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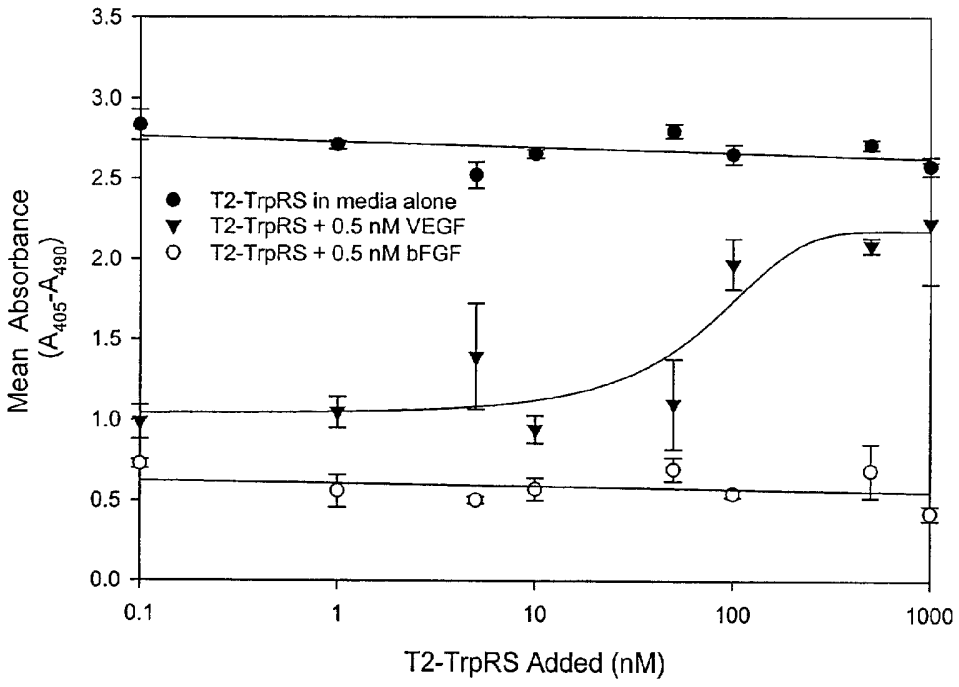
(54) Title: METHODS OF TREATING NEUROPILIN-MEDIATED DISEASES



(57) Abstract: The vascular endothelial cell growth factor receptor, neuropilin, is a selective binding partner for the anti-angiogenic molecule T2-TrpRS. This binding inhibits VEGF-mediated activity by effectively blocking VEGF binding to neuropilin. It is hypothesized that T2- TrpRS acts as a competitive antagonist of VEGF-neuropilin interaction with consequent disruption of angiogenesis. This discovery is exploited to provide novel methods for the treatment of diseases and conditions impacted by T2- TrpRS or T2-TrpRS derivatives via their interaction with neuropilins.

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METHODS OF TREATING NEUROPILIN-MEDIATED DISEASES

This application claims the benefit of U.S. Provisional Application Nos. 60/307,872, filed July 26, 2001, and 60/355,551, filed February 7, 2002, both of which are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

This invention relates to the identification of the vascular endothelial cell growth factor receptor, neuropilin, as a selective binding partner for the anti-angiogenic molecule T2-TrpRS. The invention particularly relates to methods of using T2-TrpRS that exploit this discovery.

BACKGROUND OF THE INVENTION

Angiogenesis, or the proliferation of new capillaries from preexisting blood vessels, is a fundamental process necessary for embryonic development, subsequent growth, and tissue repair. Angiogenesis is a prerequisite for the development and differentiation of the vascular tree, as well as for a wide variety of fundamental physiological processes including embryogenesis, somatic growth, tissue and organ repair and regeneration, cyclical growth of the corpus luteum and endometrium, and development and differentiation of the nervous system. Angiogenesis additionally occurs as part of the body's repair processes, e.g. in the healing of wounds and fractures. Angiogenesis is also a factor in tumor growth, because a tumor just continuously stimulate growth of new capillary blood vessels in order to grow. Angiogenesis is an essential part of the growth of human solid cancer, and abnormal angiogenesis is associated with other diseases such as rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman *et al., Science* 235: 442-447 (1987)).

Several factors are involved in angiogenesis. Both acidic and basic fibroblast growth factor molecules that are mitogens for endothelial cells and other cell types. Angiotropin and angiogenin can induce angiogenesis, although their functions are unclear. A highly selective mitogen for vascular endothelial cells is vascular endothelial growth factor (VEGF) (Ferrara *et al., Endocr. Rev.* 13: 19-32 (1992)).

The neuropilin (NRP) family is composed of two homologous members, neuropilin-1 (NRP1) and neuropilin-2 (NRP2). NRP1 was first identified in certain classes of neurons as a 140-kDa transmembrane glycoprotein expressed on the tips of actively growing axons

in the developing nervous system. NRP2 was subsequently identified by expression cloning. NRP's are high-affinity receptors for several classes of the class 3 semaphorins, secreted proteins with potent axon repulsive activity that prevent improper innervation during embryogenesis. NRP's have also been demonstrated to be receptors for VEGF (Rossignol *et al., Genomics* 70: 211-222 (2000)).

In mice, NRP1 is expressed in the nervous, cardiovascular, and skeletal systems. Capillaries and blood vessel endothelial cells (EC) and the mesenchymal cells surrounding blood vessels express NRP1. After birth, NRP1 expression in neural tissues is dimished, but NRP1 expression persists in many adult tissues and notably in the placenta and heart. NRP1 and NRP2 have been reported to be the only VEGF receptors expressed at the surface of tumor cells, suggesting that NRP could directly mediate as yet unidentified signals upon VEGF binding to tumor cells (Soker *et al., Cell* 92: 735-745 (1998)).

Several lines of evidence suggest that NRP1 might be a mediator of angiogenesis. NRP1 is expressed in EC *in vitro* and is associated with blood vessels *in vivo* (Kitasukawa *et al., Development* 121: 4309-4318 (1995); Soker *et al., Cell* 92: 735-745 (1998)). It binds $VEGF_{165}$, which is a potent mitogenic and chemotactic factor for EC *in vitro* and an angiogenic factor *in vivo* (Soker *et al.*, 1998). Overexpression of NRP1 in transgenic mice results in embryonic death due to excess blood vessels and hemorrhaging (Kitasukawa *et al.*, 1995). Homozygous NRP1 knockouts are embryonic lethal due to defects in the cardiovascular system, impaired neural vascularization, and disruption of vascular networks (Kitasukawa *et al., Neuron* 19: 995-1005 (1997)).

Aminoacyl-tRNA synthetases, which catalyze the aminoacylation of tRNA molecules, are ancient proteins that are essential for decoding genetic information during the process of translation. In higher eukaryotes, nine aminoacyl- tRNA synthetases associate with at least three other polypeptides to form a supramolecular multienzyme complex (Mirande *et al., Eur. J. Biochem.* 147: 281-89 (1985)). Each of the eukaryotic tRNA synthetases consists of a core enzyme, which is closely related to the prokaryotic counterpart of the tRNA synthetase, and an additional domain that is appended to the amino-terminal or carboxyl-terminal end of the core enzyme (Mirande, *Prog. Nucleic Acid Res. Mol. Biol.* 40: 95-142 (1991)).

Trytophanyl- tRNA synthetase (TrpRS) catalyzes a reaction that involves aminoacylation of tRNA to incorporate tryptophan into protein. TrpRS has been found to be secreted under apoptotic conditions in cell culture. Structural analysis has revealed that

TrpRS contains structures similar to the CXC-chemokine family. However, TrpRS lacks the ELR motif which is essential for PMN receptor binding and cytokine activities (Wakasugi *et al., Science* 284: 147-151 (1999)). In human, TrpRS exists as two forms, the full length TrpRS and the truncated TrpRS (mini-TrpRS), wherein the NH_2-terminal 48 amino acids are deleted due to alternate mRNA splicing (Fig. 1) (Wakasugi *et al., Proc. Natl. Acad. Sci.* 99: 173-177 (2002)).

Studies conducted by Schimmel *et al.* indicate that the full-length TrpRS is an inactive protein, whereas mini-TrpRS inhibits VEGF-induced cell proliferation and migration (Wakasugi *et al., Proc. Natl. Acad. Sci.* 99: 173-177 (2002)). In a chick CAM assay, mini-TrpRS has been shown to block the angiogenic activity of VEGF. Thus, removal of the first 48 amino acid residues exposes the anti-angiogenic activity of TrpRS. TrpRS and mini-TrpRS are further described in International Application Nos. PCT/US01/08966 and PCT/US01/08975, both filed March 21, 2001, the disclosures of which are incorporated herein by reference in their entireties.

Treatment of TrpRS with PMN elastase results into two additional products, the 47 (super mini-TrpRS or T1-TrpRS) and 44 (T2-TrpRS) kDa fragments. Terminal amino acid analysis has revealed Ser-71 and Ser-94, respectively, as the NH_2-terminal residues for these fragments. These molecules, T1-TrpRS and T2-TrpRS, have been shown to be potent antagonists of *in vivo* angiogenesis (Otani *et al., Proc. Natl. Acad. Sci.* 99: 178-183, 2002). T1-TrpRS and T2-TrpRS are further described in U.S. Provisional Application No. 60/270,951 filed on February 23, 2001, for "Tryptophanyl- tRNA Synthetase Derived Polypeptides Useful for the Regulation of Angiogenesis" as well as U.S. Patent Application No. 10/080,839, filed February 22, 2002, and International Application No. PCT/US02/05185, filed February 22, 2002, the disclosures of which are incorporated herein by reference in their entireties.

The Schimmel laboratory has previously noted that TrpRS contains a small stretch of residues in which 13 of 39 residues are identical to semaphorin-E, a ligand for the NRP receptors (Fig. 2A). While intriguing, this homology does not necessarily imply functional significance. Furthermore, the Schimmel lab has previously noted that the related tyrosyl-Trna synthetase (TyrRS) may function in a manner similar to the CXC chemokines (Wakasugi *et al., Science* 284: 147-151 (1999)), and they hypothesized that the angiogogenic and angiostatic activities of TyrRS and TrpRS may be via chemokine receptors.

SUMMARY OF THE INVENTION

The present disclosure supports for the first time a specific and surprising interaction between T2-TrpRS and neuropilin and that this binding inhibits VEGF-mediated activity. Furthermore, it is demonstrated herein that this interaction effectively blocks VEGF binding to neuropilin. Thus, it is hypothesized that T2-TrpRS acts as a competitive antagonist of VEGF-neuropilin interaction with consequent disruption of angiogenesis. This hypothesis is supported by the finding described herein that T2-TrpRS down-regulates VEGF-induced receptor tyrosine kinase activity and HUVEC protection against apoptosis but has no effect on fibroblast growth-factor mediated biological activity on HUVEC.

According, the present invention provides a method of treating a neuropilin-mediated disease in a mammal, comprising administering to the mammal an amount of T2-TrpRS or a derivative thereof effective to treat the disease. In a preferred embodiment, the T2-TrpRS or a derivative thereof comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO:3. In a more preferred embodiment, the T2-TrpRS or a derivative thereof comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO:3. In an even more preferred embodiment, the T2-TrpRS or a derivative thereof comprises an amino acid sequence having at least 99% sequence identity to SEQ ID NO:3. In an even further preferred embodiment, T2-TrpRS is administered to said mammal.

In a preferred embodiment, the mammal is a primate. In a more preferred embodiment, the primate is a human.

In a preferred embodiment, the disease is cancer. In a more preferred embodiment, the cancer is selected from the group consisting of lung, colon, breast, and prostate cancer.

In another preferred embodiment, the disease is injury to the nervous system.

In another preferred embodiment, disease is rheumatoid arthritis.

In another preferred embodiment, disease is a development anomaly.

In a preferred embodiment, the T2-TrpRS or derivative is administered as a recombinant protein. In a more preferred embodiment, the protein is administered orally, intravenously, intramuscularly, intraperitoneally, or intrathecally.

In a preferred embodiment, the T2-TrpRS or derivative is administered by administering a vector comprising a nucleotide sequence encoding the T2-TrpRS or derivative. In a more preferred embodiment, the vector is a plasmid, a lipid formulation, or a viral vector. Preferably, the vector is administered orally, intravenously, intramuscularly, intraperitoneally, or intrathecally.

The present invention also provides a method for identifying an antagonist to neuropilin, comprising: (a) designing molecules that are expected to bind to a neuropilin based on the chemistry and three-dimensional structure of neuropilin and the chemistry and three-dimensional structure of T2-TrpRS; (b) contacting the molecules with neuropilin in an *in vitro* or cellular assay; and (c) identifying which molecules bind to neuropilin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of full-length TrpRS and mini-TrpRS. Numbers on the left and right represent the NH_2 and COOH terminal residues.

Figure 2A shows a partial primary amino acid sequence alignment of T2-TrpRS (SEQ ID NO:1) and SEM (SEQ ID NO:2). Identical residues are depicted in bold.

Figure 2B shows the complete amino acid sequence of T2-TrpRS (SEQ ID NO:3).

Figure 3A shows that T2-TrpRS specifically binds to NRP-1 and NRP-2 but not Flt-1 and KDR.

Figure 3B shows that the binding of T2-TrpRS is reversible by VEGF but not bFGF.

Figure 4A shows that T2-TrpRS does not inhibit RTK activity.

Figure 4B shows that T2-TrpRS inhibits VEGF-induced, but not bFGF-induced, activity on HUVEC.

Figure 4C shows that T2-TrpRS, but not full-length TrpRS, inhibits VEGF-induced RTK activity on HUVEC.

Figure 5 shows that T2-TrpRS blocks VEGF-mediated HUVEC survival but has no effect on bFGF protection of HUVEC apoptosis.

Figure 6: Specific and saturable binding of biotinylated T2-TrpRS to HUVECs is shown by subtracting the total binding from non-specific binding. Binding constants were determined using the equation in Example 4. Number of T2-TrpRS binding sites were calculated using a standard curve as outlined in Example 4. Data points represent the means (+ SEM) of three observations.

Figure 7A: Luciferase dsRNA, NRP1/a1 mismatch control and NRP1/a1 were electrophorated into 10^6 HUVEC. After 24 hrs, cells were lysed and lysates electrophoresed on a SDS PAGE. Gels were transferred to nitrocellulose membranes and silencing of NRP-1 expression by the dsRNA oligonucleotides was detected by Western blot using C19 antibody that recognized the cytoplasmic domain of NRP-1.

Figure 7B: Experimental conditions were identical to Figure 7A except that 40 μg of the dsRNA oligonucleotides were used. Cells were plated on tissue culture dishes and at the indicated time intervals, cells were lysed and the amount of NRP-1 expression was detected as described in Example 5.

Figure 8: Binding of T2-TrpRS to HUVEC with RNAi was conducted as indicated in Example 5. For endothelial cell (EC) sample, cells were electroporated in the presence of PBS or with the indicated concentrations of dsRNAi oligonucleotides. Cells were incubated with either 300 nM biotin T2-TrpRS for total binding determination and 300 nM biotin T2-TrpRS plus 15 μM of T2-TrpRS for non specific binding determination. Data showed here is the specific binding of T2-TrpRS where total binding was subtracted from the non specific binding. Data points represent the means (+ SEM) of three observations.

Figure 9A shows that the chromogenic substrate S-2765 is specific to Fxa and non-reactive to HUVEC membrane, the phospholipids PC/PS nor T2-TrpRS.

Figure 9B shows that T2-TrpRS stimulates Fxa activities in the presence of HUVEC membrane fragments but not phospholipids or buffer alone (graph labelled no membrane).

Figure 10 shows that T2-TrpRS recombinant protein inhibits *in vivo* angiogenesis in a dose-dependent manner (average matrigel plug weight was 10 mg and the amount of VEGF used in these experiments was 0.5 nM).

Figure 11A: Northern analysis of RNA extracted from cells transduced with 500 PPC of Av3T2TrpRS shows presence of expected 1.4 Kb band.

Figure 11B: Western analysis of conditioned media (CM) and cell lysate (CL) shows presence of T2TrpRS protein in both the CM and CL fractions.

Figure 12 is a map of pAvCsT2TrpRS.

Figure 13 is a map of pNDSQ3.1.

DEFINITIONS

"Neuropilin" means neuropilin 1 (NRP1), neuropilin 2 (NRP2) and their isoforms, which are referred to collectively as neuropilin (Rossignol *et al., Genomics* 70: 211-222 (2000)).

"TrpRS" means tryptophanyl-tRNA synthetase.

"Truncated tRNA synthetase polypeptides" means polypeptides that are shorter than the corresponding full-length tRNA synthetase.

"Cell culture" encompasses both the culture medium and the cultured cells.

The phrase "isolating a polypeptide from the cell culture" encompasses isolating a soluble or secreted polypeptide from the culture medium as well as isolating an integral membrane protein from the cultured cells.

"Cell extract" includes culture media, especially spent culture media from which the cells have been removed. A cell extract that contains the DNA or protein of interest should be understood to mean a homogenate preparation or cell-free preparation obtained from cells that express the protein or contain the DNA of interest.

"Plasmid" is an autonomous, self-replicating extrachromosomal DNA molecule and is designated by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37 °C. are ordinarily used, but can vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment. The nucleotides present in various DNA and RNA fragments are designated herein by the standard single letter designations (A, T, C, G, U) used in the art.

"Polynucleotide" embodying the present invention can be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA can be double-stranded or single-stranded, and if single stranded can be the coding strand or non-coding (anti-sense) strand.

The term "Polynucleotide encoding a polypeptide" encompasses a polynucleotide that includes only coding sequence for the polypeptide as well as a polynucleotide that includes additional coding and/or non-coding sequence.

"Oligonucleotides" refers to either a single stranded polynucleotide or two complementary polynucleotide strands that can be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an A TP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Amino acid residue" refers to an amino acid that is part of a polypeptide. The amino acid residues described herein are preferably in the L" isomeric form. However, residues in the D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. All amino acid residue sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. A dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or to an amino-terminal group such as NH_2 or to a carboxyl-terminal group such as COOH.

In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in this art and can be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.,* Watson *et al. Molecular Biology of the Gene,* 4th Edition, 1987, The Benjamin/Cummings Pub. Co., p.224). Such substitutions are preferably made in accordance with those set forth as follows:

Original residue	Conservative substitution(s)
Ala	Gly; Ser
Arg	Lys
Asn	Gln; His
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala; Pro
His	Asn; Gln
Ile	Leu; Val

Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Tyr; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Other substitutions are also permissible and can be determined empirically or in accord with known conservative substitutions.

As used herein, a polypeptide "variant" or "derivative" refers to a polypeptide that is a mutagenized form of a polypeptide or one produced through recombination but that still retains a desired activity, such as the ability to bind to a ligand or a nucleic acid molecule or to modulate transcription. In particular, a derivative of T2-TrpRS refers to an alteration of the native T2-TrpRS to one produced through amino acid substitution, addition, or deletion. Thus, a derivative of T2-TrpRS includes a polypeptide in which one or more wild-type amino acids are substituted with alternate amino acids, and includes primary sequence changes. It also includes truncations of T2-TrpRS that retain T2-TrpRS's ability to specifically bind to neuropilin and thereby inhibit VEGF-mediated activity. Such truncations may include N-terminal deletions, C-terminal deletions, and internal deletions of amino acids.

As used herein, the term "nucleic acid cassette" refers to the genetic material of interest which can express a protein, or a peptide, or RNA after it is incorporated transiently, permanently or episomally into a cell. The nucleic acid cassette is positionally and sequentially oriented in a vector with other necessary elements such that the nucleic acid in the cassette can be transcribed and, when necessary, translated in the cell.

"Complementing plasmid" describes plasmid vectors that deliver nucleic acids into a packaging cell line for stable integration into a chromosome in the cellular genome.

"Delivery plasmid" is a plasmid vector that carries or delivers nucleic acids encoding a therapeutic gene or gene that encodes a therapeutic product or a precursor thereof or a regulatory gene or other factor that results in a therapeutic effect when delivered *in vivo* in or

into a cell line, such as, but not limited to a packaging cell line, to propagate therapeutic viral vectors.

A variety of vectors are described herein. For example, one vector is used to deliver particular nucleic acid molecules into a packaging cell line for stable integration into a chromosome. These types of vectors are generally identified herein as complementing plasmids. A further type of vector described herein carries or delivers nucleic acid molecules in or into a cell line *(e.g.,* a packaging cell line) for the purpose of propagating therapeutic viral vectors; hence, these vectors are generally referred to herein as delivery plasmids. A third "type" of vector described herein is used to carry nucleic acid molecules encoding therapeutic proteins or polypeptides or regulatory proteins or are regulatory sequences to specific cells or cell types in a subject in need of treatment; these vectors are generally identified herein as therapeutic viral vectors or recombinant adenoviral vectors or viral Ad-derived vectors and are in the form of a virus particle encapsulating a viral nucleic acid containing an expression cassette for expressing the therapeutic gene.

The terms "homology" and "identity" are often used interchangeably. In this regard, degree of homology or identity can be determined, for example, by comparing sequence information using a GAP computer program. The GAP program utilizes the alignment method of Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970), as revised by Smith and Waterman, *Adv. Appl. Math.* 2:482 (1981). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program can include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745 (1986), as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure,* National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Whether any two nucleic acid molecules have nucleotide sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical" can be determined using known computer algorithms such as the "FAST A" program, using for example, the default parameters as in Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 (1988). Alternatively the BLAST function of the National Center for Biotechnology Information database can be used to determine identity. In general, sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be calculated using published

techniques. (See, *e.g.: Computational Molecular Biology,* Lesk, A.M., ed., Oxford University Press, New York, (1988); Smith, D.W., ed., *Biocomputing: Informatics and Genome Projects,* Academic Press, New York, (1993); Griffm, A.M., and Griffin, H.G., eds., *Computer Analysis of Sequence Data, Part I,* HumanaPress, New Jersey, (1994); vonHeinje, G., *Sequence Analysis in Molecular Biology,* Academic Press, (1987); and Gribskov, M. and Devereux, J., eds., *Sequence Analysis Primer,* M Stockton Press, New York, (1991)). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo, H. & Lipton, D., *SIAMJ. Applied Math.* 48:1073 (1988)). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Martin J. Bishop, ed., *Guide to Huge Computers,* Academic Press, San Diego, (1994), and Carillo, H. & Lipton, D., *SIAMJ. Applied~ath.* 48:1073 (1988). Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., *et al., Nucleic Acids Research* 12(1):387 (1984), BLASTP, BLASTN, FASTA (Atschul, S.F., *et al., J. Malec. Bioi.* 215:403 (1990)).

The term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide. For example, a test polypeptide can be defined as any polypeptide that is 90% or more identical to a reference polypeptide. As used herein, the term at least "90% identical to" refers to percent identities from 90 to 99.99 relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polynucleotide length of 100 amino acids are compared. No more than 10% (i.e., 10 out of 100) amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons can be made between a test and reference polynucleotides. Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they' can be clustered in one or more locations of varying length up to the maximum allowable, e.g. 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, or deletions.

The terms "gene therapy" and "genetic therapy" refer to the transfer of heterologous DNA to the certain cells, target cells, of a mammal, particularly a human, with a disorder or conditions for which such therapy is sought. The DNA is introduced into the selected target cells in a manner such that the heterologous DNA is expressed and a therapeutic product

encoded thereby is produced. Alternatively, the heterologous DNA can in some manner mediate expression of DNA that encodes the therapeutic product, it can encode a product, such as a peptide or RNA that in some manner mediates, directly or indirectly, expression of a therapeutic product. Genetic therapy can also be used to nucleic acid encoding a gene product replace a defective gene or supplement a gene product produced by the mammal or the cell in which it is introduced. The introduced nucleic acid can encode a therapeutic compound, such as a growth factor inhibitor thereof, or a tumor necrosis factor or inhibitor thereof, such as a receptor therefor, that is not normally produced in the mammalian host or that is not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous DNA encoding the therapeutic product can be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof.

"Heterologous DNA" is DNA that encodes RNA and proteins that are not normally produced *in vivo* by the cell in which it is expressed or that mediates or encodes mediators that alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes. Heterologous DNA can also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers drug resistance, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies. Antibodies that are encoded- by heterologous DNA can be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced. Hence, "heterologous DNA" or "foreign DNA", refers to a DNA molecule not present in the exact orientation and position as the counterpart DNA molecule found in the corresponding wild-type adenovirus. It can also refer to a DNA molecule from another organism or species *(i.e.,* exogenous) or from another Ad serotype.

"Therapeutically effective DNA product" is a product that is encoded by heterologous DNA so that, upon introduction of the DNA into a host, a product is expressed that effectively ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures said disease. Typically, DNA encoding the desired heterologous DNA is cloned into a plasmid vector and introduced by routine methods, such as calcium-phosphate mediated DNA uptake or microinjection, into producer cells, such as packaging cells. After amplification in

producer cells, the vectors that contain the heterologous DNA are introduced into selected target cells.

"Expression or delivery vector" refers to any plasmid or virus into which a foreign or heterologous DNA can be inserted for expression in a suitable host cell- *i.e.,* the protein or polypeptide encoded by the DNA is synthesized in the host cell's system. Vectors capable of directing the expression of DNA segments (genes) encoding one or more proteins are referred to herein as "expression vectors." Also included are vectors that allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

"Gene" is a nucleic acid molecule whose nucleotide sequence encodes RNA or polypeptide. A gene can be either RNA or DNA. Genes can include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

"Isolated" with reference to a nucleic acid molecule, polypeptide, or other biomolecule, means that the nucleic acid or polypeptide has separated from the genetic environment from which the polypeptide or nucleic acid was obtained. It can also mean altered from the natural state. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell is considered isolated. Also intended as an "isolated polypeptide" or an "isolated polynucleotide" are polypeptides or polynucleotides that have been purified, partially or substantially, from a recombinant host cell or from a native source. For example, a recombinantly produced version of a compounds can be substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31-40 (1988). The terms "isolated" and "purified" are sometimes used interchangeably. Such polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

By "isolated polynucleotide" is meant that the nucleic acid is free of the coding sequences of those genes that, in the naturally occurring genome of the organism (if any) immediately flank the gene encoding the nucleic acid of interest. Isolated DNA can be single-stranded or double-stranded, and can be genomic DNA, cDNA, recombinant hybrid DNA, or synthetic DNA. It can be identical to a native DNA sequence, or can differ from such sequence by the deletion, addition, or substitution of one or more nucleotides.

"Isolated" or "purified" as it refers to preparations made from biological cells or hosts means any cell extract containing the indicated DNA or protein including a crude extract *of* the DNA or protein of interest. For example, in the case *of* a protein, a purified preparation can be obtained following an individual technique or a series *of* preparative or biochemical techniques and the DNA or protein *of* interest can be present at various degrees *of* purity in these preparations. The procedures can include for example, but are not limited to, ammonium sulfate fractionation, gel filtration, ion exchange change chromatography, affinity chromatography, density gradient centrifugation and electrophoresis.

A preparation of DNA or protein that is "substantially pure" or "isolated" means a preparation free from naturally occurring materials with which such DNA or protein is normally associated in nature. "Essentially pure" should be understood to mean a "highly" purified preparation that contains at least 95 % of the DNA or protein of interest.

"Packaging cell line" is a cell line that provides a missing gene product or its equivalent.

"Adenovirus viral particle" is the minimal structural or functional unit of a virus. A virus can refer to a single particle, a stock of particles or a viral genome. The adenovirus (Ad) particle is relatively complex and can be resolved into various substructures.

"Post-transcription regulatory element (PRE)" is a regulatory element found in viral or cellular messenger RNA that is not spliced, i.e. intronless messages. Examples include, but are not limited to, human hepatitis virus, woodchuck hepatitis virus, the TK gene and mouse histone gene. The PRE can be placed before a polyA sequence and after a heterologous DNA sequence

"Pseudotyping" describes the production of adenoviral vectors having modified capsid protein or capsid proteins from a different serotype than the serotype of the vector itself. One example is the production of an adenovirus 5 vector particle containing an Ad37 fiber protein. This can be accomplished by producing the adenoviral vector in packaging cell lines expressing different fiber proteins.

"Promoters of interest herein" can be inducible or constitutive. Inducible promoters will initiate transcription only in the presence of an additional molecule; constitutive promoters do not require the presence of any additional molecule to regulate gene expression. a regulatable or inducible promoter can also be described as a promoter where the rate or extent of RNA polymerase binding and initiation is modulated by external stimuli. Such stimuli include, but are not limited to various compounds or compositions, light, heat, stress and chemical energy sources. Inducible, suppressible and repressible promoters are considered regulatable

promoters. Preferred promoters herein, are promoters that are selectively expressed in ocular cells, particularly photoreceptor cells.

"Receptor" refers to a biologically active molecule that specifically binds to (or with) other molecules. The term "receptor protein" can be used to more specifically indicate the proteinaceous nature of a specific receptor.

"Recombinant" refers to any progeny formed as the result of genetic engineering. This can also be used to describe a virus formed by recombination of plasmids in a packaging cell.

"Transgene" or "therapeutic nucleic acid molecule" includes DNA and RNA molecules encoding an RNA or polypeptide. Such molecules can be "native" or naturally derived sequences; they call also be "non-native" or "foreign" that are naturally or recombinantly derived. The term "transgene," which can be used interchangeably herein with the term "therapeutic nucleic acid molecule," is often used to describe a heterologous or foreign (exogenous) gene that is carried by a viral vector and transduced into a host cell. Therapeutic nucleotide nucleic acid molecules include antisense sequences or nucleotide sequences that can be transcribed into antisense sequences. Therapeutic nucleotide sequences (or transgenes) all include nucleic acid molecules that function to produce a desired effect in the cell or cell nucleus into which said therapeutic sequences are delivered. For example, a therapeutic nucleic acid molecule can include a sequence of nucleotides that encodes a functional protein intended for delivery into a cell which is unable to produce that functional protein.

"Promoter region" refers to the portion of DNA of a gene that controls transcription of the DNA to which it is operatively linked. The promoter region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of the RNA polymerase. These sequences can be *cis* acting or can be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, can be constitutive or regulated.

"Operatively linked" means that the sequences or segments have been covalently joined into one piece of DNA, whether in single or double stranded form, whereby control sequences on one segment control expression or replication or other such control of other segments. The two segments are not necessarily contiguous, however.

"Complex" as used herein refers to the product of a specific binding reaction such as an antibody-antigen or receptor-ligand reaction. Exemplary complexes are immunoreaction products.

"Label" and" Indicating means" in their various grammatical forms refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal to indicate the presence of a complex. Any label or indicating means can be linked to or incorporated in an expressed protein, polypeptide, or antibody molecule that is part of an antibody or monoclonal antibody composition of the present invention or used separately, and those atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well known in clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel proteins methods and/or systems.

DETAILED DESCRIPTION OF THE INVENTION

While the role of neuropilin as a mediator of VEGF activity has been suggested (Soker *et al., Cell* 92: 735-745 (1998)), the present disclosure supports for the first time a specific interaction between T2-TrpRS and neuropilin and that this binding inhibits VEGF-mediated activity. The current invention therefore offers improvements toward modulation of neuropilin-mediated activity for therapeutic purposes.

First, the finding that T2-TrpRS acts by binding to neuropilin indicates that it can be an effective inhibitor of disease processes that act through neuropilin. For example, it is known that neuropilins serve as co-receptors with the plexins in mediating the activity of the semaphorins. Thus, the present invention includes methods for the treatment of all diseases impacted by T2-TrpRS or T2-TrpRS derivatives via their interaction with neuropilins. The T2-TrpRS or derivatives can be delivered as a recombinant protein or by gene therapy vectors including but not limited to adenoviral, AAV, HSV vector, retroviral, lentiviral, and plasmid vectors. The invention further includes small molecules or other drugs based on this interaction. The present invention further provides for the use of surrogate markers such as overproduction of VEGF to target specific cancers for treatment and evaluation of T2-TrpRS-based treatment.

Accordingly, one aspect of the present invention is directed to the use of T2-TrpRS, its derivatives, modifications, or small molecules or drugs based on this interaction for the treatment of cancer because neuropilin-1 is known to be expressed by newly formed blood

vessels and by tumor cells, including but not limited to metastatic prostate tumor cells, breast carcinomas, and melanoma (Soker *et al., Cell* 92:735-745 (1998); Latil *et al., Int. J. Cancer* 89: 167-171 (2000)). Neuropilin expression by tumor cells promotes tumor angiogenesis and progression (Miao *et al., FASEB J.* 14: 2532-2539 (2000)). Furthermore, increased expression of endothelial neuropilin is associates with neuroblastoma stages I-IV (Fakhari *et al., Cancer* 94(1): 258-263 (2001)).

Another aspect of the present invention is directed to the use of T2-TrpRS, its derivatives, modifications, or small molecules or drugs based on this interaction for the treatment of nervous system injury (Pasterkamp *et al., Brain Res. Rev.* 35: 36-54 (2001)). This includes the treatment of traumatic brain and spinal cord injury including but not limited to neuronal cell apoptosis and scar formation, neural angiogenesis, and peripheral nerve regeneration. For example, prolonged binding of semaphorin 3A (sem3A) to neuropilin 1 induces cell death (Bagnard *et al., J. of Neurosci.* 21(10): 3332-41 (2001)). Thus, T2-TrpRS can act as an antagonist of sem3A-nuropilin-1 interaction to prevent apoptosis and promote cell survial, and possibly proliferation of neuronal stem cells. A second example is that the interaction between semaphorin IV (semIV) and neurophilin-2 defines specific repulsive axon guidance events *in vivo* (Kolodkin et *al., Neuron* 21: 1079-92 (1998)); thus, T2-TrpRS can bind to neuropilin-2 and therapeutically modify this process.

Another aspect of the present invention is directed to the use of T2-TrpRS, its derivatives, modifications, or small molecules or drugs based on this interaction for the treatment of rheumatoid arthritis. Neuropilin up-regulation has been detected in the synovial tissues of rheumatoid arthritis patients and this correlated with increased vascular density (Ikeda *et al., J. of Path.* 191: 426-433 (2000)).

Another aspect of the present invention is directed to the use of T2-TrpRS, its derivatives, modifications, or small molecules or drugs based on this interaction for the treatment of organogenesis and development anomalies. Both semaphorin III (nerves, bones, and heart) (Behar *et al., Nature* 383: 525-528 (1996)) and neuropilin-1 (blood vessel) (Kawasaki *et al., Development* 126: 4895-4902 (1999)) have been shown to be critical cues for appropriate embryonic development. Furthermore, a double knockout of NRP-1 and NRP-2 revealed more severe vascular defects, suggesting that both are required for appropriate blood vessel development (Takashima *et al., Proc. Natl. Acad. Sci.* 99: 3657-3662 (2002)).

Another aspect of the present invention is directed to the use of T2-TrpRS, its derivatives, modifications, or small molecules or drugs based on this interaction for the

treatment of ocular-related diseases such as Proliferate Diabetic Retinopathy and Age Related Macular Degeneration, because neuropilin-1 and KDR/Flk-1 have been found to co-localized in the area of neovascularized vessels of the retina (Maramatsu *et al., Inves Ophthalmol Vis. Sci.* 42(6): 1172-8 (2001); Ikeda *et al., Inves Ophthalmol Vis. Sci.* 41(7): 1649-56 (2000)).

Another aspect of the present invention is directed to the use use of T2-TrpRS, its derivatives, modifications, or small molecules or drugs based on this interaction for the treatment of angiogenesis diseases associated with inappropriate arterial-venous junctions or conversion such as in vein graft stenosis, because neuropilin-1 is preferentially express by arterial and neuropilin-2 is preferentially expressed by venous blood vessels (Herzog *et al., Mech. Dev.* 109 (1): 115-9 (2001)).

Second, discovery of the mechanism of action of T2-TrpRS creates the opportunity to model the molecule and design even more potent antagonists of angiogenesis to treat angiogenesis-related diseases and potentially other diseases mediated by neuropilin. It is also envisioned that this discovery enables identification of small molecule antagonists by further defining the interaction. For example, using peptides and site-directed mutagenesis to identify the sequences or residue(s) that are important for T2-TrpRS binding to neuropilin would allow the creation of novel molecules with a better binding interaction. By understanding the charge-charge interactions and hydrophobicity/hydrophilicity required for binding, it would be possible to design a small molecule agonist or antagonist of the interaction.

The N-terminal of T2-TrpRS contains a region of high sequence of homology to semaphorin E (SemE), a binding partner for neuropilin (see Figure 2A). However, there are interesting differences in the primary sequence of T2-TrpRS and SemE that can be used as the starting point for additional constructs. For example, altering the T2-TrpRS Tyr_{100} -> Lys or T2-TrpRS Phe_{107} ->Ile could alter the charge-to-charge interaction between the binding surfaces and enhance T2-TrpRS binding to neuropilin. The findings described herein indicate a common neuropilin binding element between the semaphorins and VEGF. Further analysis will facilitate the identification of functional specific agonists and antagonists of neuropilin ligands.

Third, neuropilin belongs to a large class of receptors that contain CUB and blood coagulation factor V/VIII (F V/VIII) domains. CUB domains are widespread in other proteins and are important in developmental regulation. CUB domains have been found in several classes of proteases, such as spermadhesins, procollagen C-proteinase, and the complement system proteins. Database mining indicates that there are other proteins that share sequence homology to the CUB domain of neuropilin. We have identified these as endothelial and

smooth muscle cell-derived neuropilin-like molecule (ESDN), neuropilin and tolloid-like 1 and 2 (NETO1 and NETO2) (Kobuke *et al., J. Biol. Chem.* 276(36): 34105-14 (2001); Stohr *et al. Gene* 286: 223-31 (2002)). In addition to the CUB domains, other proteins e.g. blood coagulation factor V and VIII, which play an important role in the homeostasis and pathophysiology of the vascular system, share the same b1/b2 domain structure of neuropilins.

The binding of VEGF to neuropilin has been found to reside within the b1/b2 domain and the binding is enhanced by the a1/a2 domain (J. Biol. Chem. 277(27): 24818-25 (July 5, 2002)). Because VEGF binds to neuropilin and is able to compete with T2-TrpRS for neuropilin binding, it is likely that the a1/a2 and/or b1/b2 domains of neuropilin are involve in the binding to T2-TrpRS. Therefore, factor V, factor VIII, NETO1. NETO2 and ESDN may represent additional binding partners for T2-TrpRS and represent novel activities of T2-TrpRS. For example, the blood protein factor Xa (Fxa) binds to the membrane bound EPR-1 protein that is similar to the light chain of factor Va (Altieri, *FASEB J.* 9: 860-865 (1995)); this protein can act as a cofactor for Fxa to catalyze prothrombin activation in the absence of added factor Va (Ambrosini *et al., J. Biol. Chem.* 271: 1243-1248 (1996). Because the light chain of factor Va contains the A3 domain, which shares sequence similarity to the b1/b2 domain of NRP-1, it is possible that VEGF as well as T2-TrpRS may be involved in the regulation of blood coagulation through their interactions with factor Va or factor Va-like protein such as EPR-1. We have data to demonstrate that this observation is credible; our *in vitro* data indicates that T2-TrpRS accelerates Fxa activity in the presence of HUVEC membrane but not phospholipids. Furthermore, our *in vivo* data showes that T2-TrpRS is not only a potent inhibitor of VEGF-induced angiogenesis, it also modulates the coagulation system. Visual inspection of the matrigel plug indicated that it was clear and contained very little or no blood at all. Furthermore, no thrombus was observed within the vicinity for the plug. It is possible that the unexpected high potency of the T2-TrpRS in this assay is accounted for by interaction of T2-TrpRS with neuropilin as well as these putative additional identified binding partners.

Viral Delivery systems

Viral transduction methods for delivering nucleic acid constructs to cells are contemplated herein. Suitable DNA viral vectors for use herein includes, but are not limited to an adenovirus (Ad), adeno-associated virus (AAV), herpes virus, vaccinia virus or a polio virus. A suitable RNA virus for use herein includes but is not limited to a retrovirus or Sindbis virus. It is to be understood by those skilled in the art that several such DNA and RNA viruses exist

that may be suitable for use herein. Adenoviral vectors have proven especially useful for gene transfer into eukaryotic cells and are widely available to one skilled in the art and is suitable for use herein.

Adeno-associated virus (AAV) has been used as a gene transfer system with applications in gene therapy. See U.S. Patents Nos. 5,139,941; 5,436,146; and 5,622,856. Herpes simplex virus type-1 (HSV-1) vectors are available and are especially useful in the nervous system because of its neurotropic property. See U.S. Patent No. 5,288,641. Vaccinia viruses, of the poxvirus family, have also been developed as expression vectors. Each of the above-described vectors is widely available and is suitable for use herein.
Retroviral vectors are capable of infecting a large percentage of the target cells and integrating into the cell genome. Preferred retroviruses include lentiviruses, and also include, but are not limited to, HIV, BIV and SIV. See U.S. Patents Nos.5,665,577; 5,994,136; 6,013,516; 5,672,510; 5,707,865 and 5,817,491.

Various viral vectors that can be used for gene therapy as taught herein include adenovirus (See U.S. Patent No.5,935,935), herpes virus, vaccinia, adeno-associated virus (AAV), or, preferably, an RNA virus such as a retrovirus, and also include a modified viral vector, such as an adenovirus, known as a "gutless" vector. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus, or is a lentiviral vector. The preferred retroviral vector is a lentiviral vector. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), SIV, BIV, HIV and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a zinc finger derived-DNA binding polypeptide sequence of interest into the viral vector, along with another gene that encodes the ligand for a receptor on a specific target cell, for example, the vector is made target specific.

Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing the zinc finger-nucleotide binding protein polynucleotide.

Because recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include but are not limited to Ψ2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced. The vector virions produced by this method can then be used to infect a tissue cell line, such as NIH 3T3 cells, to produce large quantities of chimeric retroviral virions.

Gutless viral vectors

In a particularly preferred embodiment of the present invention the viral vector used is a "gutless" adenoviral vector. Such vectors are devoid of all viral coding regions and contain only the essential adenovirus packaging signals and the transgene expression cassette. (See Example 6). They may be prepared by techniques known to those of skill in the art. Sandig et al. PNAS 97:1002-1007 (2000)

Nonviral Delivery systems

"Non-viral" delivery techniques for gene therapy include DNA-ligand complexes, adenovirus-ligand-DNA complexes, direct injection of DNA, $CaPO_4$ precipitation, gene gun techniques, electroporation, liposomes and lipofection. Any of these methods are available to one skilled in the art and would be suitable for use herein. Other suitable methods are available to one skilled in the art, and it is to be understood that the herein may be accomplished using any of the available methods of transfection.

Another targeted delivery system is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes, which are preferred. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large

macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley *et al., Trends Biochem. Sci.*, 6:77, 1981).

Lipofection may be accomplished by encapsulating an isolated nucleic acid molecule within a liposomal particle and contacting the liposomal particle with the cell membrane of the target cell. Liposomes are self-assembling, colloidal particles in which a lipid bilayer, composed of amphiphilic molecules such as phosphatidyl serine or phosphatidyl choline, encapsulates a portion of the surrounding media such that the lipid bilayer surrounds a hydrophilic interior. Unilammellar or multilammellar liposomes can be constructed such that the interior contains a desired chemical, drug, or, as provide herein, an isolated nucleic acid molecule.

Liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells as well as mammalian cells. In order for a liposome to be an efficient gene transfer vehicle, characteristics among the following should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino *et al., Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting uses the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal

capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

In general, the compounds bound to the surface of the targeted delivery system are ligands and receptors permitting the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest that interacts with another compound, such as a receptor.

In general, surface membrane proteins that bind to specific effector molecules are referred to as receptors. Antibodies are preferred receptors. Antibodies can be used to target liposomes to specific cell-surface ligands. For example, certain antigens expressed specifically on tumor cells, referred to as tumor-associated antigens (TAAs), may be exploited for the purpose of targeting antibody-zinc finger-nucleotide binding protein-containing liposomes directly to the malignant tumor. Since the zinc finger-nucleotide binding protein gene product may be indiscriminate with respect to cell type in its action, a targeted delivery system offers a significant improvement over randomly injecting non-specific liposomes. A number of procedures can be used to covalently attach either polyclonal or monoclonal antibodies to a liposome bilayer. Antibody-targeted liposomes can include monoclonal or polyclonal antibodies or fragments thereof such as Fab, or $F(ab')_2$, as long as they bind efficiently to an the antigenic epitope on the target cells. Liposomes may also be targeted to cells expressing receptors for hormones or other serum factors.

Delivery of constructs to cells

The cells may be transfected *in vivo*, *ex vivo* or *in vitro*. The cells may be transfected as primary cells isolated from a patient or a cell line derived from primary cells, and are not necessarily autologous to the patient to whom the cells are ultimately administered. Following *ex vivo* or *in vitro* transfection, the cells may be implanted into a host. Genetic modification of

the cells may be accomplished using one or more techniques well known in the gene therapy field (see, *e.g.*, (1994) *Human Gene Therapy* 5:543-563).

Administration of a nucleic acid molecules provided herein to a target cell *in vivo* may be accomplished using any of a variety of techniques well known to those skilled in the art. The vectors of the herein may be administered orally, parentally, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. Suppositories for rectal administration of the drug can be prepared by mixing the drug with a suitable non-irritating excipient such as cocoa butter and polyethylene glycols that are solid at ordinary temperatures but liquid at the rectal temperature and therefore melt in the rectum and release the drug.

The dosage regimen for treating a disorder or a disease with the vectors and/or compositions provided is based on a variety of factors, including the type of disease, the age, weight, sex, medical condition of the patient, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined empirically using standard methods.

The pharmaceutically active compounds (*i.e.*, vectors or ligands) can be processed in accordance with conventional methods of pharmacy to produce medicinal agents for administration to patients, including humans and other mammals. For oral administration, the pharmaceutical composition may be in the form of, for example, a capsule, a tablet, a suspension, or liquid. The pharmaceutical composition is preferably made in the form of a dosage unit containing a given amount of DNA or viral vector particles (collectively referred to as "vector"). For example, these may contain an amount of vector from about 10^3-10^{15} viral vector particles, preferably from about 10^6-10^{12} viral particles. A suitable daily dose for a human or other mammal may vary widely depending on the condition of the patient and other factors, but, once again, can be determined using routine methods. The vector may also be administered by injection as a composition with suitable carriers including saline, dextrose, or water.

While the nucleic acids and /or vectors herein can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more vectors or other agents. When administered as a combination, the therapeutic agents can be formulated as separate compositions that are given at the same time or different times, or the therapeutic agents can be given as a single composition.

Ligands similarly may be delivered by any suitable mode of administration, including by oral, parenteral, intravenous, intramuscular and other known routes. Any known pharmaceutical formulations is contemplated.

The following examples are to illustrate the invention, but should not be interpreted as a limitation thereon.

EXAMPLES

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by, for example, Ausubel (ed.), *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. (1994); J. Sambrook and D.W. Russell, *Molecular Cloning: A Laboratory Manual,* 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (2001); and by T. J. Silhavy, M. L. Berman, and L. W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1984).

EXAMPLE 1: Binding of T2-TrpRS to NRP is Specific and Can Be Competed by VEGF

Binding assays were performed in 96 well PolySorb plates (Fisher Scientific, Newark, DE). Wells were absorbed with 0.5 μM of test protein for 2 hrs at room temperature, washed 3 times with PBS, and incubated with 200 μl of 0.2% Tween 20 and 0.5% BSA in PBS for one hr to block non-specific binding sites. The wells were then washed 3 times with washing buffer (0.05% Tween 20, 0.5% BSA in PBS). After washing, recombinant Biotin T2-TrpRS was added and binding was allowed to occur overnight at 4°C. Wells were washed 3 times with wash buffer to remove unbound T2-TrpRS. For VEGF competition assays, T2-TrpRS (500 nM) was mixed with VEGF at the indicated concentrations and incubated on soluble NRP-1 (sNRP-1) containing plate and treated as above. Avidin-HRP (Sigma, St. Louis, MO) was used to detect the bound T2-TrpRS. The wells were washed 3 times with PBS and the amount of T2-TrpRS bound was detected colormetrically by reading A_{415}.

Binding data were analyzed using Sigma Plot. Equations used to calculate direct binding and competition binding are as follows:

Direct binding: $Y = (AX)/(B+ X)$

Where X= Concentration of protein added

Y= A_{415} values

B= Estimated K_d

A= Estimated maximum binding

Competition Binding: $Y = A/(1+ BX)$

Where the concentration of T2-TrpRS (X) added was held constant either 100 or 500 nM and B is the estimated IC_{50}.

The specificity and the affinity of T2-TrpRS binding to sNRP-1 was examined utilising ELISA based binding assays. The K_d for T2-TrpRS binding to NRP-1 was determined to be 50-160 nM in three separate binding experiments and the K_d for T2-TrpRS binding to NRP-2 was determined to be 20-100 nM in two separate binding experiments; whereas, the binding of T2-TrpRS to Flt-1 and KDR remained low and statistically indistinguishable from back ground binding (Fig. 3A). Furthermore, because VEGF can bind to NRP-1 with high affinity, VEGF as well as bFGF (used as a negative control) were used in a competition binding experiment to determine their capacities to inhibit T2-TrpRS binding to NRP-1. It was found that VEGF, but not bFGF, inhibited T2-TrpRS binding to sNRP-1 with an $IC_{50} \sim 100$ nM (Fig. 3B) which is similar to the K_d of T2-TrpRS binding to sNRP-1. Despite the fact that K_d and IC_{50} values are incomparable, it is apparent that the saturable binding observed with T2-TrpRS to sNRP-1 is readily reversible with VEGF. Thus, it is postulated that these molecules share at least one domain on the same binding surface on the sNRP-1 molecule.

Table 1: Summary of the T2-TrpRS Direct Binding Experiments

Protein	K_d (nM)
NRP-1	50 - 160 nM
NRP-2	20- 100 nM
Flt-1	None
KDR	None

Summary of the competition binding experiments: VEGF competes with T2-TrpRS for binding to NRP-1: $IC_{50} \sim 100$ nM.

EXAMPLE 2: T2-TrpRS Inhibits VEGF-induced Receptor Tyrosine Kinase Activity

Receptor tyrosine kinase (RTK) assays were conducted according to method by Schraag *et al., Anal. Biochem.* 221: 233-239 (1993) with minor modifications. Briefly, HUVECs (passages 5-7) membrane fragments were prepared from endothelial cells grown to ~90% confluency. Cells were detached from the surface with 10mM EDTA in PBS and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in 5 ml of extraction buffer (PBS, Protease inhibitors, 0.2nM Na_3VO_4, 5mM mercaptoethanol, 1mM EDTA) and lysed by homogenizing for 2 min. The extract was transferred to microcentrifuge tubes and centrifuged for 5 minutes at maximum speed (14,000 rpm). The supernatant was centrifuged twice at 100,000 g to pellet the cell membrane. Cell membrane was resuspended in PBS and total protein concentration was determined using the BCA protein determination method (BioRad, Hercules, CA). Cell membrane was flash freeze and kept frozen until use. The RTK activity of the extracts was determined using Oncogen's protein tyrosine kinase assay kit (Oncogene, Boston, MA). Essentially, the membrane preparations were incubated on plates containing polymeric tyrosine residues that serve as substrates for RTKs. After a 30-minute incubation, the reaction was terminated and the amount of phosphorylated tyrosine quantitated by binding to an HRP-conjugated phosphotyrosine specific antibody. Experiments were done in triplicate.

T2-TrpRS was examined in the RTK activity assay and found to have no effect on basal RTK activity in the range of concentrations tested (Fig. 4A). However, T2-TrpRS was a potent inhibitor of VEGF-induced RTK activity and suppressed VEGF activity with an IC_{50} of ~100nM (Fig. 4A). T2-TrpRS blocked VEGF induced RTK stimulation (Fig. 4A and B) but had no effect on bFGF-induced RTK activity (Fig. 4B). The readings were converted into relative percent of RTK activity to allow comparison between VEGF and FGF. Full length TrpRS was also tested and found to have no effect on VEGF-induced membrane RTK activity (Fig. 4C).

Table 2: Summary of the RTK Activity Experiments

Protein	%RTK Stimulation
VEGF	100
T2-TrpRS	2
VEGF + T2-TrpRS	50
VEGF + Full Length TrpRS	100

bFGF	100
bFGF + T2-TrpRS	100
Membrane only	0

VEGF = 0.5 nM

BFGF = 0.5 nM

T2-TrpRS = 100 nM

Membrane used: 0.1 mg/ml

% calculation assumed that VEGF or bFGF stimulation is 100% and membrane alone is 0%.

EXAMPLE 3: T2-TrpRS Induces Apoptosis on Human Endothelial Cells

Apoptosis assays were conducted according to methods described by Gerber *et al., J. Biol. Chem.* 273: 30336-30343 (1998); Kanellis *et al., Am. J. Physiol. Renal Physiol.* 278: F905-915 (2000); Kwak *et al., FEBS Lett.* 448: 249-253 (1999); and Mesri *et al., Am. J. Pathol.* 158: 1757-1765 (2001) with minor modifications. Briefly, Human umbilical vein endothelial cells (HUVECs) (Vec Technologies, Inc. Rensselaer, NY) at passage 7 were seeded at 10^4 per well in a 96-well flat bottom plate (CoStar, Corning, New York) in MCDB131 medium (Vec Technologies, Inc. Rensselaer, NY) and incubated in a 37^0C, 5% CO_2 humidified incubator overnight. Cells were incubated in MCDB131 and 10% FBS (BioWittaker, Walkersville, MD) for 24 hours. Wells were washed twice with 37^0C HBSS prior to the addition of the treatments. All treatments were added in triplicate in a final volume of 200 µl of M199 per well. The cells were incubated at 37^0C for 24 hr and the degree of apoptosis was determined by ELISA. The Cell Death Detection ELISA^{PLUS} (Roche Molecular Biochemicals, Germany) is a photometric enzyme-immunoassay for the *in vitro* determination of cytoplasmic histone-associated-DNA fragments after cell death. Briefly, a 20 µl aliquot of cell lysate was placed into a streptavidin-coated MTP plate followed by the addition of 80 µl of an anti-histone-biotin and anti-DNA-POD mixture (Roche Molecular Biochemicals, Germany). After a 2 hour incubation the unbound antibodies were washed off. The amount of nucleosomes retained by the POD (the DNA components of the nucleosomes) in the immunocomplex was determined photometrically at 405 nm on a plate reader (Spectra Max, Molecular Devices) with ABTS as the substrate (reference wavelength at 490 nm). The numerical values obtained from A_{405} minus the reference A_{490} readings are used as the apoptosis index. Experiments were done in triplicates.

The effect of T2-TrpRS on HUVEC apoptosis alone or in combination with 0.5 nM VEGF or bFGF was examamined. When T2-TrpRS was added at concentrations between 0.01-1,000nM, alone or in the presence of either VEGF or bFGF, T2-TrpRS consistently enhanced the level of endothelial cell apoptosis in the presence of VEGF, but not bFGF (Fig. 5). At a low concentration of T2-TrpRS and 0.5nM VEGF, the apoptosis index was ~1.0, as the concentration of T2-TrpRS increased, the apoptosis index increased to a maximum of 2.2 (Fig. 5). The concentration in which T2-TrpRS induced 50% of cell death (IC_{50}) was estimated to be ~100nM. The results are summarized in the following table.

Table 3: Summary of the Apoptosis Experiments

Protein	%HUVEC apoptosis
VEGF	0
T2-TrpRS	100
VEGF + T2-TrpRS	50
bFGF	0
bFGF + T2-TrpRS	0
M199 only	100

VEGF = 0.5 nM

BFGF = 0.5 nM

T2-TrpRS = 100 nM

% calculation assumed that VEGF or bFGF alone offered 100% protection from cell death and cell grown in M199 media alone is 100% apoptotic.

EXAMPLE 4: Specific Binding of T2-TrpRS to Endothelial Cells

The nonradioactive cell binding assay was adapted from method by Vieira A. (*Molecular Biotechnology* 247(10): 247-50 (1998)). The following buffers were prepared before the experiment: ST Buffer (1 nM EDTA, 50 mM NaCl, 100 mM Tris-HCL, 0.1% SDS, 1% Triton X-100, 0.2% BSA, pH 7.4); Buffer A (200 mM HEPES, 100 mM potassium acetate, 85 mM sucrose, 1 mM magnesium acetate, pH 7.4); Buffer B1 (200 mM HEPES, 100 mM potassium acetate, 75 mM sucrose, 1 mM magnesium acetate, pH 7.4); Buffer B2 (200 mM HEPES, 100 mM potassium acetate, 1 mM magnesium acetate, pH 7.4); Binding Buffer (200 mM HEPES, 100 mM potassium acetate, 1 mM magnesium acetate, 0.2 mg/ml BSA, pH 7.4).

The ligand-capture plates were prepared as follow: The ELISA plate was coated with 100 µl of 10 nM anti 6 His tag monoclonal Ab diluted in PBS for 2 hrs at room temperature wells were blocked with 200 µl PBS, 0.5% BSA, 0.2% Tween 20 for 2 hrs at room temperature.

Perforated HUVEC's were prepared as follows: Passage cells at 70% confluency in a 150 mm dish (~3.5 x 10^6 cells). Cells were washed three times with cold Buffer A, then scraped and collected in a siliconized tube containing 40 ml of ice-cold Buffer A. Cells were resuspended and centrifuged at 1000 g for 5 min. at 4°C, the supernatant was discarded and the pellet was resuspended in 2 volume of Buffer B1. Cells were frozen and kept in liquid nitrogen.

Biotin T2-TrpRS was prepared as follows: Biotinylation of T2-TrpRS was conducted as outlined in the protocol provided by the manufacture (Pierce, Rockford, IL). Briefly, 100 µl of the stock T2-TrpRS (2 mg/ml) was incubated with 50 fold molar excess of the Sulfo-NHS-biotin for 2 hrs on ice. The unbound biotin was removed by a desalting column and the amount of biotin bound to T2-TrpRS was calculated to be 1:1 molar ratio using the HABA method for determining biotin incorporation.

The binding assay was done as follows: Cells were thawed on ice followed by a 5 second centrifuge at 3500 g. The supernatant was removed and cells were resuspended in ice-cold Buffer B2 at the concentration of ~5 x 10^6 cells /ml. For total binding, the biotinylated T2-TrpRS was added and for non-specific binding, biotin-T2-TrpRS was added with 100 fold molar excess of unlabelled T2-TrpRS. The final volume of 100 µl contained 500,000 cells reaction mixture tubes was gently mixed and incubate at 4°C for 3 hr.

The binding of T2-TrpRS to HUVECs was detected as follows: The reaction mixture tubes were centrifuged for 5 seconds at 14,000 rpm to pellet cells. Pellet was washed three times in 1 ml of ice-cold Binding Buffer then centrifuged for 5 seconds to remove supernatant. Cells were lysed by adding 200 µl of ST Buffer. 50 µl of cell lysates was added to the wells of the ligand capture plate and incubated overnight with gentle rocking. To detect binding, wells were washed with 200 µl PBS, 0.5% BSA, 0.05% Tween 20 three times. The amount of T2-TrpRS bound was detected by adding Avidin-HRP followed by p-nitropheyl phosphate to develop a color reaction which was measured at 415 nm with an ELISA plate reader. The amount of T2-TrpRS bound was converted into number of moles using the standard curve and divided by the number of cells to determine to number of T2-TrpRS binding site per cell.

The amount of T2-TrpRS bound was compared based on a standard curve, which was generated as follows: A standard curve was generated for each binding experiment to determine the amount of T2-TrpRS bound per cell. A range of biotin-T2-TrpRS concentrations (20, 10, 2,

1, 0.2, 0.1, 0.02, 0.01, and 0 nM) was incubated on the ligand-capture plate along side the samples. The amount of biotin-T2-TrpRS bound was determined as outlined above. A graph was generated with biotin-T2-TrpRS concentrations on the X axis, and A_{415} measurements on the Y axis. The points were plotted using the equation: $y=y_o + a \ln x$ where y_o (intercept at the y axis) and a (slope of the line) values were extrapolated from the linear line.

The specificity and the affinity of T2-TrpRS binding to HUVECs was examined utilising perforated cell based binding assays. The binding assays indicated that T2-TrpRS binding to HUVECs was saturable and specific. Total binding of T2-TrpRS binding to HUVECs was determined. In the presence of 50-100 fold molar excess of unlabeled T2-TrpRs, non-specific binding was demonstrated. Subtracting total from non-specific binding curves, the specific binding of T2-TrpRS to HUVECs was determined to be biphasic. The high affinity, low capacity site was calculated to have a binding constant of 3 nM and 18,000 sites/cell, whereas the low affinity, high capacity site was 200 nM and 60,000 sites/cell (Figure 6).

EXAMPLE 5: dsRNA Interference to Suppress NRP-1 Expression

The siRNAs chosen had a G/C content of about 50% and consisted of 21-nt sense and antisense strands that paired to produce a 19-nt duplex region with 2-nt, dTdT, overhangs at each 3' terminus. BLAST searches (NCBI database) against the human EST library showed that each siRNA was specific for the targeted gene. Purified, annealed siRNA duplexes were purchased from Dharmacon Research, Inc. (Lafayette, CO). The siRNAs used in this study are given in the table below. Specificity was controlled by incorporating four mis-matches in the N1 duplex to create a NRP-1a1 control RNAi. The luc control duplex (targeted to the luciferase gene) was used to show that an unrelated dsRNAi also has no effect on NRP-1 expression.

Transfection procedure: Human umbilical vein endothelial cells (HUVEC) (Vec Technologies, Rensselaer, NY) were passaged in MCDB-131 complete media (Vec Technologies). At passage 5-7 the cells were trypsinized, spun down and resuspended in PBS at 2 x 10^6 cells/ml. Ten micrograms of sheared salmon sperm DNA, 40 µg of ds RNA and one million cells were combined and placed in an electroporation cuvette (0.4 cm electrode gap). Samples were electroporated at 0.3 kV and 500 µF. After transfection, cuvettes were placed on ice for one minute. Samples were then combined with 0.5 ml of MCDB-131 complete media and transferred to a 60 mm dish containing complete media. Dishes were placed in a CO_2 incubator at 37^{0}C overnight. On average there was approximately 50-70% cell survival the following morning.

Name	Target Region	SiRNA Duplex	SEQ ID NO:
dsRNA/a1	a1 region of human Nrp-1	CCCUCACUUCGAUUUGGACdTdT dTdTGGGAGUGAAGCUAAACCUG	4 5
dsRNA/a1 Control	a1 region of human Nrp-1	CCGACACUUCGGCUUGGACdTdT dTdTGGCUGUGAAGCCGAACCUG	6 7
Luc Control	luciferase	CACUUACGGCAUUUCGCAGdTdT dTdTGUGAAUGCCGUAAAGCGUC	8 9

The ability of dsRNA/a1 to suppressed NRP-1 expression on the cells surface was examined. The Nrpa1 but not the Nrpa1 and Luc controls silenced the expression of NRP-1 in a dose dependent manner (Fig. 7A). Furthermore, the time course study showed that the NRP-1 expression can be silenced for up to 48 hrs post transfection (Fig. 7B)

Binding to T2-TrpRS to cells that have been treated with various concentrations of dsRNA was also examined. When cells were transfected with dsRNA/a1, at 10 μg, the number of T2-TrpRS binding sites/cell decreased to 40,000 and further decreased to 20,000 binding sites/cell when treated with 40 μg dsRNA/a1, compaired to cells untreated with dsRNA. The number of T2-TrpRS binding to cells remained unchanged in the negative control, the dsRNA/a1 control and dsRNA Luciferase (Fig. 8). These results indicate that neuropilin functions as a mediator of T2-TrpRS activity.

EXAMPLE 6: T2-TrpRS Enhances Factor Xa Activities in the Presence of HUVEC Membrane

To test for Factor Xa (Fxa) activities, the test conditions were set up where 10 nM of Fxa (Haematologic Tech, Essez Junction, VT) was incubated with either 20 mM of phospholipids (4 parts phosphatidylcholine and 1 part phosphatidylserine (Sigma, St. Louis, MO) or 20 μg of HUVEC membrane fragments (described in Example 3) in an assay buffer containing 0.05 M Tris-HCl, pH 7.3, 0.2% BSA and 25 mM $CaCl_2$. For negative control, 10 mM of Fxa was incubated with assay buffer alone. T2-TrpRS was added into these test conditions at various concentrations and incubated at 37°C for 5 minutes. To measure Fxa activities, 1 mM of the chromogenic substrate S-2765 (DiaPharma, West Chester, OH) was added into each sample. After 3 minutes, the reactions were stopped with 20% of acetic acid and Fxa activities were measured by reading the A_{405}.

In the control experiments (Fig. 9A), S2765 was demonstrated to be specific to Fxa and not reactive to membrane fragments nor T2-TrpRS, even though this chromogenic substrate has been demonstrated to be reactive to other serine proteases. However, when Fxa activity was measured in the presence T2-TrpRS and membrane, at 100 nM, T2-TrpRS induced a 75% enhancement of Fxa activity when compared to Fxa in combination with buffer alone (Fig. 9B). Thus, it is clear that T2-TrpRS interacted with a component on membrane to enhance Fxa catalytic activities. Because Fxa is vital in regulation of blood coagulation as well as an active chemoattractant for smooth muscle cells (Gasic *et al., PNAS USA* 89: 2317-2320 (1992)) and endothelial cells (Mackie, The Oxford Text Book of Medicine 3rd Ed., 3613-3627 (1996)) migration, the ability of T2-TrpRS to modulate Fxa activites may influence these process and contribute to the overall effectiveness of the molecule as an anti-angiogenesis agent.

EXAMPLE 7: T2-TrpRS is a Potent Inhibitor of VEGF Induced Angiogenesis *in vivo*

Female nude (nu/nu) mice were purchased from Taconic (Model # NCRNU-M, Taconic, Germantown, NY) and grouped into 10 per group. The mice were kept for one week in an animal quarantine room before being released to holding rooms. To evaluate the anti-angiogenic property of T2-TrpRS *in vivo*, Matrigel Assays were performed. Various concentrations of the recombinant T2-TrpRS protein were mixed with 0.45 ml matrigel (Fisher Scientific, Neward, DE) in the presence of VEGF (3mg/ml) (R&D, Minneapolis, Mn) and 20 U/ml heparin (Sigma, St. Louis, MO). Briefly, matrigel was mixed with test substances and preloaded into a 1.0 ml tuberculin syringes with G27 needles. Animals were anaesthetised and 0.45 ml of the mixed Matrigel were injected subcutaneously into the dorsal caudal portion of the midline (1 injection/animal) using a G27 needle. The Matrigel rapidly formed a solid gel that persisted for over 7 days. On Day 7, all animals were euthanized by CO_2 and matrigel plugs were harvested for haemoglobin measurement.

Haemoglobin measurement of the matrigel plug was a means to evaluate the angiogenic index. Matrigel plugs were dissected free of animal tissue and snapped frozen with dry ice and dried overnight. The dry weight was recorded and the plug was rehydrated with 0.5 ml of 0.5% Tween-20 in distilled water. After homogenisation, the mixture was centrifuged at 14,000 rpm in a desktop centrifuge (Eppendorf 5415C, Brinkmann Instruments, Inc, Westbury, NY) for 30 min. Supernatant was collected and the A_{405} was determined and converted to µg haemoglobin per mg matrigel using a haemoglobin standard curve (Sigma, St. Louis, MO). All data are

presented as mean ± SEM, n=10. Data were analysed by student t-test and p values ≤ 0.05 were considered to be statistically significant. Each experiment was repeated at least twice.

The *in vivo* assays indicated that T2-TrpRS is a potent suppressor of VEGF-induced angiogenesis. In the presence of 50 nM VEGF, 10 nM of T2-TrpRS brought about the onset of anti-angiogenesis activity and at 1000 nM, the matrigel plug was clear of hemoglobin. Because the formation of blood vessels is directly proportional to the % of hemoglobin content per given matrigel, T2-TrpRS demonstrated that it is a potent anti-angiogenic molecule. Indeed, the concentration in which T2-TrpRS induced 50% of anti-angiogenic effect (IC_{50}) was estimated to be ~100nM (Fig. 10).

EXAMPLE 8: Generation of Gene Therapy Vectors Expressing T2-TrpRS

The 1.2 kb *Nhe*I fragment, containing cDNA encoding human T2-TrpRS was cloned into the adeno-shuttle plasmid pAvCsxlx digested with *Spe*I to generate the adenoviral shuttle plasmid pAvT2TrpRS (Figure 12). The recombinant adenovirus encoding human T2-TrpRS was constructed by a rapid vector generation protocol using Cre recombinase-mediated recombination of two lox-site containing plasmids, pNDSQ3.1 (containing a lox site 5′ to the right hand portion of the adenoviral vector genome; Figure 13), and the adenoviral shuttle plasmid pAvT2TrpRS (containing a loxt site 3′ to the left end of the viral genome and the T2TrpRS expression cassette). The pNDSQ3.1 (digested with *Cla*I), pAvT2TrpRS (digested with *Pvu*II), and the Cre-encoding plasmid, pC-Cre3.1, were cotransfected using $CaPO_4$ (Promega's Profection kit) into S8 cells (A549 cells stably transfected with E1/E2a regions under dexamethasone inducible promoters (Gorziglia *et al., J. Virol.* 6: 4173-4178 (1996)). The transfected cells were treated with dexamethasone (0.33 μM) and the plasmids were joined by Cre-mediated recombination, generating the adenovirus encoding T2-TrpRS (Av3T2TrpRS). The Av3CsNull vector was generated in a similar manner, but lacks a transgene.

To amplify the virus, the cells were harvested a week after transfection and passaged until a cytopathic effect (CPE) was observed. For passaging, cells were freeze (dry ice)/thawed (37^0C) 5 times to obtain a CVL (crude viral lysate), which was centrifuged to remove the cell debris and then used to infect fresh S8 cells in the presence of dexamethasone (0.33 μM). Cells were harvested when CPE was observed, typically after one week. For purification of the vector, cell pellets were freeze/thawed 5 times (as above) and the cell debris was pelleted by centrifugation at 3,000 rpm for 10 min at 4^0C. The supernatant was loaded on a discontinuous Cesium Chloride gradient (1.25 g/ml CsCl and 1.4 g/ml CsCl) and centrifuged for 1 hr at

28,000 rpm (in a SW28 swing bucket rotor). The bottom viral band was pulled from the gradient and centrifuged on a CsCl continuous gradient (1.33 g/ml CsCl) overnight at 60,000 rpm (in an NVT-65 rotor). The purified viral band was pulled from the gradient, glycerol was added to a final concentration of 10% and the mixture was dialyzed in 200 mM Tris pH 8.0, 50 mM Hepes, 10% glycerol for ≥16 hr at 4°C. The concentration of vector was determined by spectrophotometric analysis (Mittereder *et al., J. Virol.* 70: 7498-7509 (1996)) and the vector was then aliquoted and stored at – 70°C. The genomic organization and purity of Av3T2TrpRS was verified by DNA analysis of isolated viral DNA by restriction endonuclease digestion. DNA was isolated from Av3T2TrpRS by first digesting the virus with Proteinase-K followed by Phenol/Chloroform extraction and ethanol precipitation of the viral DNA. The viral DNA was then digested with *Bam*HI (Gibco BRL, Rockville, MD), *Eco*RV, *Not*I and *Pvu*I (NEB, Beverly, MA) and applied to a 0.4% agarose/TAE gel. The DNA restriction analysis revealed the expected sizes.

EXAMPLE 9: Expression of T2TrpRS Protein by the Adenoviral Vector *in vitro*

A) Northern Analysis:

S8 cells were infected with AV3T2TrpRS at 100, 500, and 1000 particles per cell (ppc). The cells were harvested 72 hours after infection and RNA was isolated from these cells using RNAzol B (Cat# CS-104, Tel-Test Inc., Friendswood, TX) according to the manufacturer provided protocol. Ten micrograms of total RNA resuspended in DEPC dH_2O (Cat# 9920, Ambion, Austin, TX) was mixed with 12.5 volumes of RNA Sample Loading Buffer with Ethidium Bromide (Cat# 2-631636, 5Prime→ 3Prime, Boulder, CO) and heated at 56°C for 15 minutes and quickly cooled on ice. Samples were run via Easycast horizontal electrophoresis (Cat# B2CE, Owl Separartion Systems, Portsmouth, NH) on a 1% agarose (Cat# 15510-0827, Gibco BRL, Rockville, MD) gel containing 14 ml 37% formaldehyde (Cat# F-1635, Sigma, St. Louis, MO) and 10 ml 10X MOPS buffer (Cat# 3400-1005, Digene, Beltsville, MD) for 3-5 hours at 100 Volts. The RNA was transferred to a Nytran membrane (0.45μm) (Cat# 77412, Schleicher & Schuell, Keene, NH) according to the protocol in Molecular Cloning (Maniatis, pp 7.46-7.48, 1989) via capillary transfer overnight at room temperature. After the completion of the transfer, the membrane was UV crosslinked and blocked in prehybridization solution (8 ml of 1M $NaPO_4$, 32μl 0.5M EDTA, 0.5% BSA, 11.2 ml 10% SDS) at 65°C for 2 hours. After prehybridization, 20μCi of the random primed T2TrpRs ^{32}P probe was added directly to the membrane in prehybridization solution and incubated overnight at 65°C. The following day the

membrane was washed with 2X SSC (Cat# 15557-044 Gibco BRL, Rockville, MD) + 0.1% SDS (Cat# 15553-035 Gibco BRL, Rockville, MD) one time at room temperature for 30 minutes. After the initial wash, the membrane was washed two additional times with 0.2X SSC + 0.1% SDS for 30 minutes at 64°C with shaking. Following the washes, the membrane was exposed to Kodak X-OMAT AR film (Cat# 1651579 Kodak, Rochester, NY) at -80°C for 24 hours to detect the signal. The results of the Northern analysis revealed that Av3T2TrpRS vector transduced cells produced T2TrpRS mRNA in a dose dependent manner, whereas at similar doses the negative control vector, Av3CsNull, transduced cells did not produce detectable amount of T2TrpRS mRNA (Figure 11A).

B) Western Analysis:

S8 cells were infected with AV3T2TrpRS at 500 ppc. Conditioned media (25 ml) and cells were harvested 72 hours post-infection. The cell pellet was lysed using 300µl lysis buffer containing protease inhibitor cocktail. The cell lysate (1.35 µl) and the conditioned media (16.3 µl) were mixed with the corresponding amounts of NuPAGE LDS Sample Buffer 4X (6.3µl) (Cat# NP0007 Invitrogen, Carlsbad, CA) and NuPAGE Sample Reducing Agent 10X (2.5µl) (Cat# NP0004 Invitrogen, Carlsbad, CA) and heated at 70°C for 10 minutes. SDS PAGE analysis was conducted on a 4-12% linear gradient Bis-Tris ready gel (Cat# NP0321 Invitrogen, Carlsbad, CA). The proteins were transferred to a Nitrocellulose Membrane (0.45µm) (Cat# LC2001 Invitrogen, Carlsbad, CA) using the XCell Blot II Module (Cat# EI9051 Invitrogen, Carlsbad, CA) according to the manufacturer provided protocol. The transfer was conducted for 1 hour at 30 Volts. After the completion of the transfer, the membrane was blocked with 5% non-fat dry milk (NFDM) in TSE + 0.05% Tween-20 buffer for 2 hours at room temperature or 4°C overnight with shaking. The membrane was incubated with His-probe (H-15) rabbit polyclonal IgG (200µg/ml) diluted 1:200 (Cat# sc-803 Santa Cruz Biotechnology, Santa Cruz, CA) for one hour at room temperature with shaking. After four 5 minute washes with TSE + 0.05% Tween-20, the membrane was incubated with a 1:5000 dilution of anti-rabbit IgG HRP-linked $F(Ab')_2$ secondary antibody (Cat# NA9340, Amersham Pharmacia, Piscataway, NJ) for 1 hour, followed by four additional washes and a five minute incubation in chemiluminescence detection reagent mix (Cat# 34075 Supersignal West Dura Extended Duration Substrate, Pierce, Rockford, IL). The blot was exposed to a Kodax Biomax MR X-ray film (Cat# 876-1520, Kodak, Rochester, NY). The results of the Western analysis revealed the presence of T2TrpRS protein in the conditioned media and the cell lysate of the cell transduced with the Av3T2TrpRS vector. No T2TrpRS protein was detected in either conditioned media or

cell lysate from the Av3CsNull vector transduced cells (Figure 11B). The T2TrpRS protein was purified from the conditioned media of the cells transduced with Av3T2TrpRS vector using Ni-NTA agarose beads. This semi-purified protein was found biologically active in a variety of assays including receptor tyrosine kinase (RTK) assay using HUVEC membrane and apoptosis assay explained in previous examples.

EXAMPLE 10: Generation of Bovine Immunodeficiency Virus (BIV) Based Lentiviral vectors Encoding Human T2TrpRS

For generation of BIV-based lentiviral vectors encoding human T2TrpRS, the shuttle plasmid was generated by linearizing the parent shuttle plasmid, pBIVUniversalCMV, with *Nhe*I and ligating it with the 1.2 Kb T2TrpRS-*Nhe*I fragment. The sequence of the shuttle plasmid, pBIVUniversalCMVT2TrpRS, was confirmed by restriction analysis and DNA sequencing. The lentiviral supernatant was obtained by co-transfecting 293T cells with pBIVUniversalCMVT2TrpRS, packaging construct and env construct by $CaPO_4$ transient transfection method per manufacturer protocol. Briefly, 293T cells were seeded in 10cm tissue culture plates in appropriate medium (DMEM+10% Heat Inactivated FBS+1%Penn/Strep). The following day, the cells were serum stimulated by changing the medium. 15 μg each of the packaging plasmid and the shuttle plasmid and 4.5 μg of the envelope plasmid were diluted in distilled water and mixed with calcium phosphate. DNA solution was added drop-wise to the 2X HBSS solution while vortexing. DNA precipitate was then added to the cells and the cells were incubated at 37^0C, 5% CO_2, overnight. Next day, the medium was aspirated and fresh medium was added to the plates. Supernatants containing the lentiviral vector, BIVCMVT2TrpRS, were harvested the following day, spun down at 2000 rpm for 8 minutes, and filtered through a 0.45 μm filter. Fresh medium was added to the cells and 24 hours later the harvest was repeated as explained earlier and the cells were discarded. For *in vitro* expression of the T2TrpRS by the BIVCMVT2TrpRS, Cf2Th cells (canine thymus cells) are transduced with the vector and T2TrpRS RNA and protein are expressed.

It is to be understood that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims. All publications cited herein are hereby incorporated herein by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method of treating a neuropilin-mediated disease in a mammal, comprising administering to said mammal an amount of T2-TrpRS or a derivative thereof effective to treat said disease.
2. The method of claim 1, wherein said T2-TrpRS or a derivative thereof comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO:3.
3. The method of claim 1, wherein said T2-TrpRS or a derivative thereof comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO:3.
4. The method of claim 1, wherein said T2-TrpRS or a derivative thereof comprises an amino acid sequence having at least 99% sequence identity to SEQ ID NO:3.
5. The method of claim 1, wherein T2-TrpRS is administered to said mammal.
6. The method of claim 1, wherein said mammal is a primate.
7. The method of claim 6, wherein said primate is a human.
8. The method of claim 1, wherein said disease is cancer.
9. The method of claim 8, wherein said cancer is selected from the group consisting of lung, colon, breast, and prostate cancer.
10. The method of claim 1, wherein said disease is injury to the nervous system.
11. The method of claim 1, wherein said disease is rheumatoid arthritis.
12. The method of claim 1, wherein said disease is a development anomaly.
13. The method of claim 1, wherein said T2-TrpRS or derivative is administered as a recombinant protein.
14. The method of claim 13, wherein said protein is administered orally, intravenously, intramuscularly, intraperitoneally, or intrathecally.
15. The method of claim 1, wherein said T2-TrpRS or derivative is administered by administering a vector comprising a nucleotide sequence encoding said T2-TrpRS or derivative.
16. The method of claim 15, wherein said vector is a plasmid, a lipid formulation, or a viral vector.
17. The method of claim 15, wherein said vector is administered orally, intravenously, intramuscularly, intraperitoneally, or intrathecally.
18. A method for identifying an antagonist to neuropilin, comprising:

(a) designing molecules that are expected to bind to a neuropilin based on the chemistry and three-dimensional structure of neuropilin and the chemistry and three-dimensional structure of T2-TrpRS;

(b) contacting said molecules with neuropilin in an *in vitro* or cellular assay; and

(c) identifying which molecules bind to neuropilin.

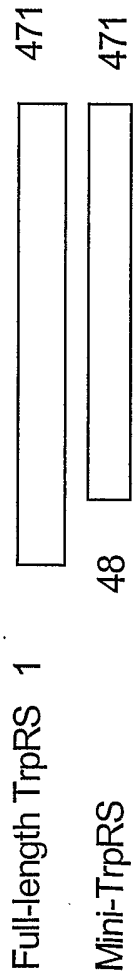


Figure 1

T2-TrpRS: 94-SAKGIDYDKLIVRFGSS – KID KELINR I ERAT GQRPHHFL.....
SEM: 410-S IYP IHKRP LIVRIGTDYKYTKIAVDRVNAADGRYHV LFL.....

Figure 2A

T2-TrpRS:

94-SAKGIDY DKLIVRFGSS KIDKELINRI ERATGQRPHH FLRRGIFFSH RDMNQVLDAY
ENKKPFYLYT GRGPSSEAMH VGHLIPFIFT KWLQDVFNVP LVIQMTDDEK YLWKDLTLDQ
AYSYAVENAK DIIACGFDIN KTFIFSDLDY MGMSSGFYKN VVKIQKHVTF NQVKGIFGFT
DSDCIGKISF PAIQAAPSFS NSFPQIFRDR TDIQCLIPCA IDQDPYFRMT RDVAPRIGYP KPALLHSTFF
PALQGAQTKM SASDPNSSIF LTDTAKQIKT KVNKHAFSGG RDTIEEHRQF GGNCDVDVSF
MYLTFFLEDD DKLEQIRKDY TSGAMLTGEL KKALIEVLQP LIAEHQARRK EVTDEIVKEF
MTPRKLSFDF Q-471

Figure 2B

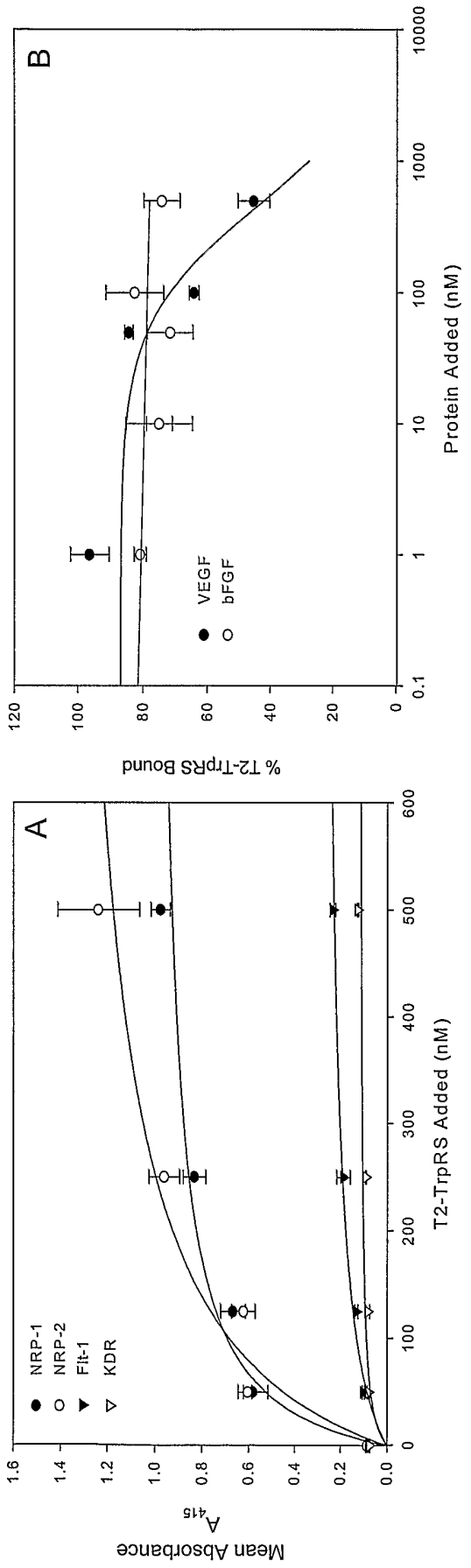


Figure 3A

Figure 3B

Figure 4A

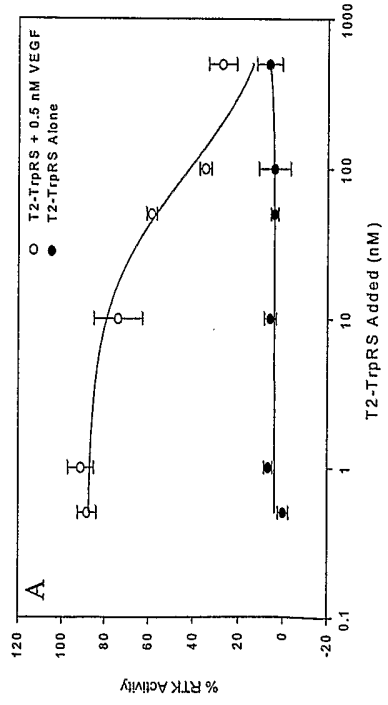
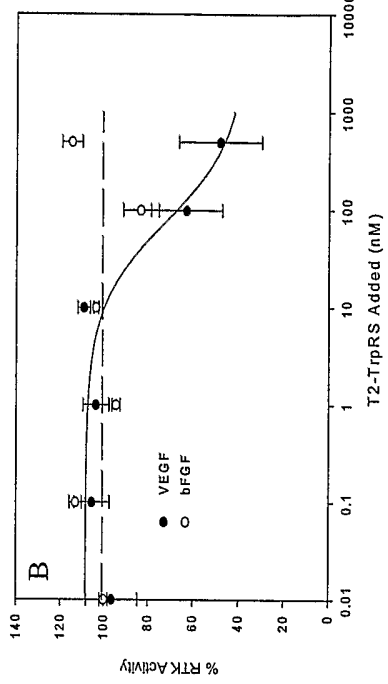


Figure 4B



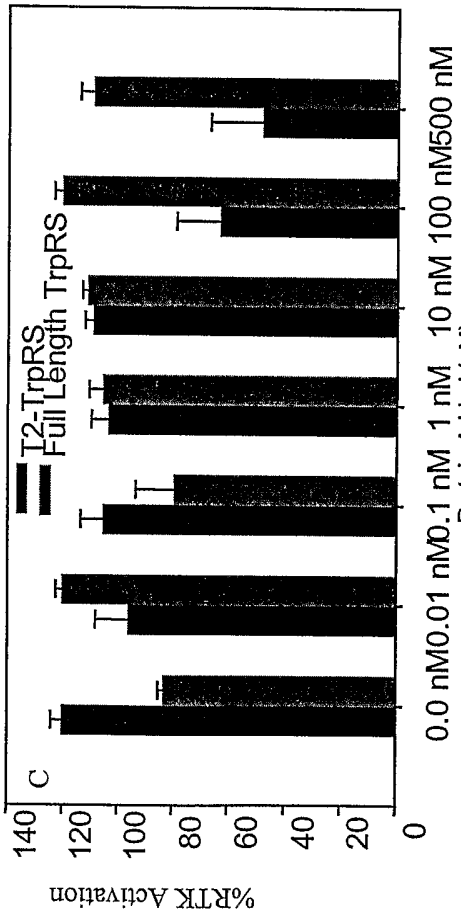


Figure 4C

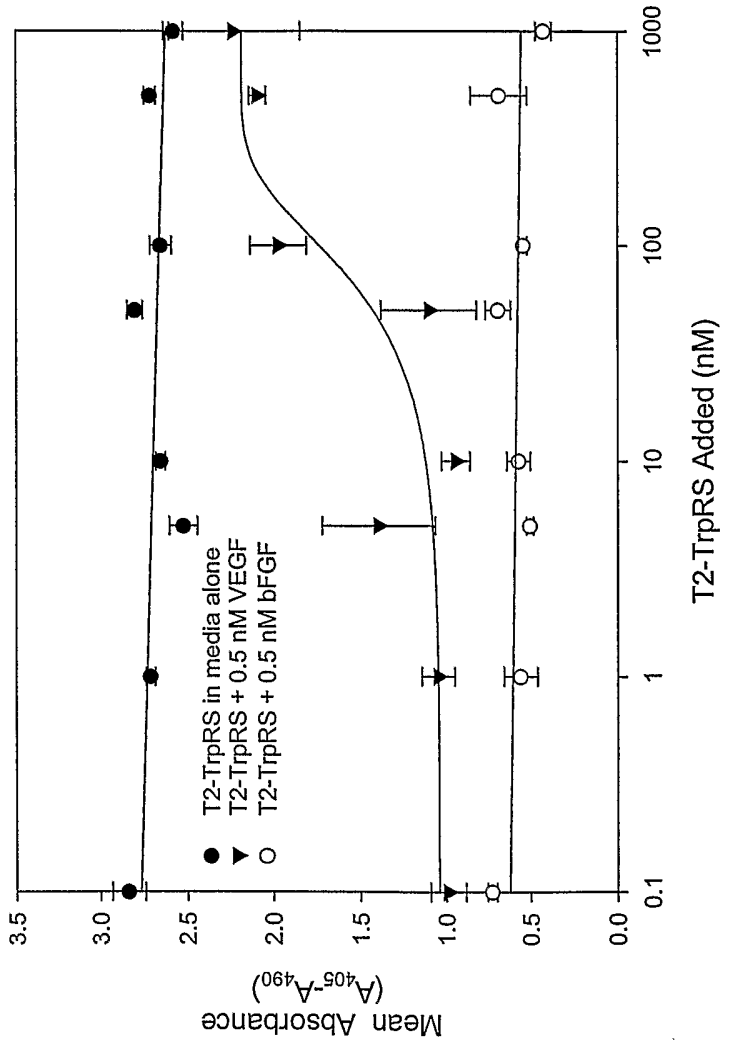

Mean Absorbance
(A_{405}-A_{490})
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.1
1
10
100
1000
T2-TrpRS Added (nM)
T2-TrpRS in media alone
T2-TrpRS + 0.5 nM VEGF
T2-TrpRS + 0.5 nM bFGF

Figure 5

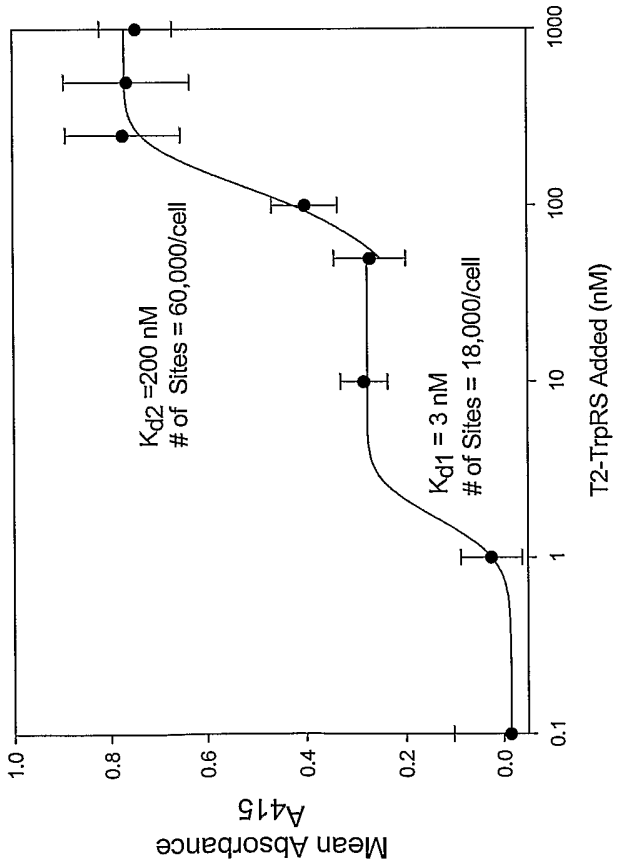

Mean Absorbance
A415
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
1000
T2-TrpRS Added (nM)
K_{d2} =200 nM
# of Sites = 60,000/cell
K_{d1} = 3 nM
# of Sites = 18,000/cell

Figure 6

Figure 7A

Figure 7B

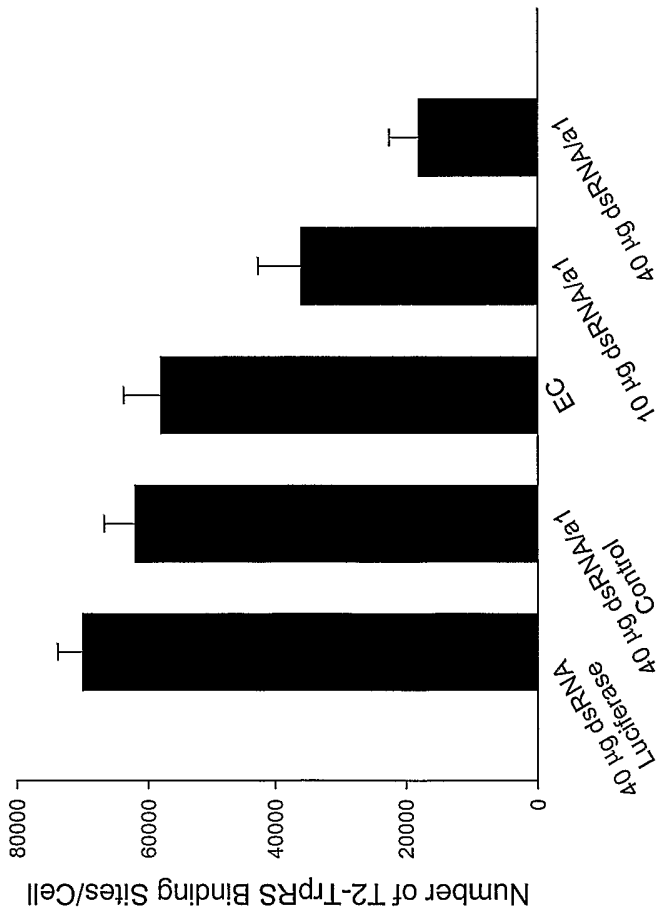

Number of T2-TrpRS Binding Sites/Cell
80000
60000
40000
20000
0
40 µg dsRNA Luciferase
40 µg dsRNAi Control
EC
10 µg dsRNAi
40 µg dsRNAi

Figure 8

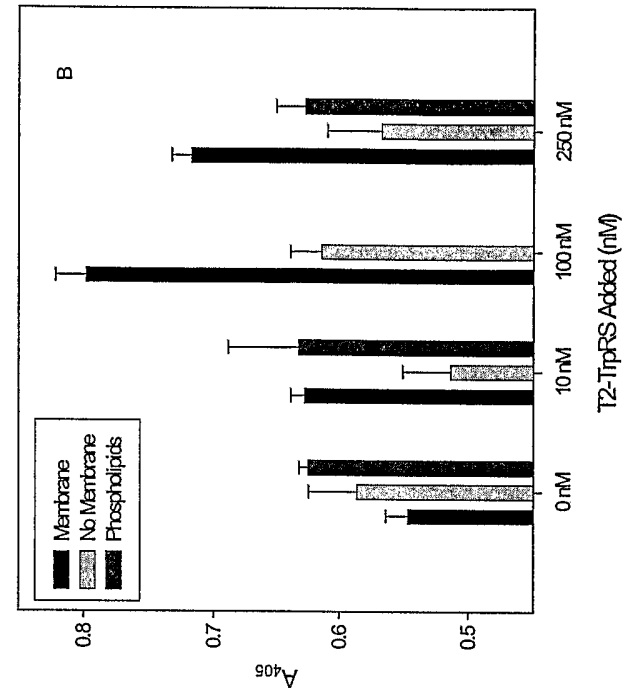


Figure 9B

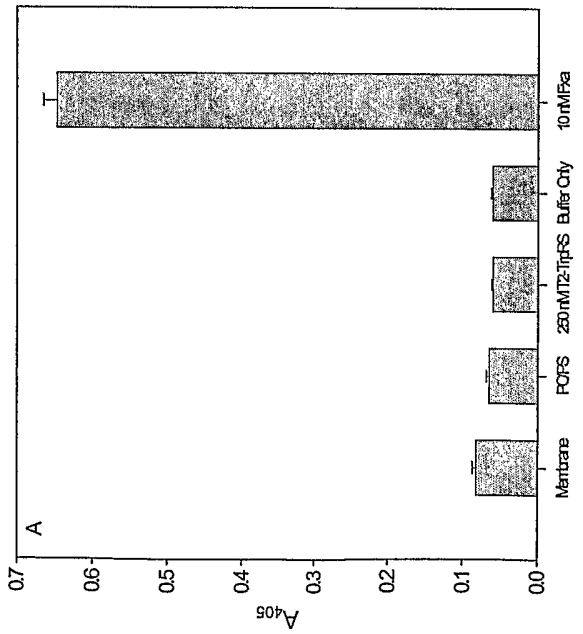


Figure 9A

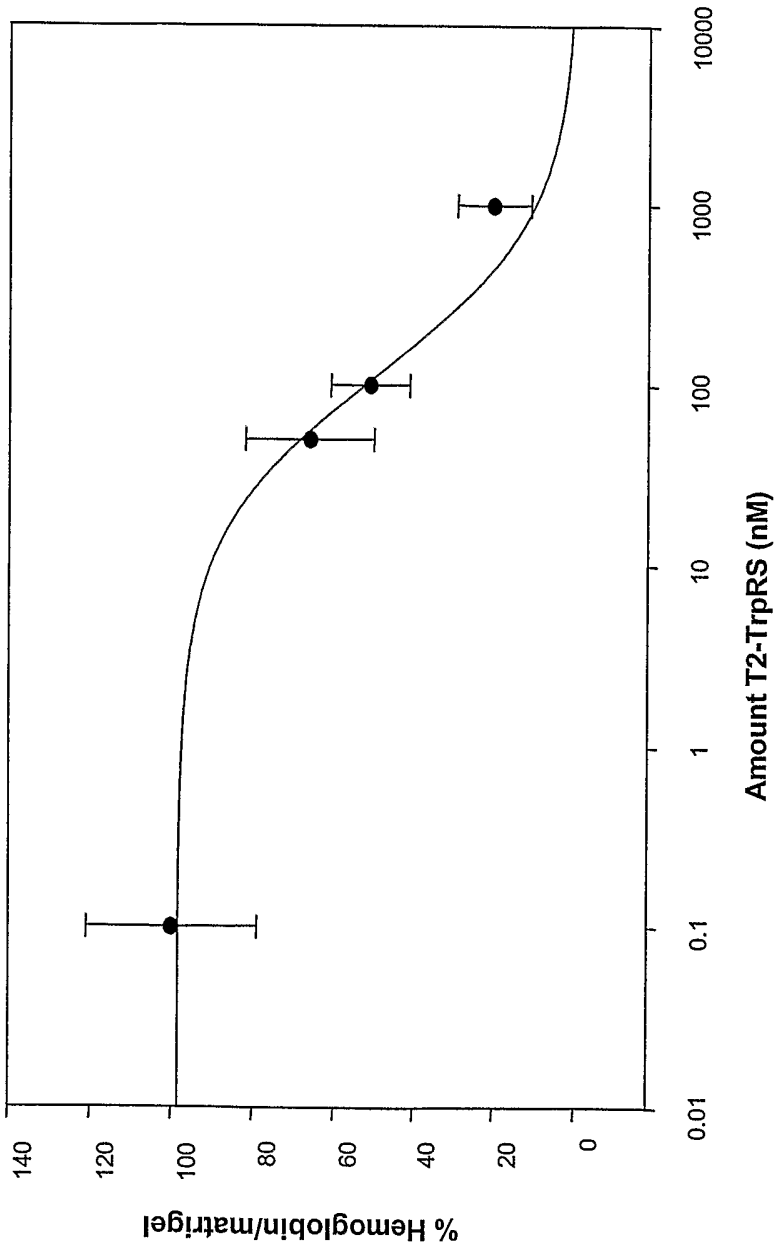

% Hemoglobin/matrigel
140
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
Amount T2-TrpRS (nM)

Figure 10

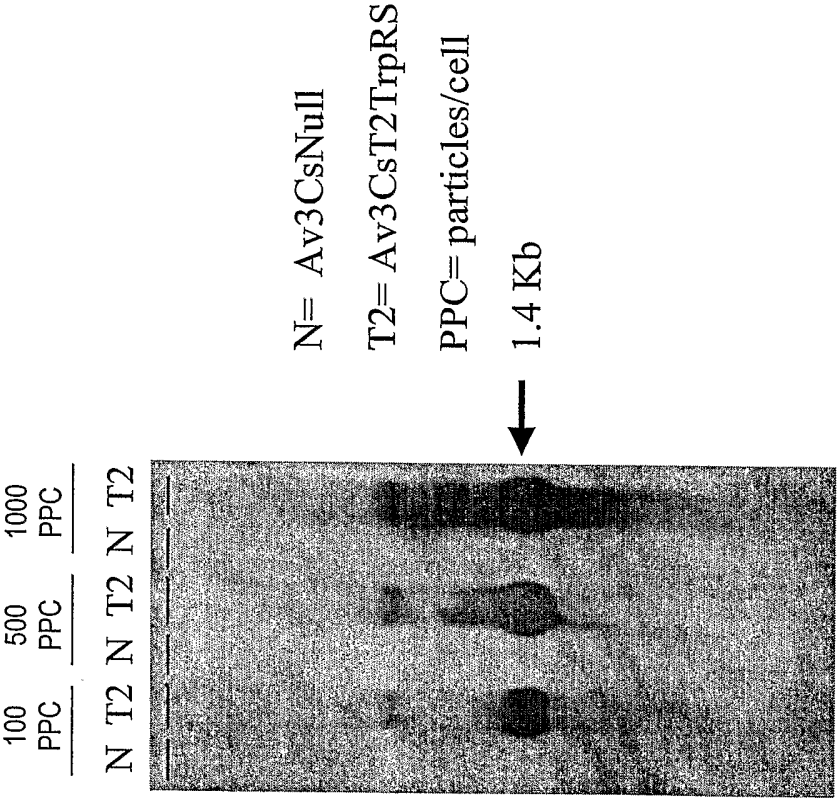
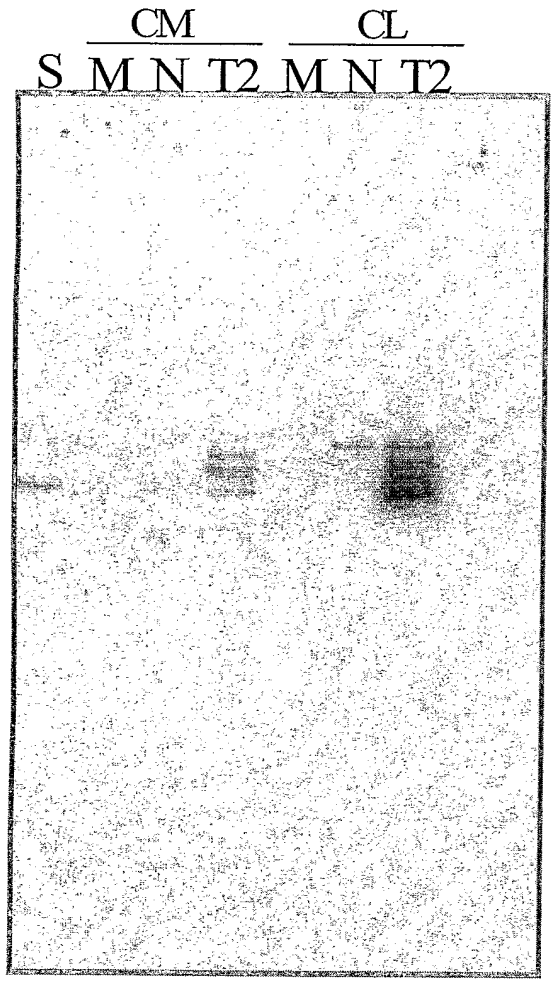

N= Av3CsNull
T2= Av3CsT2TrpRS
PPC= particles/cell
1.4 Kb
100 PPC
500 PPC
1000 PPC
N T2 N T2 N T2

Figure 11A



S= purified recombinant protein (20 ng)

CM= conditioned media

CL= cell lysate

M= Mock

N= Av3CsNull

T2= Av3T2TrpRS

Figure 11B

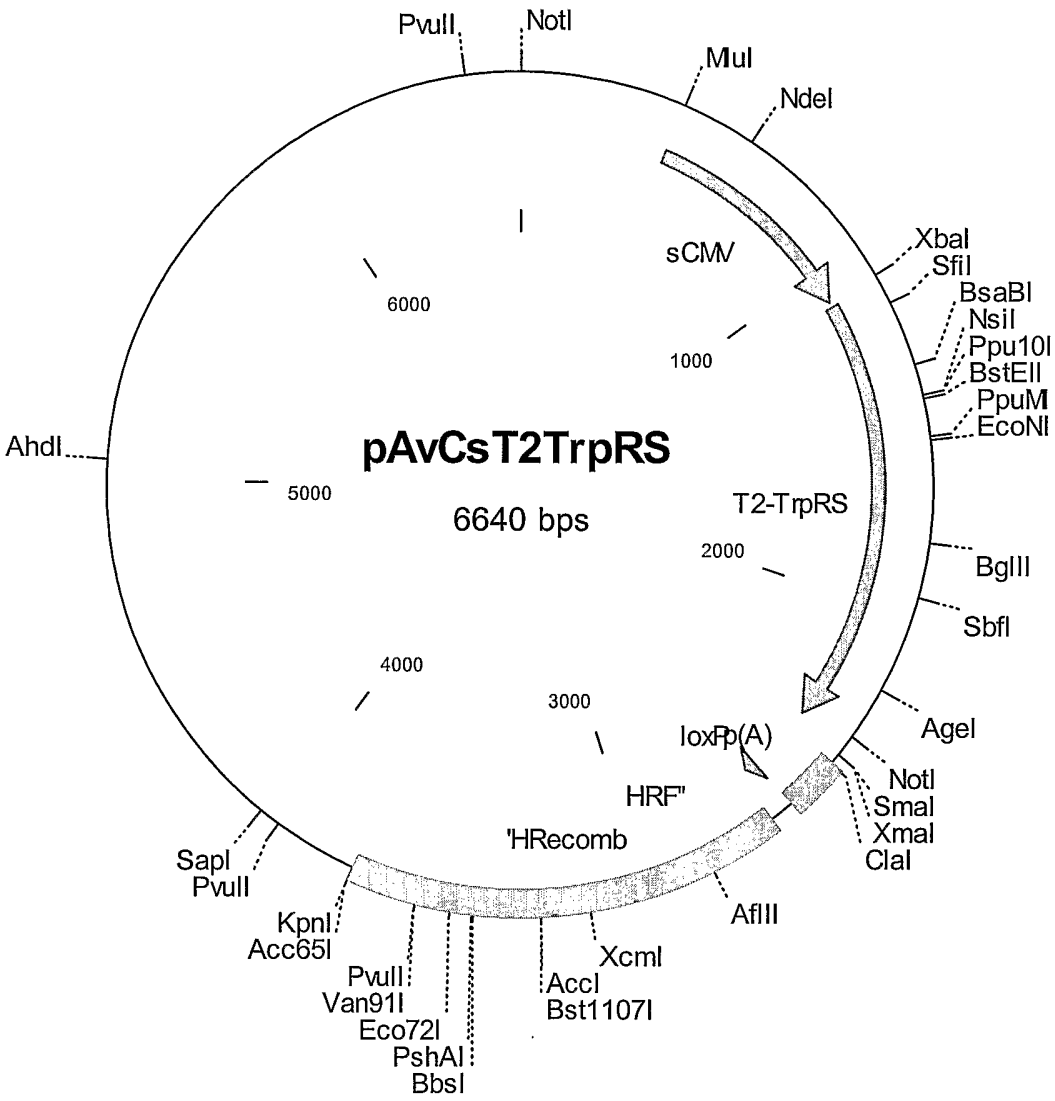
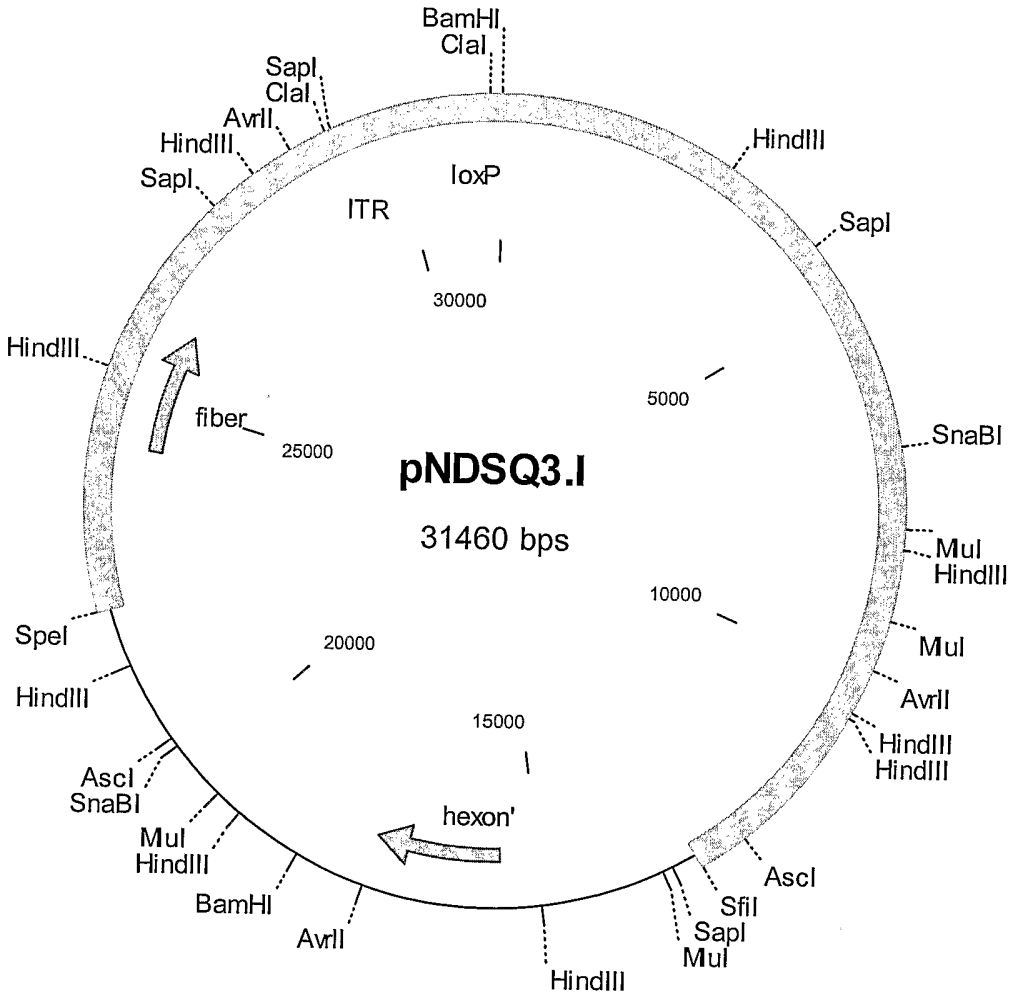

pAvCsT2TrpRS
6640 bps
sCMV
T2-TrpRS
loxP
p(A)
HRF"
'HRecomb
1000
2000
3000
4000
5000
6000
PvuII
NotI
MuI
NdeI
XbaI
SfiI
BsaBI
NsiI
Ppu10I
BstEII
PpuMI
EcoNI
BglII
SbfI
AgeI
NotI
SmaI
XmaI
ClaI
AflII
XcmI
AccI
Bst1107I
PvuII
Van91I
Eco72I
PshAI
BbsI
KpnI
Acc65I
SapI
PvuII
AhdI

Figure 12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ClaI
SapI
ClaI
AvrII
HindIII
SapI
HindIII
SapI
loxP
ITR
30000
HindIII
fiber
25000
pNDSQ3.I
31460 bps
5000
SnaBI
MuI
HindIII
10000
MuI
AvrII
HindIII
HindIII
SpeI
20000
HindIII
15000
AscI
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MuI
HindIII
hexon'
BamHI
AvrII
HindIII
AscI
SfiI
SapI
MuI

Figure 13

SEQUENCE LISTING

<110> Novartis AG
Liau, Gene
Ho, David

<120> METHODS OF TREATING NEUROPILIN-MEDIATED DISEASES

<130> 4-32014A

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<151> 2001-07-26

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35

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Asp Met Asn Gln Val Leu Asp Ala Tyr Glu Asn Lys Lys Pro Phe Tyr
50 55 60

Leu Tyr Thr Gly Arg Gly Pro Ser Ser Glu Ala Met His Val Gly His
65 70 75 80

Leu Ile Pro Phe Ile Phe Thr Lys Trp Leu Gln Asp Val Phe Asn Val
85 90 95

Pro Leu Val Ile Gln Met Thr Asp Asp Glu Lys Tyr Leu Trp Lys Asp
100 105 110

Leu Thr Leu Asp Gln Ala Tyr Ser Tyr Ala Val Glu Asn Ala Lys Asp
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Ile Ile Ala Cys Gly Phe Asp Ile Asn Lys Thr Phe Ile Phe Ser Asp
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Leu Asp Tyr Met Gly Met Ser Ser Gly Phe Tyr Lys Asn Val Val Lys
145 150 155 160

Ile Gln Lys His Val Thr Phe Asn Gln Val Lys Gly Ile Phe Gly Phe
165 170 175

Thr Asp Ser Asp Cys Ile Gly Lys Ile Ser Phe Pro Ala Ile Gln Ala
180 185 190

Ala Pro Ser Phe Ser Asn Ser Phe Pro Gln Ile Phe Arg Asp Arg Thr
195 200 205

Asp Ile Gln Cys Leu Ile Pro Cys Ala Ile Asp Gln Asp Pro Tyr Phe
210 215 220

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Arg Met Thr Arg Asp Val Ala Pro Arg Ile Gly Tyr Pro Lys Pro Ala
225                 230                 235                 240

Leu Leu His Ser Thr Phe Phe Pro Ala Leu Gln Gly Ala Gln Thr Lys
                245                 250                 255

Met Ser Ala Ser Asp Pro Asn Ser Ser Ile Phe Leu Thr Asp Thr Ala
            260                 265                 270

Lys Gln Ile Lys Thr Lys Val Asn Lys His Ala Phe Ser Gly Gly Arg
        275                 280                 285

Asp Thr Ile Glu Glu His Arg Gln Phe Gly Gly Asn Cys Asp Val Asp
    290                 295                 300

Val Ser Phe Met Tyr Leu Thr Phe Phe Leu Glu Asp Asp Asp Lys Leu
305                 310                 315                 320

Glu Gln Ile Arg Lys Asp Tyr Thr Ser Gly Ala Met Leu Thr Gly Glu
                325                 330                 335

Leu Lys Lys Ala Leu Ile Glu Val Leu Gln Pro Leu Ile Ala Glu His
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