENHSDKLKISTWSDMQVVE	C	A	C	S
	53	SSVASVTQAASHHSSIMKILSTSGANKSLELVPC	C	TAKQYSSLQLVVMDSSNTATVKTLPNMVVES	C	G	C	R
	54	KVGYLEQYPHTHLAALTTSATPC	C	SPTHMSSLSLLYFDDNHNLVLSVIPNMSVEG	C	S	C	S
	55	PNPMPAQLNATNHAIIQSLLHSLKPDEVPPPC	C	VPTETSPLSILYMDVDKVIVIREYADMRVDS	C	G	C	R
	56	AKPMLASGKASNHAMLQSLFAAEPVC	C	APTNLKSLNFLYRDEKGRTVIRNYSKHLIGS	C	S	C	L
Family 2a/b		X[ST]		[PA]VAXX	C	X	C	XXCXXXX[STDAI][DEY]C
	57	PTMTRVLQGVLPALPQVV	C	NYRDVRFESIRLPGCPRGVNPVVSYAVALS	C	Q	C	ALCRRSTTDC
	58	PTMTRVLQGVLPALPQVV	C	NYRDVRFESIRLPGCPRGVNPVVSYAVALS	C	Q	C	ALCRRSTTDC
	59	PTHTRVLQGVLPALPQVV	C	NYRDVRFESIRLPGCPRGVNPVVSYAVALS	C	Q	C	ALCRRSTTDC
	60	YTRDLVYKDPARPKIQKT	C	TFKELVYETVRVPGCAHHADSLYTYPVATQ	C	H	C	KCDSDSTDCT
	61	ETWEKPILEPPYIEAHHRV	C	TYNETKQVTVKLPNCAPGVDPPYTYPVAIR	C	D	C	GACSTATTEC
	62	FTQDSVYKSSLKSYPQQA	C	NFRDVVYETVHLPGCPSGMDLHFTYPVALS	C	E	C	SKCNTDSTDC
	63	ITKDPSYKSPLSTVYQRV	C	TYRDVRYETVRLPDCRPGVDPHVTFPVALS	C	D	C	NLCTMDTSDC
	64	ITKDPSYKSPLSTVYQRV	C	TYRDVRYETVRLPDCRPGVDPHVTFPVALS	C	D	C	NLCTMDTSDC
	65	PTMMRVLQAVLPPLPQVV	C	TYRDVRFESIRLPGCPRGVDPVVSFPVALS	C	R	C	GPCRRSTSDC
	66	MTRDINGKLFLPKYALSQDV	C	TYRDFIYRTVEIPGCPLHVHPYFSYPVALS	C	K	C	GKCNTDYSDC
	67	KTSESGTHGFPPRVQNSKV	C	TLVTTSTRKVVLDDCDDGADESVKFVMVPHGTD	C	E	C	SAVPLEQHHS
Family 3		[KRL]G[LIVT][DE]XXX[YW]XSX	C					
	68	RGIDKRHWNSQ	C	RTTQSYVRALTHDSKKRIGWRFIRIDTS	C	V	C	TLTIKRGR
	69	RGIDARHWNSY	C	TTTHTYVRALTKE-GNQASWRFIRIDTA	C	V	C	VISRITENFG
	70	RGIDSRHWNSY	C	TTTQSFVRALTKEGNQASWRFIRIDTA	C	V	C	VISRKTGNF
	71	RGIDSRLWNSY	C	TTTRTFVRALTMEGNQASWRFIRIDTA	C	V	C	VIIRKTDNF
	72	RGIDSRLWNSY	C	TTTQTFVRALTMEGNQASWRFIRIDTA	C	V	C	VIIRKTDNF
	73	RGTDSRHWNSY	C	TTTQTFVRALTMEGNQASWRFIRIDTA	C	V	C	VIIRKTDNF
	74	RGTDSRHWNSY	C	TTTQTFVRALTMEGNRASWRFIRIDTA	C	V	C	VISRKTDNF
	75	RGIDSKHWNSY	C	TTTHTFVRALTMDGKQAAWRFIRIDTA	C	V	C	VLSRKAVRRA
	76	RGIDSKHWNSY	C	TTTHTFVRALTTDDKQAAWRFIRIDTA	C	V	C	VLSRKAARRG
	77	RGIDDKHWNSQ	C	KTSQTYVRALTSENNKLVGWRWIRIDTS	C	V	C	ALSRKIGRT
	78	RGVDRRHWVSE	C	KAKQSYVRALTADAQGRVGWRWIRIDTA	C	V	C	TLLSRTGRA
	79	RGIDSKHWVSY	C	TNTHTYVRALTSYKNQIAWRFIRINAA	C	V	C	VLSRNSWRHG
Family 4								
	80	NTSSVK	C	QPSRVHHRSVKVAKVEYVRKKPKLKEVQVRLEEHLE	C	A	C	ATSLNPDYRE
	81	NNRNVQ	C	RPTQVQLRPVQVRKIEIVRKKPIFKKATVTLEDHLA	C	K	C	ETVAAARPVT
	82	ACCLHNCNECQ	C	VPSKVTKKYHEVLQLRPKTGVRGLHKSLTDVALEHHEE	C	D	C	VCRGSTGG
	83	GCCGTVNWRSCT	C	NSGKTVKKYHEVLQFEPGHIKRRGRAKTMALVDIQLDHHER	C	D	C	ICSSRPPR
	84	GDENLH	C	VPVETANVTMQLLKIRSGDRPSYVELTFSQHVR	C	E	C	RHSPGRQSPD
	85	NNRNVQ	C	RPTQVQLRPVQVRKIEIVRKKPIFKKATVTLEDHLA	C	K	C	EIVAAARAVT
	86	TDESLE	C	TATGKRSVGREIMRLSPHKGTSEKEVMQFTEHTD	C	E	C	RPRSASGVNS
	87	TDESLK	C	TPVGKHTVDLQIMRVNPRTQSSKMEVMKFTEHTA	C	E	C	RPRRKQGEPD
	88	NDEGLE	C	VPTEESNITMQIMRIKPHQGQHIGEMSFLQHNK	C	E	C	RPKKDRARQE
	89	PDDGLE	C	VPTGQHQVRMQILMIRYPSSQLGEMSLEEHSQ	C	E	C	RPKKKDSAVK
	90	NSEGLQ	C	MNTSTSYLSKTLFEITVPLSQGPKPVTISFANHTS	C	R	C	MSKLDVYRQV
	91	NEESLI	C	MNTSTSYISKQLFEISVPLTSVPELVPVKVANHTG	C	K	C	LPTAPRHPYS
	92	NDESLE	C	VPTEEVNVSMELLGASGSGSNGMQRLSFVEHHK	C	D	C	RPRPTTTPPT
	93	NGDGQI	C	TAVETRNTTVTVSVTGVSSSSGTNSGVSTNLQRISVTEHTK	C	D	C	IGRTTTTPTT
	94	NDEALE	C	VPTETRNVTMEVLRVKQRVSQHNFQLSFTEHTK	C	E	C	RPKAEVKAKK
	95	NDESLE	C	VPTEEANVTMEFMGVGVSSTGSSVSTQHLEFVEHTK	C	D	C	PRGGQQTTPP
	96	NNRNVQ	C	RPTQVQLRLVQVRKIEIVRKRPVFKKATVTLEDHLA	C	K	C	ETVVAARPVT
Family 5		[FY]S[RQS]A[FY]PTP						
	97	FSRAYPTPLQSKKAMLVPKNITSEATC	C	VAKEGERVVVDNIKLTNHTE	C	W	C	NTCYHHKS

Figure 10.6

	98	FSRAYPTPLRSKQTMLVPKNITSEATC	C	VAKEGERVTTKDGFPVTNHTE	C	H	C	STCYYHKS
	99	FSRAYPTPLRSKKTMLVQKNVTSESTC	C	VAKSYNRVTVHGGFKVENHTA	C	H	C	STCYYHKS
	100	FSRAYPTPLRSKKTMLVQKNVTSESTC	C	VAKSLTRVMVHGNVRVENHTQ	C	H	C	STCYYHKF
Family 6								
	101	SVPEGMV	C	KPSKSVHLTVLRWRCQRRGGQRCGWIPIQYPIISE	C	K	C	SC
	102	SVPEGMV	C	KPAKSTHITLLRWRCVARRGALKCAWIPVQYPIITE	C	K	C	SCAN
	103	SFPEGMS	C	KPVKAVTKTFLRWYCQGFMRQKYCTWIQVQYPIISQ	C	K	C	SC
	104	SVPEGMV	C	KPPKSSHLTVLRWRCVQRKGGLKCAWIPVQYPVISE	C	K	C	SCPN
	105	SWPPGMA	C	RPAQLAHLKLLAWHCWAARPPGPPHCAWRQVPYPVVVA	C	K	C	SCR
	106	SLPEGMF	C	KPVQSVSVTLLRWHCQQGSRALKRCAWIRAHYPVISQ	C	A	C	AC
	107	SIPDRADFL	C	RPDVFNQEQMGTLTALRWDCCWEVVETIVRGRRGRTFRRLSRRYHCGWRRIRFPIVCD	C	D	C	NCPGNRPRI
	108	SWPPGMN	C	RPNDQKLLKILKWTCISDPLGKRWNEFRESIFADKRKRRLRRHKIKRHLSQKRKTNKVIRPKRMKRLVKRYLYRTSKYVSGYLCQWKPIDYTVHQS	C	T	C	SCQG
	109	SWPPGMA	C	KQSDKKTLKVLKWTCLSDPLGEKWNAFRELMHEDRKRRRLRRHQFKRHLSQKRKIGVKKVKKPKQLKRLVKRYLYKTSKYASGYLCQWKPIDYTVYES	C	T	C	SC
Family 7								
	110	QRTGR	C	TQQRSVVRLVTYDLEKGVFFCENVRTCCG	C	P	C	RS
	111	GFSGR	C	TQQRSVVRLVTYNLEKDGFLCESFRTCCG	C	P	C	RNY
Family 2c/8			C C	XXXXXXXXXXXXXCXX[GN]XXXXXXXXXXXX	C	X	C	(X)nC (n=2-4)
	112	ASYIQVSGSKIWQMERSCMC	C	QESGEREAAVSLFCPKVKPGERKFKKVLTKAPLE	C	M	C	RPCTSIEESG
	113	NSQVQPSVITPTGFLKECYC	C	RESFLKEKVITLTHCYDPDGTRLTSPGEMGSMDIRLREPTE	C	K	C	FKCGDFTR
	114	VDGRC	C	TPQQTRTVKIRFRCDDGETFTKSVMMIQS	C	R	C	NYNCPHANEA
	115	GSVHFPGAAQHSHTSCSH	C	LPAKFTTMHLPLNCTELSSVIKVVHLVEE	C	Q	C	KVKTEHEDGH
	116	TDGRC	C	TPHRTTTLPVEFKCPDGEVMKKMMMFIKT	C	A	C	HYNCPGDNDI
	117	VDGRC	C	TPQLTRTVKMRFRCEDGETFSKNVMMIQS	C	K	C	NYNCPHANEA
	118	SSLYIPGSDPTPLVLCNS	C	MPARKRWAPVVLWCLTGSSASRRRVKISTMLIEG	C	H	C	SPKA
	119	NSFYVPGWPAGLSQPCTS	C	APSRSRRISLPLRCRSGHLAWQEVELVEE	C	E	C	ETRYDRNTVE
	120	NSFYIPRHIRKEEGSFQSCSF	C	KPKKFTTMMVTLNCPELQPPTKKKRVTRVKQ	C	R	C	ISIDLD
	121	NSFYIPRHVKKEEESFQSCAF	C	KPQRVTSVLVELECPGLDPPFRLKKIQKVKQ	C	K	C	MSVNLS
	122	EKTAKYNHDILLLEHSCLC	C	REENYELRDIVLDCPDGSTIPYQYKHITT	C	S	C	LDICQLYTTF
	123	GTFVMYSAKAQALDHSCSC	C	KEEKTSQREVVLSCPNGGSLTHTYTHIES	C	Q	C	QDTVCGLPTG
	124	GDSSSMYSLEGNTVEHRCQC	C	QELRTSLRNVTLHCTDGSSRAFSYTEVEE	C	G	C	MGRRCP
	125	PGASKYSAEAQAMQHQCTC	C	QERRVHEETVPLHCPNGSAILHTYTHVDE	C	G	C	TPFCV
	126	ISAASFNIITQQVDARCSC	C	RPLHSYEQQLELPCPDPSTPGRRLVLTLQVFSH	C	V	C	SSVACG
	127	SQASRSEPLVSFSTVLKQPFRSSCHC	C	RPQTSKLKALRLRCSGGMRLTATYRYILS	C	H	C	EECNS
	128	SDGRC	C	TPHNTKTIQAEFQCSPGQIVKKPVMVIGT	C	T	C	HTNCPKNNEA
	129	PSATIYNINIESHLRFCKC	C	RENGVRNLSVPLYCSGNGTEIMYTLQEPID	C	T	C	QWN
	130	PSASIYNYNINTYARFCKC	C	REVGLQRRSVQLFCTNATWVPYTVQEPTD	C	A	C	QWS
	131	PGQGC	C	QGLRLKRRKFTFECSDGTSFAEEVEKPTK	C	G	C	ALCA
	132	AGGQC	C	GPLRSKRRKYSFECTDGSSFVDEVEKVVK	C	G	C	TRCV
	133	GPQC	C	QPTRSKRRKYVFQCTDGSSFVEEVERHLE	C	G	C	LACS
	134	GSQC	C	AAKIVRRRKVRMVCSNNRKYIKNLDIVRK	C	G	C	TKKCY
	135	LPLPVLPNWIGGGYGTKYWSRRSSQEWR	C	VNDKTRTQRIQLQCQDGSTRTYKITVVTA	C	K	C	KRYTRQHNES
	136	GPARLLPNAIGRGKWWRPSGPDFR	C	IPDRYRAQRVQLLCPGGEAPRARKVRLVAS	C	K	C	KRLTRFHNQS
	137	PSSTHVMPEEPYLQSQCDC	C	SYRLDPESPVRILNLRCLGCHTEPVVLPVIHS	C	Q	C	SSCQGGDFSK
	138	ASKAMYSIDINDVQDQCSC	C	SPTRTEPMQVALHCTNGSVVYHEVLNAME	C	K	C	SPRKCSK
	139	MDNRC	C	IPYKSKTIDVSFQCPDGLGFSRQVLWINA	C	F	C	NLSCRNPNDI
	140	LDKRC	C	IPNKSKMITIQFDCPNEGSFKWKMLWITS	C	V	C	QRNCREPGDI
	141	SSGSKYNTLTDMHEKFCTC	C	SIKSYHPISVKMICDDGHTFTQKHEVPSN	C	G	C	SPCSEFSDSA
	142	ESSAFPSRYSVLVASGYRHNITSVSQC	C	TISGLKKVKVQLQCVGSRREELEIFTARA	C	Q	C	DMCRLSRY
	143	ESSAFPSRYSVLVASGYRHNITSSSQC	C	TISSLRKVRVWLQCVGNQRGELEIFTARA	C	Q	C	DMCRFSRY
	144	QNDASCRDLVNGYRCICPPGYAGDH	C	ERDIDECASNPCLNGGHCQNEINRFQ	C	L	C	PTGFSGNLCQ
	145	QHGGTCKDLVNGYQCVCPRGFGGRH	C	ELERDECASSPCHSGGLCEDLADGFH	C	H	C	PQGFSGPLCE

Figure 11



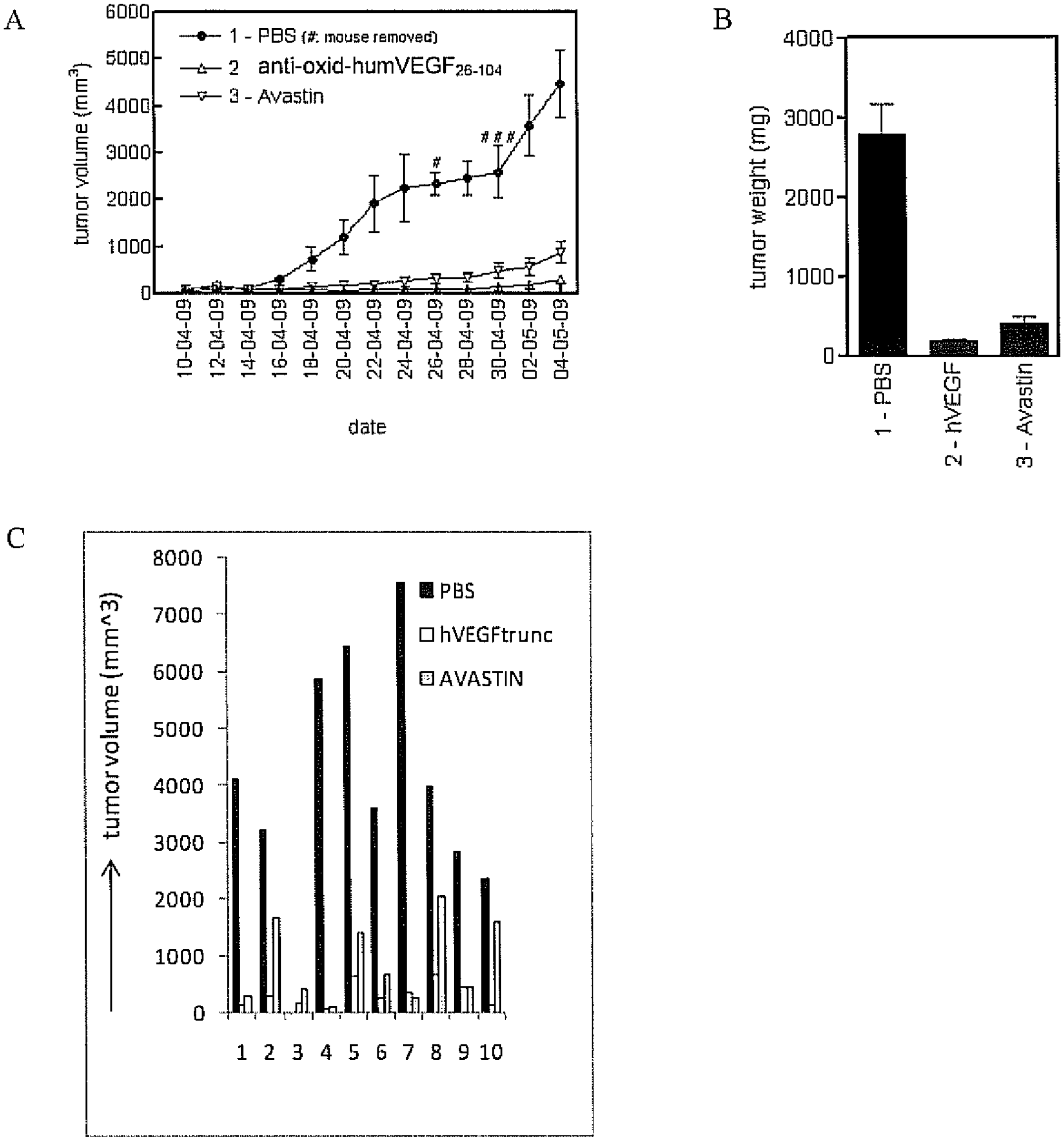

A
6000
5000
4000
3000
2000
1000
0
tumor volume (mm³)
1 - PBS (#: mouse removed)
2 anti-oxid-humVEGF26-104
3 - Avastin
#
###
10-04-09
12-04-09
14-04-09
16-04-09
18-04-09
20-04-09
22-04-09
24-04-09
26-04-09
28-04-09
30-04-09
02-05-09
04-05-09
date
B
4000
3000
2000
1000
0
tumor weight (mg)
1 - PBS
2 - hVEGF
3 - Avastin
C
8000
7000
6000
5000
4000
3000
2000
1000
0
tumor volume (mm^3)
■ PBS
□ hVEGFtrunc
▨ AVASTIN
1
2
3
4
5
6
7
8
9
10

Figure 12

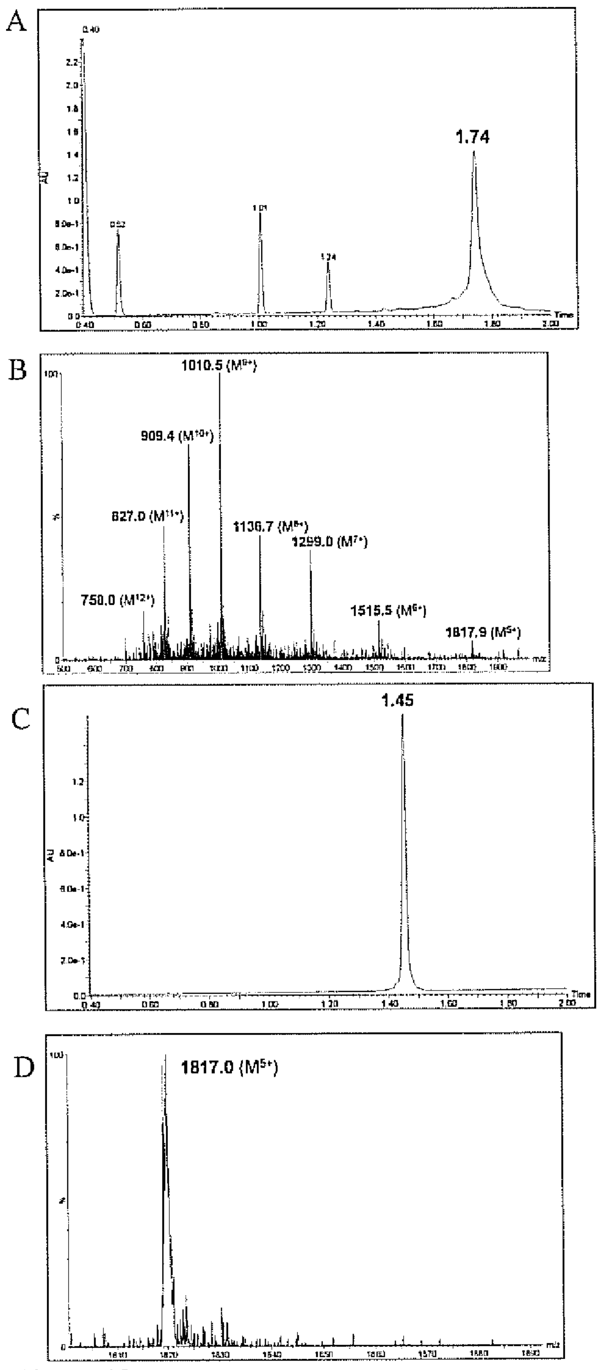
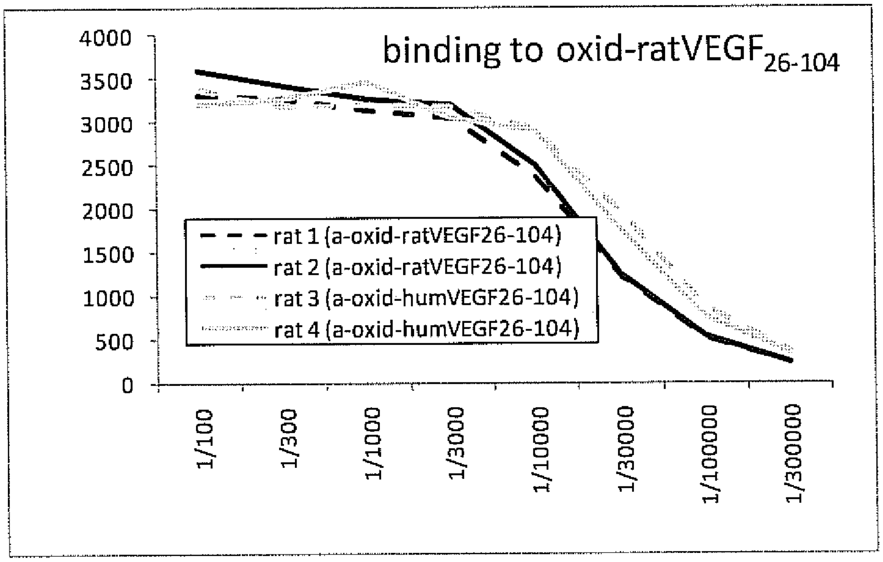

A
0.40
0.52
1.01
1.24
1.74
AU
Time
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
B
1010.5 (M9+)
909.4 (M10+)
827.0 (M11+)
1136.7 (M8+)
1299.0 (M7+)
750.0 (M12+)
1515.5 (M6+)
1817.9 (M5+)
100
%
0
m/z
C
1.45
AU
Time
D
1817.0 (M5+)
100
%
m/z

Figure 13

A


binding to oxid-ratVEGF26-104
4000
3500
3000
2500
2000
1500
1000
500
0
rat 1 (a-oxid-ratVEGF26-104)
rat 2 (a-oxid-ratVEGF26-104)
rat 3 (a-oxid-humVEGF26-104)
rat 4 (a-oxid-humVEGF26-104)
1/100
1/300
1/1000
1/3000
1/10000
1/30000
1/100000
1/300000

B

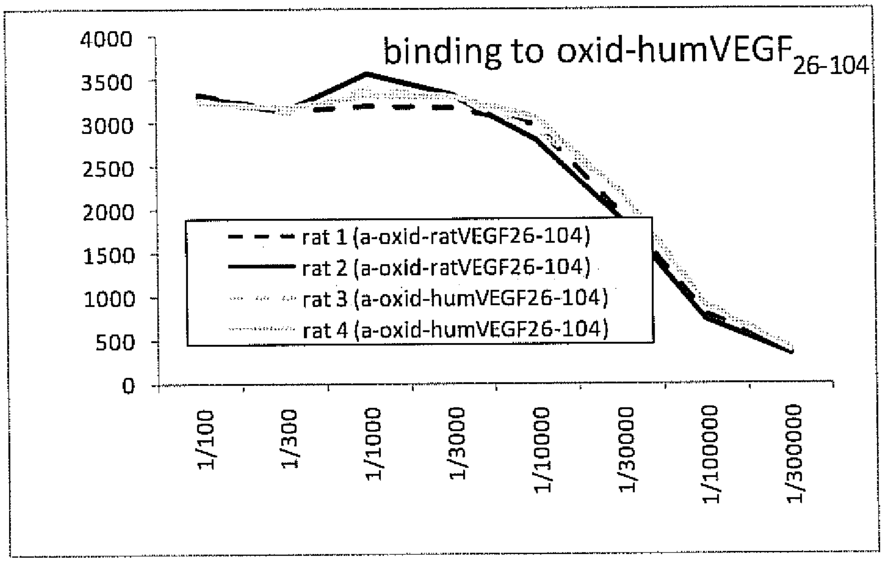

binding to oxid-humVEGF26-104
4000
3500
3000
2500
2000
1500
1000
500
0
rat 1 (a-oxid-ratVEGF26-104)
rat 2 (a-oxid-ratVEGF26-104)
rat 3 (a-oxid-humVEGF26-104)
rat 4 (a-oxid-humVEGF26-104)
1/100
1/300
1/1000
1/3000
1/10000
1/30000
1/100000
1/300000

Figure 14

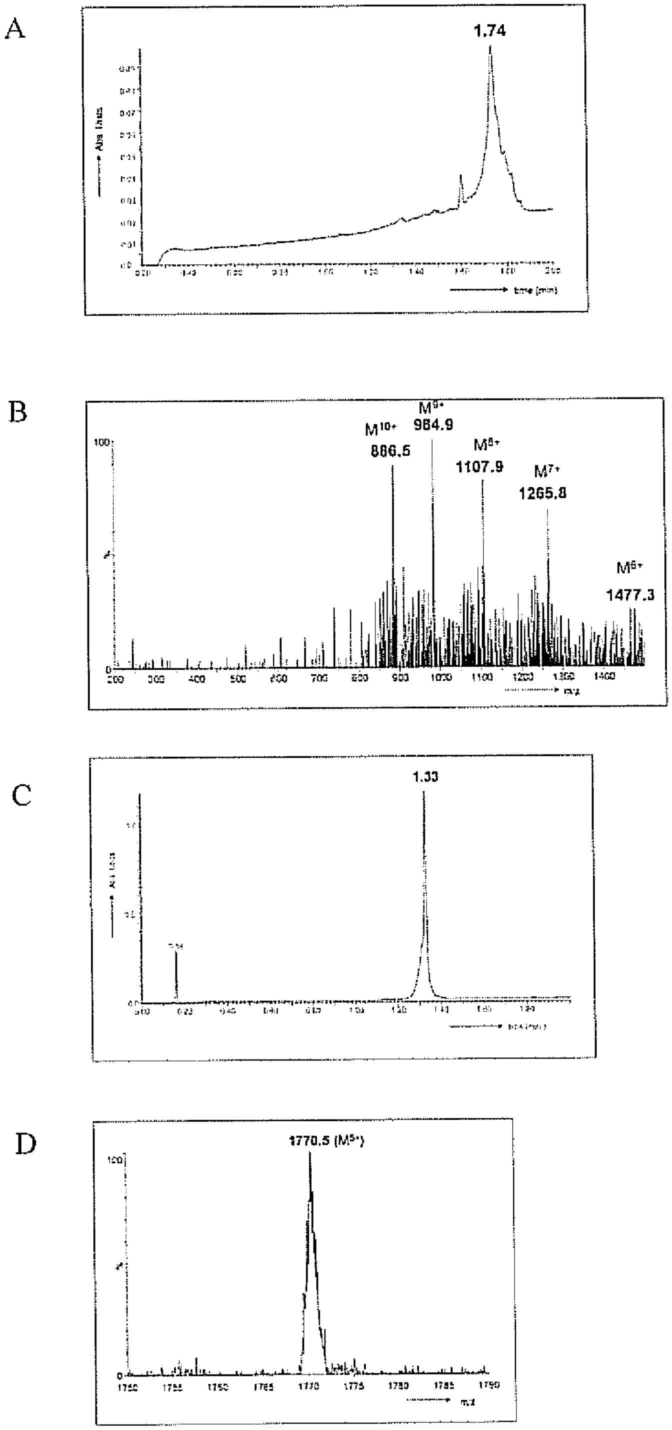

A
1.74
Abs Units
time (min)
B
M^{10+}
886.5
M^{9+}
984.9
M^{8+}
1107.9
M^{7+}
1265.8
M^{6+}
1477.3
100
%
0
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
m/z
C
1.33
D
1770.5 (M^{5+})
100
%
0
1750
1755
1760
1765
1770
1775
1780
1785
1790
m/z

Figure 15

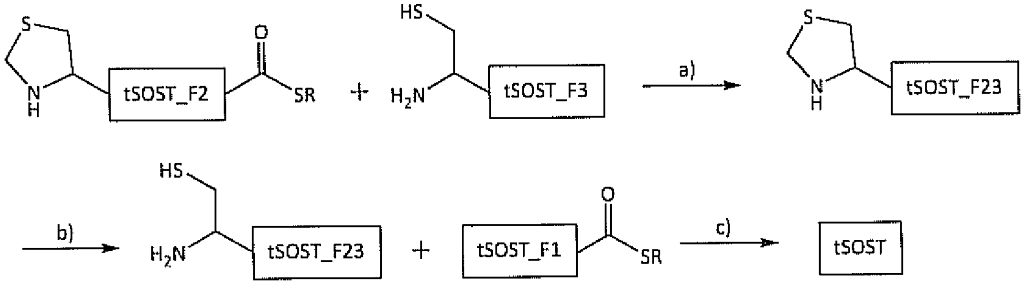


Figure 16

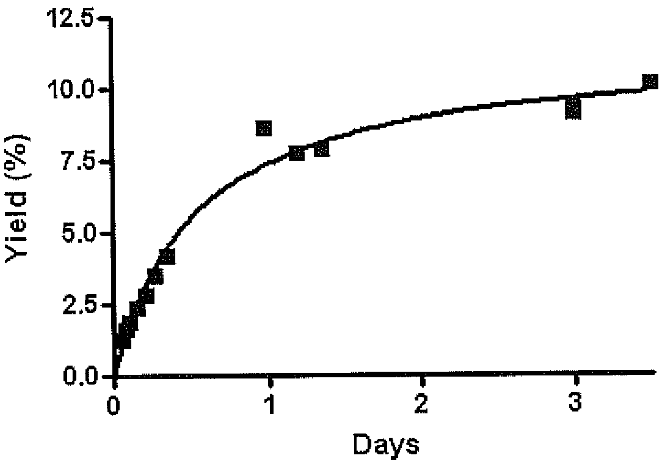


Figure 17

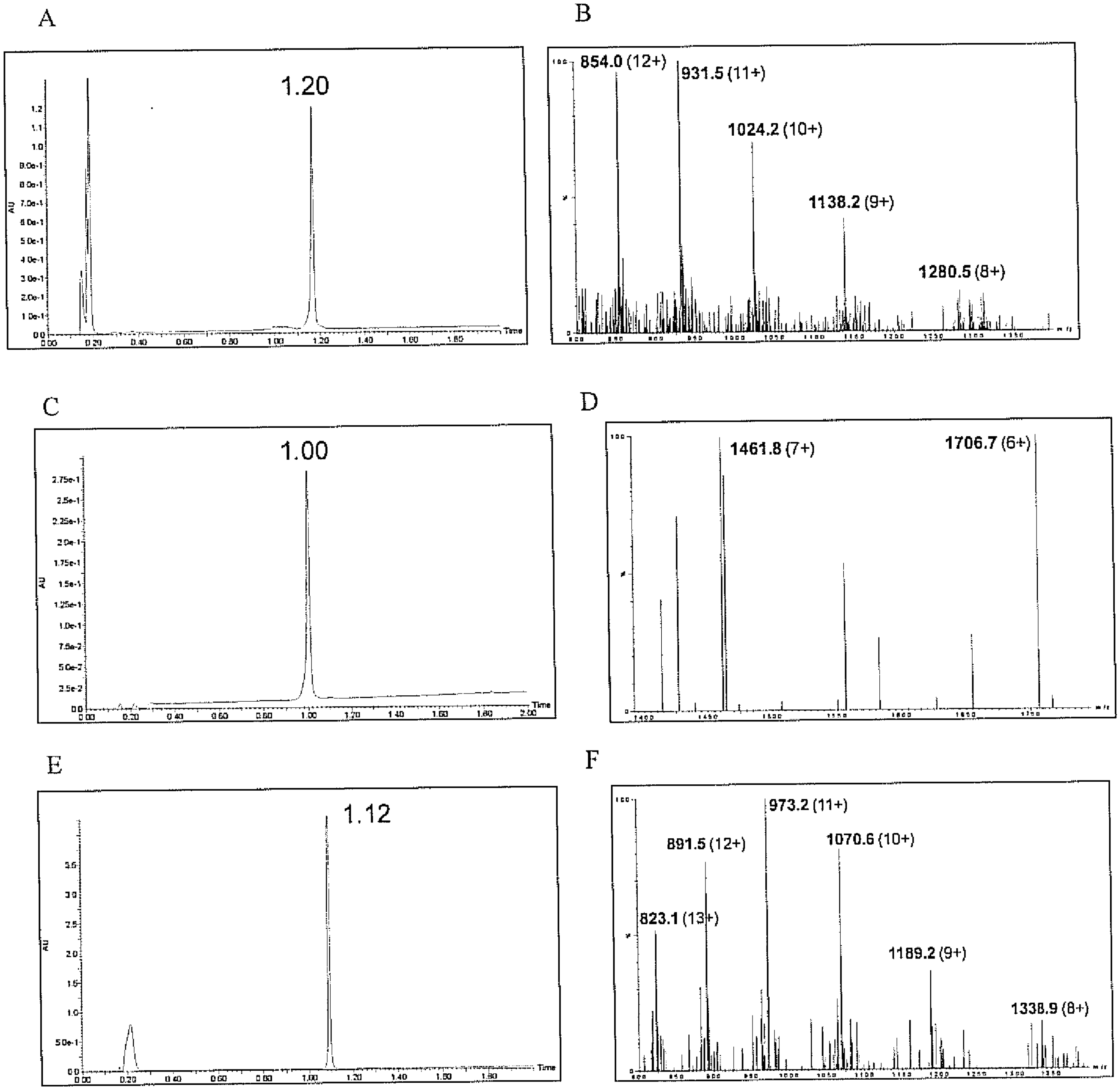

A
1.20
AU
Time
B
854.0 (12+)
931.5 (11+)
1024.2 (10+)
1138.2 (9+)
1280.5 (8+)
C
1.00
AU
Time
D
1461.8 (7+)
1706.7 (6+)
E
1.12
AU
Time
F
973.2 (11+)
891.5 (12+)
1070.6 (10+)
823.1 (13+)
1189.2 (9+)
1338.9 (8+)

Figure 18

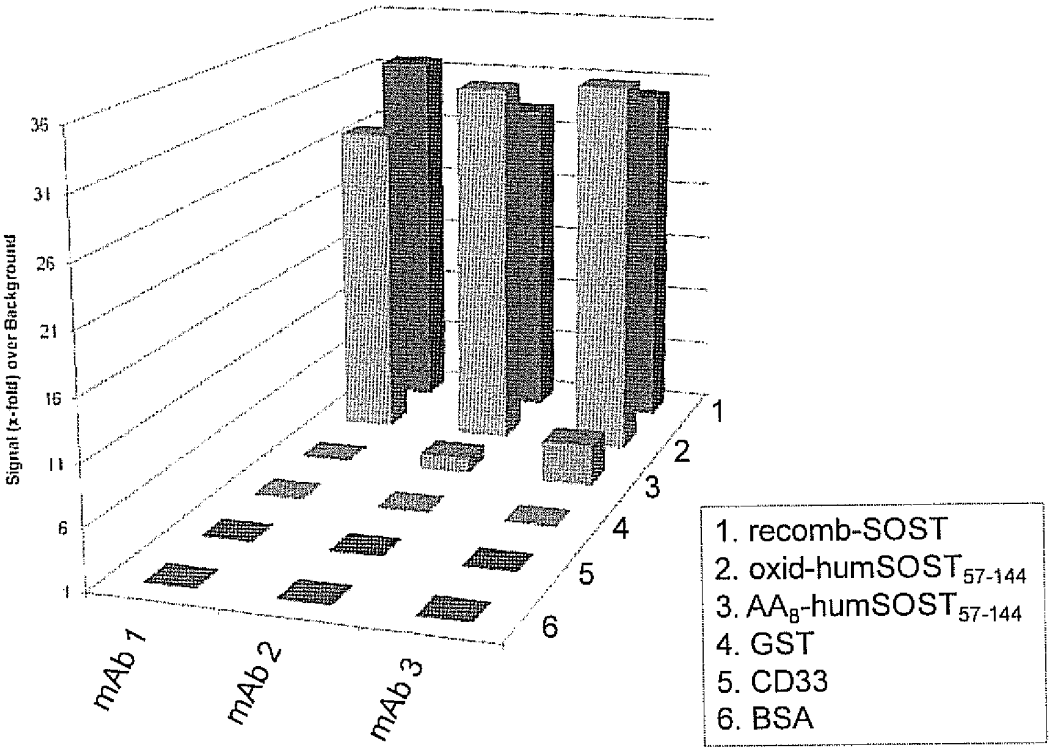

Signal (x-fold) over Background
36
31
26
21
16
11
6
1
mAb 1
mAb 2
mAb 3
1
2
3
4
5
6
1. recomb-SOST
2. oxid-humSOST57-144
3. AA8-humSOST57-144
4. GST
5. CD33
6. BSA

Figure 19

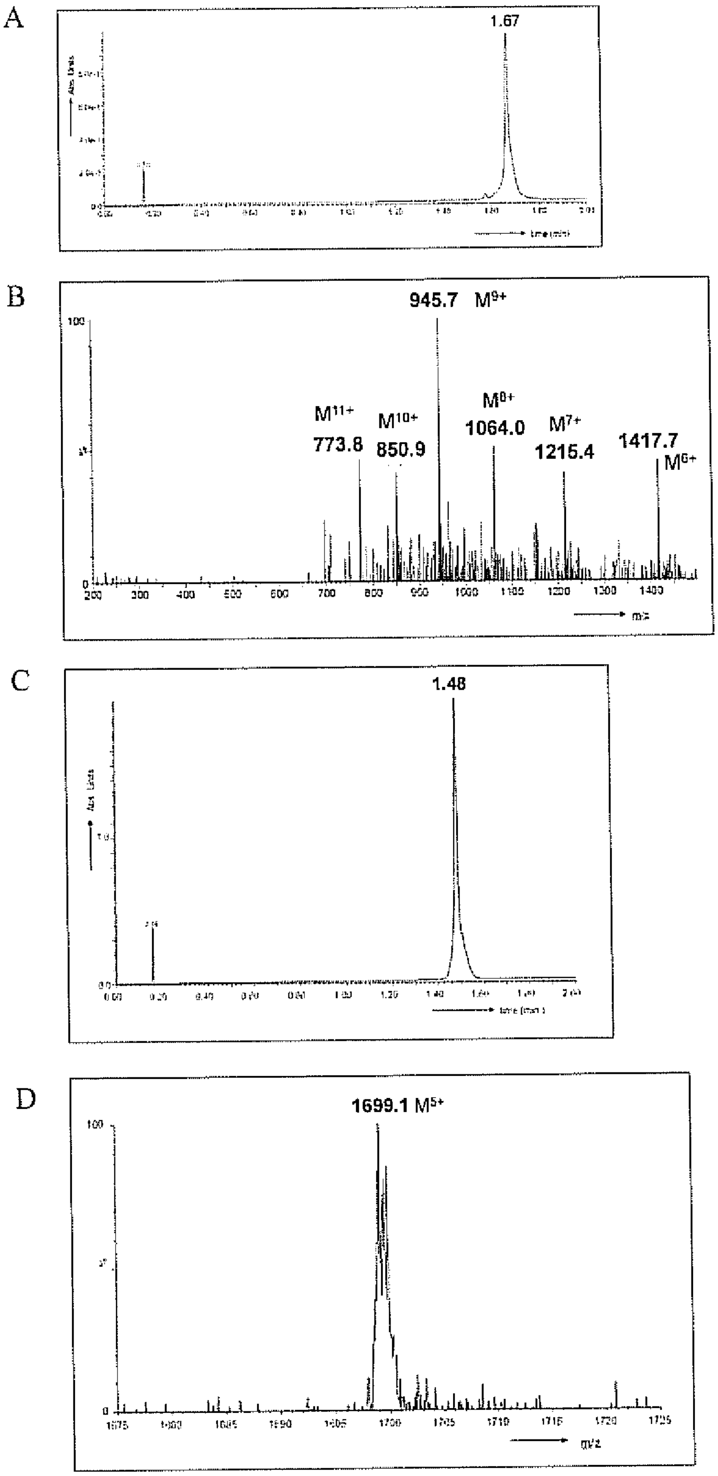

A
1.67
Abs. Units
time (min)
B
945.7 M9+
100
M11+
773.8
M10+
850.9
M8+
1064.0
M7+
1215.4
1417.7
M6+
%
0
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
m/z
C
1.48
Abs. Units
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
time (min)
D
1699.1 M5+
100
%
0
1675
1680
1685
1690
1695
1700
1705
1710
1715
1720
1725
m/z

Figure 20

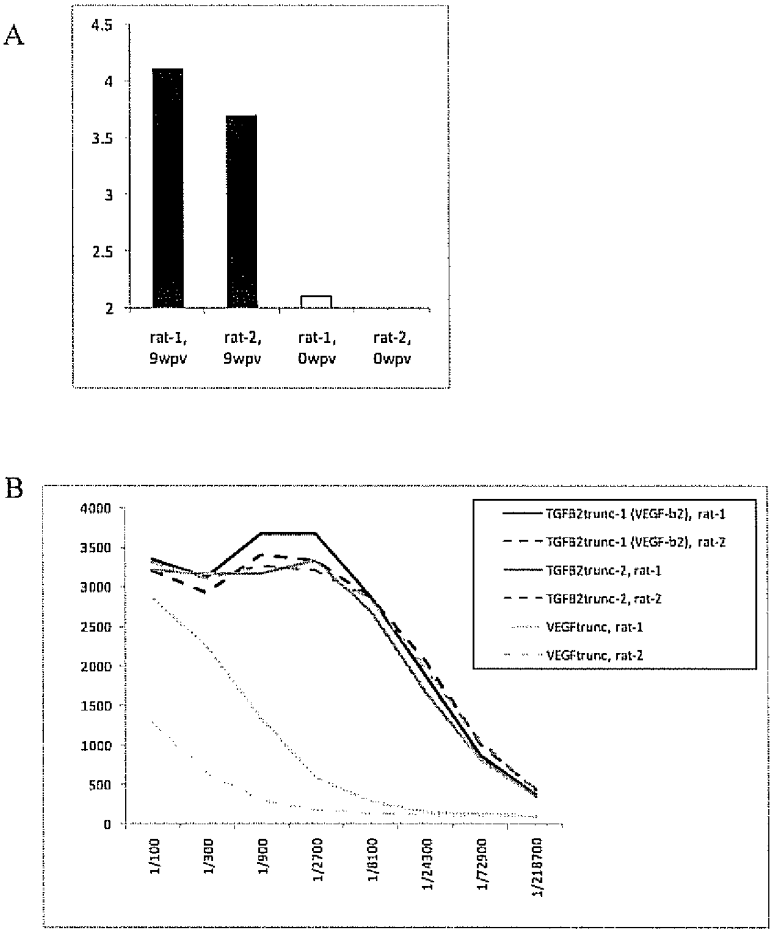

A
4.5
4
3.5
3
2.5
2
rat-1, 9wpv
rat-2, 9wpv
rat-1, 0wpv
rat-2, 0wpv
B
4000
3500
3000
2500
2000
1500
1000
500
0
1/100
1/300
1/900
1/2700
1/8100
1/24300
1/72900
1/218700
TGFB2trunc-1 (VEGF-b2), rat-1
TGFB2trunc-1 (VEGF-b2), rat-2
TGFB2trunc-2, rat-1
TGFB2trunc-2, rat-2
VEGFtrunc, rat-1
VEGFtrunc, rat-2

Figure 21