(12) PATENT **(11)** Application No. **AU 200127315 B2**
(19) AUSTRALIAN PATENT OFFICE **(10)** Patent No. **782067**

(54) Title
TWEAK receptor

(51)[7] International Patent Classification(s)
A61K 038/17 **C07K 014/705**
A61K 039/395 **C07K 016/28**
A61P 035/00 **C12N 015/12**

(21) Application No: **200127315** (22) Application Date: **2000.12.19**

(87) WIPO No: **WO01/45730**

(30) Priority Data

(31) Number	(32) Date	(33) Country
60/172878	**1999.12.20**	**US**
60/203347	**2000.05.10**	**US**

(43) Publication Date : **2001.07.03**
(43) Publication Journal Date : **2001.09.13**
(44) Accepted Journal Date : **2005.06.30**

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(56) Related Art
WO 1998/055508
WO 1999/061471
WO 1998/035061

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 June 2001 (28.06.2001)

PCT

(10) International Publication Number
WO 01/45730 A2

(51) International Patent Classification[7]: A61K 38/17, 39/395, C07K 14/705, 16/28, C12N 15/12, A61P 35/00

(21) International Application Number: PCT/US00/34755

(22) International Filing Date:
19 December 2000 (19.12.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/172,878 20 December 1999 (20.12.1999) US
60/203,347 10 May 2000 (10.05.2000) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/45730 A2

(54) Title: TWEAK RECEPTOR

(57) Abstract: The present invention provides the TWEAK receptor and methods for identifying and using agonists and antagonists of the TWEAK receptor. In particular, the invention provides methods of screening for agonists and antagonists and for treating diseases or conditions mediated by angiogenesis, such as solid tumors and vascular deficiencies of cardiac or peripheral tissue.

TITLE

TWEAK RECEPTOR

REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial Number 60/172,878, filed 20 December 1999, and U.S. Provisional Application Serial Number 60/203,347, filed 10 May 2000, both of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to the discovery of the functional receptor (TWEAKR) for the TWEAK protein. More particularly, the invention relates to the use of TWEAKR agonists and antagonists in methods of treatment, and to screening methods based on TWEAKR and the TWEAK-TWEAKR interaction.

BACKGROUND OF THE INVENTION

A. Angiogenesis

Angiogenesis is a multi-step developmental process that results in the formation of new blood vessels off of existing vessels. This spatially and temporally regulated process involves loosening of matrix contacts and support cell interactions in the existing vessels by proteases, followed by coordinated movement, morphological alteration, and proliferation of the smooth muscle and endothelial cells of the existing vessel. The nascent cells then extend into the target tissue followed by cell-cell interactions in which the endothelial cells form tubes which the smooth muscle cells surround. In a coordinated fashion, extracellular matrix proteins of the vessel are secreted and peri-endothelial support cells are recruited to support and maintain structural integrity (see, e.g., Daniel et al., Ann. Rev. Physiol. 2000(62):649, 2000). Angiogenesis plays important roles in both normal and pathological physiology.

Under normal physiological conditions, angiogenesis is involved in fetal and embryonic development, wound healing, organ regeneration, and female reproductive remodeling processes including formation of the endometrium, corpus luteum, and placenta. Angiogenesis is stringently regulated under normal conditions, especially in adult animals, and perturbation of the regulatory controls can lead to pathological angiogenesis.

Pathological angiogenesis has been implicated in the manifestation and/or progression of inflammatory diseases, certain eye disorders, and cancer. In particular, several lines of evidence support the concept that angiogenesis is essential for the growth and persistence of solid tumors and their metastases (see, e.g., Folkman, N. Engl. J. Med. 285:1182, 1971; Folkman et al., Nature 339:58, 1989; Kim et al., Nature 362:841, 1993; Hori et al., Cancer Res., 51:6180, 1991). Angiogenesis inhibitors are therefore useful for the prevention (e.g., treatment of premalignant conditions), intervention (e.g., treatment of small tumors), and regression (e.g., treatment of large tumors) of cancers (see, e.g., Bergers et al., Science 284:808, 1999).

There is a need for additional compositions and methods of modulating angiogenesis for the prevention, abrogation, and mitigation of disease.

B. TWEAK

The TWEAK protein, which has also been called TREPA and Apo3L, is a member of the tumor necrosis factor (TNF) family and is expressed in a wide variety of human tissues (Chicheportiche et al., J. Biol. Chem., 272(51):32401, 1997; see also Wiley, PCT Publication No. WO 98/35061, 13 August 1998). Like most TNF family members, TWEAK is a Type II membrane protein with an extracellular C-terminal domain. Although TWEAK was originally described as a weak inducer of apoptosis, this induction of cell death was later shown to be indirect (Schneider et al., Eur. J. Immunol. 29:1785, 1999).

Lynch et al. demonstrated that TWEAK directly induces endothelial cell proliferation and angiogenesis (J. Biol. Chem., 274(13):8455, 1999). Picomolar concentrations of recombinant soluble TWEAK induce proliferation in multiple endothelial cell lines and in aortic smooth muscle cells, and reduce the requirement for serum and growth factors in culture. Moreover, TWEAK induces a strong angiogenic response in a rat corneal pocket assay. Since TNF family members initiate biological responses by signaling through members of the TNF receptor family, there has been great interest in identifying and characterizing the TWEAK receptor.

Marsters et al. reported that TWEAK binds to and signals through a death-domain containing receptor known variously as DR3, Apo3, WSL-1, TRAMP, or LARD (Marsters et al., Current Biology 8(9):525, 1998). Schneider et al., however, showed that TWEAK binds to and signals in Kym-1 cells but that Kym-1 cells do not express the receptor DR3 (Schneider et al., Eur. J. Immunol. 29:1785, 1999). These results suggest the existence of a yet to be identified TWEAK receptor.

Because TWEAK induces angiogenesis in vivo, there is a particular need to identify the major functional TWEAK receptor. Once identified, the TWEAK receptor may be used to screen for and develop TWEAK receptor agonists and antagonists for the modulation of angiogenesis and the treatment of human disease.

SUMMARY OF THE INVENTION

The present invention is based upon the identification and biological characterization of the major functional TWEAK receptor. As described below, cDNA encoding the TWEAK receptor was molecularly cloned from a human endothelial cell expression library.

Although DNA and deduced amino acid sequences corresponding to the TWEAK receptor identified herein have been reported (see, e.g., Kato et al., PCT Publication No. WO 98/55508, 10 December 1998 and Incyte, PCT Publication No. WO 99/61471, 02 December 1999), it was not heretofore appreciated that these sequences encode a receptor for TWEAK or that the encoded polypeptide is involved in modulating angiogenesis. Similarly, investigators have recently claimed methods of making and using TWEAK receptor antagonists to treat immunological disorders, but without identifying the major TWEAK receptor or its role in angiogenesis (Rennert, PCT Publication No. WO 00/42073, 20 July 2000). These deficiencies have been addressed, as described herein, by identification of the major TWEAK receptor (TWEAKR) and characterization of its biological activities. The identification of TWEAKR has led to the development of compositions for the modulation of angiogenesis, and also provides screening tools for the identification of diagnostics and therapeutics.

The invention provides methods of modulating angiogenesis in a mammal in need of such treatment comprising administering a therapeutically-effective amount of a composition comprising a TWEAK receptor antagonist or TWEAK receptor agonist. The composition preferably comprises a pharmaceutically acceptable carrier and the mammal is preferably a human.

In some more preferred embodiments the composition inhibits angiogenesis and comprises a TWEAK receptor antagonist, such as a soluble TWEAK receptor fragment, an antagonistic antibody, or an antagonist that disrupts the interaction between the TWEAK receptor and a TRAF molecule. In some most preferred embodiments the antagonist comprises amino acids 28-79 of SEQ ID NO:7 or amino acids 28-309 of SEQ ID NO:7. The TWEAK receptor antagonists are preferably used to treat a mammal that has a disease or condition mediated by angiogenesis, more preferably a disease or condition characterized by ocular neovascularization or a solid tumor. In some embodiments, the mammal is further treated with radiation or with a second chemotherapeutic agent.

In some more preferred embodiments the composition promotes angiogenesis and comprises a TWEAK receptor agonist, such as an agonistic antibody. The TWEAK receptor agonists are preferably used to treat a vascularization deficiency in cardiac or peripheral tissue, to enhance wound healing or organ transplantation, or in conjunction with bypass surgery or angioplasty.

The invention also provides antagonists comprising a soluble TWEAK receptor fragment for use in medicine, preferably comprising amino acids 28-79 of SEQ ID NO:7 or amino acids 28-309 of SEQ ID NO:7, as well as nucleic acids encoding soluble TWEAK receptor fragments. And the invention provides for the use of a composition comprising a TWEAK receptor antagonist or TWEAK receptor agonist for the preparation of a medicament for modulating angiogenesis in a mammal in need of such treatment.

The invention further provides methods of identifying a compound that is capable of modulating angiogenesis, including: (a) identifying a test compound that binds to a TWEAK receptor extracellular domain, wherein the test compound is not TWEAK; (b) identifying a test compound that affects the interaction between a TWEAK and a TWEAK receptor; and (c) identifying a test compound that modulates the interaction between a TWEAK receptor and a TRAF. The invention encompasses compounds identified according to these methods.

The invention also provides a method for targeting a detectable label or chemotherapeutic to vascular tissue comprising contacting the vascular tissue with an antibody that binds TWEAK receptor. In some preferred embodiments the antibody is conjugated to a radioisotope, chemiluminescent or fluorescent compound, or enzyme. In some preferred embodiments the antibody is conjugated to a cytotoxin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a sequence alignment of the human and murine TWEAK receptor polypeptide sequences. The top sequence is the murine TWEAK receptor polypeptide (SEQ ID NO:5), and the bottom sequence is the human TWEAK receptor polypeptide (SEQ ID NO:4).

Figure 2 shows the effect of TWEAKR-Fc on PMA-induced HRMEC wound closure.

Figure 3 shows the effect of TWEAKR-Fc on EGF-induced HRMEC wound closure.

Figure 4 shows the effect of human TWEAKR-Fc on TWEAK-induced (100 ng/ml) HUVEC proliferation.

Figure 5 shows the effect of human TWEAKR-Fc on FGF-2-induced (10 ng/ml) HUVEC proliferation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the TWEAK receptor and methods for identifying and using agonists and antagonists of the TWEAK receptor. The invention provides methods of screening for agonists and antagonists and for treating diseases or conditions mediated by angiogenesis.

A. Abbreviations and Terminology Used in the Specification

"4-1BB" and **"4-1BB ligand"** (4-1BB-L) are polypeptides described, inter alia, in U.S. Patent No. 5,674,704, including soluble forms thereof.

"bFGF" is basic fibroblast growth factor.

"BSA" is bovine serum albumin.

"CD40 ligand" (CD40L) is a polypeptide described, inter alia, in U.S. Patent No. 5,716,805, including soluble forms thereof.

"CHO" is a Chinese hamster ovary cell line.

"DMEM" is Dulbecco's Modified eagle Medium, a commercially available cell culture medium.

"ELISA" is Enzyme-Linked Immunosorbent Assay.

"Flt3L" is Flt3 ligand, a polypeptide described, inter alia, in U.S. Patent No. 5,554,512, including soluble forms thereof.

"HRMEC" are primary human renal microvascular endothelial cells.

"HUVEC" is a line of human umbilical vein endothelial cells.

"PBS" is phosphate buffered saline.

"PMA" is phorbol 12-myristate-13-acetate.

"RTKs" are receptor tyrosine kinases.

"Tek," which has also been called Tie2 and ork, is an RTK that is predominantly expressed in vascular endothelium. The molecular cloning of human Tek (ork) has been described by Ziegler, U.S. Patent No. 5,447,860. **"Tek antagonists"** are described, inter alia, in Cerretti et al., PCT Publication No. WO 00/75323, 14 December 2000.

"TNFR" is a tumor necrosis factor receptor, including soluble forms thereof. **"TNFR/Fc"** is a tumor necrosis factor receptor-Fc fusion polypeptide.

"TRAIL" is TNF-related apoptosis-inducing ligand, a type II transmembrane polypeptide in the TNF family described, inter alia, in U.S. Patent No. 5,763,223, including soluble forms thereof.

"VEGF" is vascular endothelial growth factor, also known as VPF or vascular permeability factor.

B. Soluble TWEAK Receptor Polypeptides

As described in the examples below, the native human TWEAK receptor cDNA has the sequence

SEQ ID NO:3, which encodes a 129 residue polypeptide (SEQ ID NO:4). Examination of the DNA sequence predicts a polypeptide having an approximately 78 amino acid extracellular domain (residues 1-78 of SEQ ID NO:4, including the signal peptide), an approximately 23 amino acid transmembrane domain (residues 79-101 of SEQ ID NO:4), and an approximately 28 amino acid intracellular domain (residues 102-129 of SEQ ID NO:4). The TWEAK receptor sequence has also been reported by Kato et al., PCT Publication No. WO 98/55508, 10 December 1998 and by Incyte, PCT Publication No. WO 99/61471, 02 December 1999. As used herein, "TWEAKR" includes polypeptides having these sequences, and in particular comprising amino acids 28-79 of SEQ ID NO:7, as well as naturally occurring variants thereof.

In one aspect of the invention, a soluble TWEAK receptor fragment is used as a TWEAKR antagonist to inhibit angiogenesis and/or to inhibit the binding of TWEAK ligand to TWEAKR.

Soluble polypeptides are capable of being secreted from the cells in which they are expressed. The use of soluble forms of polypeptides is advantageous for certain applications. Purification of the polypeptides from recombinant host cells is facilitated since the polypeptides are secreted, and soluble proteins are generally suited for parenteral administration. A secreted soluble polypeptide may be identified (and distinguished from its non-soluble membrane-bound counterparts) by separating intact cells which express the desired polypeptide from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired polypeptide. The presence of the desired polypeptide in the medium indicates that the polypeptide was secreted from the cells and thus is a soluble form of the polypeptide. Soluble polypeptides may be prepared by any of a number of conventional techniques. A DNA sequence encoding a desired soluble polypeptide may be subcloned into an expression vector for production of the polypeptide, or the desired encoding DNA fragment may be chemically synthesized.

Soluble TWEAKR polypeptides comprise all or part of the TWEAKR extracellular domain, but generally lack the transmembrane domain that would cause retention of the polypeptide at the cell surface. Soluble polypeptides may include part of the transmembrane domain or all or part of the cytoplasmic domain as long as the polypeptide is secreted from the cell in which it is produced. Soluble TWEAKR polypeptides advantageously comprise a native or heterologous signal peptide when initially synthesized, to promote secretion from the cell, but the signal sequence is cleaved upon secretion. The term "TWEAKR extracellular domain" is intended to encompass all or part of the native TWEAKR extracellular domain, as well as related forms including but not limited to: (a) fragments, (b) variants, (c) derivatives, and (d) fusion polypeptides. The ability of these related forms to inhibit angiogenesis or other TWEAKR-mediated responses may be determined in vitro or in vivo, using methods such as those exemplified below or using other assays known in the art. Examples of soluble TWEAKR polypeptides are provided below. In some embodiments of the present invention a multimeric form of a soluble TWEAKR polypeptide ("soluble TWEAKR multimer") is used as an antagonist to block the binding of TWEAK to TWEAKR, to inhibit angiogenesis or other TWEAKR-mediated responses.

Soluble TWEAKR multimers are covalently-linked or non-covalently-linked multimers, including dimers, trimers, or higher multimers. Multimers may be linked by disulfide bonds formed between cysteine residues on different soluble TWEAKR polypeptides. One embodiment of the invention is directed to multimers comprising multiple soluble TWEAKR polypeptides joined via covalent or non-covalent

interactions between peptide moieties fused to the soluble TWEAKR polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting multimerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that can promote multimerization of soluble TWEAKR polypeptides attached thereto, as described in more detail below. In particular embodiments, the multimers comprise from two to four soluble TWEAKR polypeptides.

In some embodiments, a soluble TWEAKR multimer is prepared using polypeptides derived from immunoglobulins. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (Proc. Natl. Acad. Sci. USA 88:10535, 1991); Byrn et al. (Nature 344:677, 1990); and Hollenbaugh and Aruffo ("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1-10.19.11, 1992).

One preferred embodiment of the present invention is directed to a TWEAKR-Fc dimer comprising two fusion proteins created by fusing soluble TWEAKR to an Fc polypeptide. A gene fusion encoding the TWEAKR-Fc fusion protein is inserted into an appropriate expression vector. TWEAKR-Fc fusion proteins are expressed in host cells transformed with the recombinant expression vector, and allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc moieties to yield divalent soluble TWEAKR. The term "Fc polypeptide" as used herein includes native and mutein forms of polypeptides derived from the Fc region of an antibody. Truncated forms of such polypeptides containing the hinge region that promotes dimerization are also included.

One suitable Fc polypeptide, described in PCT application WO 93/10151, is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent 5,457,035 and by Baum et al., EMBO J. 13:3992, 1994. The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors. Fusion polypeptides comprising Fc moieties, and multimers formed therefrom, offer an advantage of facile purification by affinity chromatography over Protein A or Protein G columns, and Fc fusion polypeptides may provide a longer in vivo half life, which is useful in therapeutic applications, than unmodified polypeptides.

In other embodiments, a soluble TWEAKR polypeptide may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a soluble TWEAKR multimer with as many as four soluble TWEAKR polypeptides.

Alternatively, the soluble TWEAKR multimer is a fusion protein comprising multiple soluble TWEAKR polypeptides, with or without peptide linkers (spacers), or peptides that have the property of promoting multimerization. Among the suitable peptide linkers are those described in U.S. Patents 4,751,180, 4,935,233, and 5,073,627. A DNA sequence encoding a desired peptide linker may be inserted between, and in the same reading frame as, the DNA sequences encoding TWEAKR, using conventional techniques known in the art. For example, a chemically synthesized oligonucleotide encoding the linker

may be ligated between sequences encoding soluble TWEAKR. In particular embodiments, a fusion protein comprises from two to four soluble TWEAKR polypeptides, separated by peptide linkers.

Another method for preparing soluble TWEAKR multimers involves use of a leucine zipper domain. Leucine zipper domains are peptides that promote multimerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., Science 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize. Examples of leucine zipper domains suitable for producing soluble multimeric proteins are described in PCT application WO 94/10308, and the leucine zipper derived from lung surfactant protein D (SPD) described in Hoppe et al. FEBS Lett. 344:191, 1994. The use of a modified leucine zipper that allows for stable trimerization of a heterologous protein fused thereto is described in Fanslow et al., Semin. Immunol. 6:267, 1994. Recombinant fusion proteins comprising a soluble TWEAKR polypeptide fused to a leucine zipper peptide are expressed in suitable host cells, and the soluble TWEAKR multimer that forms is recovered from the culture supernatant.

For some applications, the soluble TWEAKR multimers of the present invention are believed to provide certain advantages over the use of monomeric forms. Fc fusion polypeptides, for example, typically exhibit an increased in vivo half life as compared to an unmodified polypeptide.

The present invention encompasses the use of various forms of soluble TWEAKR multimers that retain the ability to inhibit angiogenesis or other TWEAKR-mediated responses. The term "soluble TWEAKR multimer" is intended to encompass multimers containing all or part of the native TWEAKR extracellular domain, as well as related forms including, but not limited to, multimers of: (a) fragments, (b) variants, (c) derivatives, and (d) fusion polypeptides of soluble TWEAKR. The ability of these related forms to inhibit angiogenesis or other TWEAKR-mediated responses may be determined in vitro or in vivo, using methods such as those exemplified in the examples or using other assays known in the art.

Among the soluble TWEAKR polypeptides and soluble TWEAKR multimers useful in practicing the present invention are TWEAKR variants that retain the ability to bind ligand and/or inhibit angiogenesis or other TWEAKR-mediated responses. Such TWEAKR variants include polypeptides that are substantially homologous to native TWEAKR, but which have an amino acid sequence different from that of a native TWEAKR because of one or more deletions, insertions or substitutions. Particular embodiments include, but are not limited to, TWEAKR polypeptides that comprise from one to ten deletions, insertions or substitutions of amino acid residues, when compared to a native TWEAKR sequence. Included as variants of TWEAKR polypeptides are those variants that are naturally occurring, such as allelic forms and alternatively spliced forms, as well as variants that have been constructed by modifying the amino acid sequence of a TWEAKR polypeptide or the nucleotide sequence of a nucleic acid encoding a TWEAKR polypeptide.

Generally, substitutions for one or more amino acids present in the native polypeptide should be made conservatively. Examples of conservative substitutions include substitution of amino acids outside of the active domain(s), and substitution of amino acids that do not alter the secondary and/or tertiary structure of TWEAKR. Additional examples include substituting one aliphatic residue for another, such as Ile, Val,

Leu, or Ala for one another, or substitutions of one polar residue for another. such as between Lys and Arg; Glu and Asp; or Gln and Asn, or substitutions of one aromatic residue for another, such as Phe, Trp, or Tyr for one another. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are known in the art.

In some preferred embodiments the TWEAKR variant is at least about 70% identical in amino acid sequence to the amino acid sequence of native TWEAKR: in some preferred embodiments the TWEAKR variant is at least about 80% identical in amino acid sequence to the amino acid sequence of native TWEAKR. In some more preferred embodiments the TWEAKR variant is at least about 90% identical in amino acid sequence to the amino acid sequence of native TWEAKR; in some more preferred embodiments the TWEAKR variant is at least about 95% identical in amino acid sequence to the amino acid sequence of native TWEAKR. In some most preferred embodiments the TWEAKR variant is at least about 98% identical in amino acid sequence to the amino acid sequence of native TWEAKR; in some most preferred embodiments the TWEAKR variant is at least about 99% identical in amino acid sequence to the amino acid sequence of native TWEAKR. Percent identity, in the case of both polypeptides and nucleic acids, may be determined by visual inspection. Percent identity may also be determined using 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. Preferably, percent identity is determined by using a computer program, for example, the GAP computer program version 10.x available from the Genetics Computer Group (GCG; Madison, WI, see also Devereux et al., *Nucl. Acids Res.* 12:387, 1984). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, 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 for amino acids; (2) a penalty of 30 (amino acids) or 50 (nucleotides) for each gap and an additional 1 (amino acids) or 3 (nucleotides) penalty for each symbol in each gap; (3) no penalty for end gaps; and (4) no maximum penalty for long gaps. Other programs used by one skilled in the art of sequence comparison may also be used. For fragments of TWEAKR, the percent identity is calculated based on that portion of TWEAKR that is present in the fragment.

The present invention further encompasses the use of soluble TWEAKR polypeptides with or without associated native-pattern glycosylation. TWEAKR expressed in yeast or mammalian expression systems (e.g., COS-1 or COS-7 cells) may be similar to or significantly different from a native TWEAKR polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of TWEAKR polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules. Different host cells may also process polypeptides differentially, resulting in heterogeneous mixtures of polypeptides with variable N- or C-termini.

The primary amino acid structure of soluble TWEAKR polypeptides may be modified to create derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of TWEAKR may be prepared by linking particular functional groups to TWEAKR amino acid side chains or at the N-terminus or C-terminus of a TWEAKR polypeptide.

Fusion polypeptides of soluble TWEAKR that are useful in practicing the invention also include covalent or aggregative conjugates of a TWEAKR polypeptide with other polypeptides added to provide novel polyfunctional entities.

C. TWEAK Receptor Antibodies

One aspect of the present invention relates to the antigenic epitopes of the TWEAKR extracellular domain. Such epitopes are useful for raising antibodies, and in particular the blocking monoclonal antibodies described in more detail below. Such epitopes or variants thereof can be produced using techniques well known in the art such as solid-phase synthesis, chemical or enzymatic cleavage of a polypeptide, or using recombinant DNA technology.

The claimed invention encompasses compositions and uses of antibodies that are immunoreactive with TWEAKR polypeptides. Such antibodies "bind specifically" to TWEAKR polypeptides, meaning that they bind via antigen-binding sites of the antibody as compared to non-specific binding interactions. The terms "antibody" and "antibodies" are used herein in their broadest sense, and include, without limitation, intact monoclonal and polyclonal antibodies as well as fragments such as Fv, Fab, and F(ab')2 fragments, single-chain antibodies such as scFv, and various chain combinations. The antibodies of the present invention are preferably humanized, and more preferably human. The antibodies may be prepared using a variety of well-known methods including, without limitation, immunization of animals having native or transgenic immune repertoires, phage display, hybridoma and recombinant cell culture, and transgenic plant and animal bioreactors.

Both polyclonal and monoclonal antibodies may be prepared by conventional techniques. See, for example, *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet et al. (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988).

Hybridoma cell lines that produce monoclonal antibodies specific for the polypeptides of the invention are also contemplated herein. Such hybridomas may be produced and identified by conventional techniques. One method for producing such a hybridoma cell line comprises immunizing an animal with a polypeptide, harvesting spleen cells from the immunized animal, fusing said spleen cells to a myeloma cell line, thereby generating hybridoma cells, and identifying a hybridoma cell line that produces a monoclonal antibody that binds the polypeptide. The monoclonal antibodies produced by hybridomas may be recovered by conventional techniques.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., "humanized" versions of antibodies originally produced in mice or other non-human species. A humanized antibody is an engineered antibody that typically comprises the variable region of a non-human (e.g., murine) antibody, or at least complementarity determining regions (CDRs) thereof, and the remaining immunoglobulin portions derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature 332*:323, 1988), Liu et al. (*PNAS 84*:3439, 1987), Larrick et al. (*Bio/Technology 7*:934, 1989), and Winter and Harris (*TIPS 14*:139, May,

1993). Such humanized antibodies may be prepared by known techniques and offer the advantage of reduced immunogenicity when the antibodies are administered to humans.

Procedures that have been developed for generating human antibodies in non-human animals may be employed in producing antibodies of the present invention. The antibodies may be partially human or preferably completely human. For example, transgenic mice into which genetic material encoding one or more human immunoglobulin chains has been introduced may be employed. Such mice may be genetically altered in a variety of ways. The genetic manipulation may result in human immunoglobulin polypeptide chains replacing endogenous immunoglobulin chains in at least some, and preferably virtually all, antibodies produced by the animal upon immunization.

Mice in which one or more endogenous immunoglobulin genes have been inactivated by various means have been prepared. Human immunoglobulin genes have been introduced into the mice to replace the inactivated mouse genes. Antibodies produced in the animals incorporate human immunoglobulin polypeptide chains encoded by the human genetic material introduced into the animal. Examples of techniques for the production and use of such transgenic animals to make antibodies (which are sometimes called "transgenic antibodies") are described in U.S. Patent Nos. 5,814,318, 5,569,825, and 5,545,806, which are incorporated by reference herein.

D. Inhibitory Antisense, Ribozyme, and Triple Helix Approaches

Modulation of angiogenesis in a tissue or group of cells may also be ameliorated by decreasing the level of TWEAKR gene expression and/or TWEAK receptor-ligand interaction by using TWEAK receptor or ligand gene sequences in conjunction with well-known antisense, gene "knock-out," ribozyme and/or triple helix methods to decrease the level of TWEAK receptor or ligand gene expression. Among the compounds that may exhibit the ability to modulate the activity, expression or synthesis of the TWEAK receptor or ligand gene, including the ability to modulate angiogenesis, are antisense, ribozyme, and triple helix molecules. Such molecules may be designed to reduce or inhibit either unimpaired, or if appropriate, mutant target gene activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

E. Recombinant Production of TWEAK Receptor Polypeptides

TWEAKR polypeptides, including soluble TWEAKR polypeptides, fragments, and fusion polypeptides, used in the present invention may be prepared using a recombinant expression system. Host cells transformed with a recombinant expression vector ("recombinant host cells") encoding the TWEAKR polypeptide are cultured under conditions that promote expression of TWEAKR and the TWEAKR is recovered. TWEAKR polypeptides can also be produced in transgenic plants or animals, or by chemical synthesis.

The invention encompasses nucleic acid molecules encoding the TWEAKR polypeptides used in the invention, including: (a) nucleic acids that encode residues 28-79 of SEQ ID NO:7 and fragments thereof that bind TWEAK; (b) nucleic acids that are at least 70%, 80%, 90%, 95%, 98%, or 99% identical to a nucleic acid of (a), and which encode a polypeptide capable of binding TWEAK; and (c) nucleic acids that

hybridize at moderate stringency to a nucleic acid of (a), and which encode a polypeptide capable of binding TWEAK.

Due to degeneracy of the genetic code, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence. Included as embodiments of the invention are nucleic acid sequences capable of hybridizing under moderately stringent conditions (e.g., prewashing solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of 50°C, 5 X SSC, overnight) to the DNA sequences encoding TWEAKR. The skilled artisan can determine additional combinations of salt and temperature that constitute moderate hybridization stringency (see also, Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989; Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1982; and Ausubel, *Current Protocols in Molecular Biology*, Wiley and Sons, 1989 and later versions, which are incorporated herein by reference). Conditions of higher stringency include higher temperatures for hybridization and post-hybridization washes, and/or lower salt concentration. Percent identity of nucleic acids may be determined using the methods described above for polypeptides, i.e., by methods including visual inspection and the use of computer programs such as GAP.

Any suitable expression system may be employed for the production of recombinant TWEAKR. Recombinant expression vectors include DNA encoding a TWEAKR polypeptide operably linked to suitable transcriptional and translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the TWEAKR DNA sequence. Thus, a promoter nucleotide sequence is operably linked to a TWEAKR DNA sequence if the promoter nucleotide sequence controls the transcription of the TWEAKR DNA sequence. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. A sequence encoding an appropriate signal peptide (native or heterologous) can be incorporated into expression vectors. A DNA sequence for a signal peptide (referred to by a variety of names including secretory leader, leader peptide, or leader) may be fused in frame to the TWEAKR sequence so that the TWEAKR polypeptide is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells promotes extracellular secretion of the TWEAKR polypeptide. The signal peptide is cleaved from the TWEAKR polypeptide upon secretion of TWEAKR from the cell.

Suitable host cells for expression of TWEAKR polypeptides include prokaryotes, yeast and higher eukaryotic cells, including insect and mammalian cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, insect, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various other species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. In a prokaryotic host cell, such as *E. coli*, TWEAKR polypeptides may include an N-terminal methionine

residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker gene(s). A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. An appropriate promoter and a TWEAKR DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., Nature 275:615, 1978; Goeddel et al., Nature 281:544, 1979), tryptophan (trp) promoter system (Goeddel et al., Nucl. Acids Res. 8:4057, 1980; EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9, ATCC 37092) and pPLc28 (resident in *E. coli* RR1, ATCC 53082).

TWEAKR polypeptides may also be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255:2073, 1980) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7:149, 1968; Holland et al., Biochem. 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phospho-glucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (J. Biol. Chem. 258:2674, 1982) and Beier et al. (Nature 300:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of recombinant polypeptides. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. *See*, e.g., Kurjan et al., Cell 30:933, 1982; Bitter et al., *Proc. Natl. Acad. Sci. USA* *81*:5330, 1984. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from

yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., Proc. Natl. Acad. Sci. USA 75:1929, 1978. The Hinnen et al. protocol selects for Trp^+ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 µg/ml adenine and 20 µg/ml uracil.

Yeast host cells transformed by vectors containing an ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 µg/ml adenine and 80 µg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Insect host cell culture systems also may be employed to express recombinant TWEAKR polypeptides, including soluble TWEAKR polypeptides. Bacculovirus systems for production of heterologous polypeptides in insect cells are reviewed by Luckow and Summers, Bio/Technology 6:47, 1988.

Mammalian cells are particularly preferred for use as host cells. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., Cell 23:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV1/EBNA cell line derived from the African green monkey kidney cell line CV1 (ATCC CCL 70) as described by McMahan et al. (EMBO J. 10: 2821, 1991). For the production of therapeutic polypeptides it is particularly advantageous to use a mammalian host cell line which has been adapted to grow in media that does not contain animal proteins.

Established methods for introducing DNA into mammalian cells have been described (Kaufman, R.J., *Large Scale Mammalian Cell Culture*, 1990, pp. 15-69). Additional protocols using commercially available reagents, such as Lipofectamine (Gibco/BRL) or Lipofectamine-Plus, can be used to transfect cells (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413, 1987). In addition, electroporation can be used to transfect mammalian cells using conventional procedures, such as those in Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1-3, Cold Spring Harbor Laboratory Press, 1989). Selection of stable transformants can be performed using methods known in the art, such as, for example, resistance to cytotoxic drugs. Kaufman et al., Meth. in Enzymology 185:487, 1990, describes several selection schemes, such as dihydrofolate reductase (DHFR) resistance. A suitable host strain for DHFR selection can be CHO strain DX-B11, which is deficient in DHFR (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216, 1980). A plasmid expressing the DHFR cDNA can be introduced into strain DX-B11, and only cells that contain the plasmid can grow in the appropriate selective media. Other examples of selectable markers that can be incorporated into an expression vector include cDNAs conferring resistance to antibiotics, such as G418 and hygromycin B. Cells harboring the vector can be selected on the basis of resistance to these compounds.

Transcriptional and translational control sequences for mammalian host cell expression vectors can be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from polyoma virus, adenovirus 2, simian virus 40 (SV40), and human cytomegalovirus. DNA sequences

derived from the SV40 viral genome. for example. SV40 origin, early and late promoter, enhancer, splice. and polyadenylation sites can be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment, which can also contain a viral origin of replication (Fiers et al., Nature 273:113, 1978; Kaufman, Meth. in Enzymology. 1990). Smaller or larger SV40 fragments can also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Additional control sequences shown to improve expression of heterologous genes from mammalian expression vectors include such elements as the expression augmenting sequence element (EASE) derived from CHO cells (Morris et al., *Animal Cell Technology*, 1997, pp. 529-534) and the tripartite leader (TPL) and VA gene RNAs from Adenovirus 2 (Gingeras et al., J. Biol. Chem. 257:13475. 1982). The internal ribosome entry site (IRES) sequences of viral origin allows dicistronic mRNAs to be translated efficiently (Oh and Sarnow, Current Opinion in Genetics and Development 3:295, 1993; Ramesh et al., Nucleic Acids Research 24:2697, 1996). Expression of a heterologous cDNA as part of a dicistronic mRNA followed by the gene for a selectable marker (e.g. DHFR) has been shown to improve transfectability of the host and expression of the heterologous cDNA (Kaufman, Meth. in Enzymology. 1990). Exemplary expression vectors that employ dicistronic mRNAs are pTR-DC/GFP described by Mosser et al., Biotechniques 22:150. 1997, and p2A5I described by Morris et al., *Animal Cell Technology*, 1997, pp. 529-534.

A useful high expression vector, pCAVNOT, has been described by Mosley et al., Cell 59:335, 1989. Other expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (Mol. Cell. Biol. 3:280, 1983). A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (Mol. Immunol. 23:935, 1986). A useful high expression vector. PMLSV N1/N4, described by Cosman et al., Nature 312:768, 1984. has been deposited as ATCC 39890. Additional useful mammalian expression vectors are known in the art.

Regarding signal peptides that may be employed in producing TWEAKR polypeptides. the native TWEAKR signal peptide may used or it may be replaced by a heterologous signal peptide or leader sequence, if desired. The choice of signal peptide or leader may depend on factors such as the type of host cells in which the recombinant TWEAKR is to be produced. Examples of heterologous signal peptides that are functional in mammalian host cells include the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195, the signal sequence for interleukin-2 receptor described in Cosman et al., Nature 312:768 (1984); the interleukin-4 receptor signal peptide described in EP 367.566; the type I interleukin-1 receptor signal peptide described in U.S. Patent 4.968.607; and the type II interleukin-1 receptor signal peptide described in EP 460,846.

Using the techniques of recombinant DNA including mutagenesis and the polymerase chain reaction (PCR), the skilled artisan can produce DNA sequences that encode TWEAKR polypeptides comprising various additions or substitutions of amino acid residues or sequences. or deletions of terminal or internal residues or sequences, including TWEAKR fragments, variants. derivatives. and fusion polypeptides.

Transgenic animals, including mice, goats, sheep, and pigs, and transgenic plants, including tobacco, tomato, legumes, grasses, and grains, may also be used as bioreactors for the production of TWEAKR polypeptides, including soluble TWEAKR polypeptides. In the case of transgenic animals, it is particularly advantageous to construct a chimeric DNA including a TWEAKR coding sequence operably linked to cis-acting regulatory sequences that promote expression of the soluble TWEAKR in milk and/or other body fluids (see, e.g., U.S. Patent No. 5,843,705; U.S. Patent No. 5,880,327). In the case of transgenic plants it is particularly advantageous to produce TWEAKR in a particular cell type, tissue, or organ (see, e.g., US Patent No. 5,639,947; U.S. Patent No. 5,889,189).

The skilled artisan will recognize that the procedure for purifying expressed soluble TWEAKR polypeptides will vary according to the host system employed, and whether or not the recombinant polypeptide is secreted. Soluble TWEAKR polypeptides may be purified using methods known in the art, including one or more concentration, salting-out, ion exchange, hydrophobic interaction, affinity purification, HPLC, or size exclusion chromatography steps. Fusion polypeptides comprising Fc moieties (and multimers formed therefrom) offer the advantage of facile purification by affinity chromatography over Protein A or Protein G columns.

F. Methods of Treatment

Described below are methods and compositions employing the TWEAK receptor or ligand, or the genes encoding the TWEAK receptor or ligand, to promote or suppress angiogenesis in a target tissue or group of cells. The terms "treat," "treating," "treatment," "therapy," "therapeutic," and the like are intended to include preventative therapy, prophylactic therapy, ameliorative therapy, and curative therapy.

The disclosed polypeptides, compositions, and methods are used to inhibit angiogenesis or other TWEAKR-mediated responses in a mammal in need of such treatment. The term "TWEAKR-mediated response" includes any cellular, physiological, or other biological response that is caused at least in part by the binding of TWEAK ligand to TWEAKR, or which may be inhibited or suppressed, in whole or in part, by blocking TWEAK from binding to TWEAKR. The treatment is advantageously administered in order to prevent the onset or the recurrence of a disease or condition mediated by angiogenesis, or to treat a mammal that has a disease or condition mediated by angiogenesis. Diseases and conditions mediated by angiogenesis include but are not limited to ocular disorders, malignant and metastatic conditions, and inflammatory diseases.

Among the ocular disorders that can be treated according to the present invention are eye diseases characterized by ocular neovascularization including, but not limited to, diabetic retinopathy (a major complication of diabetes), retinopathy of prematurity (this devastating eye condition, that frequently leads to chronic vision problems and carries a high risk of blindness, is a severe complication during the care of premature infants), neovascular glaucoma, retinoblastoma, retrolental fibroplasia, rubeosis, uveitis, macular degeneration, and corneal graft neovascularization. Other eye inflammatory diseases, ocular tumors, and diseases associated with choroidal or iris neovascularization can also be treated according to the present invention.

The present invention can also be used to treat malignant and metastatic conditions such as solid tumors. Solid tumors include both primary and metastatic sarcomas and carcinomas.

The present invention can also be used to treat inflammatory diseases including, but not limited to, arthritis, rheumatism, and psoriasis.

Other diseases and conditions that can be treated according to the present invention include benign tumors and preneoplastic conditions, myocardial angiogenesis, hemophilic joints, scleroderma, vascular adhesions, atherosclerotic plaque neovascularization, telangiectasia, and wound granulation.

Disease states that are angiogenic-dependent include coronary or peripheral atherosclerosis and ischemia of any tissue or organ, including the heart, liver, brain, and the like. These types of diseases can be treated by compositions that promote angiogenesis.

In addition to polypeptides comprising a fragment of TWEAKR extracellular domain, soluble TWEAKR multimers, and antibodies that bind to the TWEAKR extracellular domain, other forms of TWEAKR antagonists can also be administered to achieve a therapeutic effect. Examples of other forms of TWEAKR antagonists include other antibodies such as antibodies against TWEAK, antisense nucleic acids, ribozymes, muteins, aptamers, and small molecules directed against TWEAKR or against TWEAK.

The methods according to the present invention can be tested in in vivo animal models to confirm the desired prophylactic or therapeutic activity, as well as to determine the optimal therapeutic dosage, prior to administration to humans.

The amount of a particular TWEAKR antagonist that will be effective in a particular method of treatment depends upon age, type and severity of the condition to be treated, body weight, desired duration of treatment, method of administration, and other parameters. Effective dosages are determined by a physician or other qualified medical professional. Typical effective dosages are about 0.01 mg/kg to about 100 mg/kg body weight. In some preferred embodiments the dosage is about 0.1-50 mg/kg; in some preferred embodiments the dosage is about 0.5-10 mg/kg. The dosage for local administration is typically lower than for systemic administration. In some embodiments a single administration is sufficient; in some embodiments the TWEAKR antagonist is administered as multiple doses over one or more days.

The TWEAKR antagonists are typically administered in the form of a pharmaceutical composition comprising one or more pharmacologically acceptable carriers. Pharmaceutically acceptable carriers include diluents, fillers, adjuvants, excipients, and vehicles which are pharmaceutically acceptable for the route of administration, and may be aqueous or oleaginous suspensions formulated using suitable dispersing, wetting, and suspending agents.

Pharmaceutically acceptable carriers are generally sterile and free of pyrogenic agents, and may include water, oils, solvents, salts, sugars and other carbohydrates, emulsifying agents, buffering agents, antimicrobial agents, and chelating agents. The particular pharmaceutically acceptable carrier and the ratio of active compound to carrier are determined by the solubility and chemical properties of the composition, the mode of administration, and standard pharmaceutical practice.

The compositions as described herein may be contained in a vial, bottle, tube, syringe inhaler or other container for single or multiple administrations. Such containers may be made of glass or a polymer material such as polypropylene, polyethylene, or polyvinylchloride, for example. Preferred containers may

include a seal, or other closure system, such as a rubber stopper that may be penetrated by a needle in order to withdraw a single dose and then re-seal upon removal of the needle. All such containers for injectable liquids, lyophilized formulations, reconstituted lyophilized formulations or reconstitutable powders for injection known in the art or for the administration of aerosolized compositions are contemplated for use in the presently disclosed compositions and methods.

The TWEAKR antagonists are administered to the patient in a manner appropriate to the indication. Thus, for example, a TWEAKR antagonist, or a pharmaceutical composition thereof, may be administered by intravenous, transdermal, intradermal, intraperitoneal, intramuscular, intranasal, epidural, oral, topical, subcutaneous, intracavity, sustained release from implants, peristaltic routes, or by any other suitable technique. Parenteral administration is preferred.

In certain embodiments of the claimed invention, the treatment further comprises treating the mammal with one or more additional chemotherapeutic agents. The additional chemotherapeutic agent(s) may be administered prior to, concurrently with, or following the administration of the TWEAKR antagonist. The use of more than one chemotherapeutic agent is particularly advantageous when the mammal that is being treated has a solid tumor. In some embodiments of the claimed invention, the treatment further comprises treating the mammal with radiation. Radiation, including brachytherapy and teletherapy, may be administered prior to, concurrently with, or following the administration of the second chemotherapeutic agent(s) and/or TWEAKR antagonist.

When the mammal that is being treated has a solid tumor, the method preferably includes the administration of, in addition to a TWEAKR antagonist, one or more chemotherapeutic agents selected from the group consisting of alkylating agents, antimetabolites, vinca alkaloids and other plant-derived chemotherapeutics, nitrosoureas, antitumor antibiotics, antitumor enzymes, topoisomerase inhibitors, platinum analogs, adrenocortical suppressants, hormones, hormone agonists and antagonists, antibodies, immunotherapeutics, blood cell factors, radiotherapeutics, and biological response modifiers.

In some preferred embodiments the method includes administration of, in addition to a TWEAKR antagonist, one or more chemotherapeutic agents selected from the group consisting of cisplatin, cyclophosphamide, mechloretamine, melphalan, bleomycin, carboplatin, fluorouracil, 5-fluorodeoxyuridine, methotrexate, taxol, asparaginase, vincristine, and vinblastine, lymphokines and cytokines such as interleukins, interferons (including alpha, beta, or delta), and TNF, chlorambucil, busulfan, carmustine, lomustine, semustine, streptozocin, dacarbazine, cytarabine, mercaptopurine, thioguanine, vindesine, etoposide, teniposide, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, mitomycin, L-asparaginase, hydroxyurea, methylhydrazine, mitotane, tamoxifen, and fluoxymesterone.

In some preferred embodiments the method includes administration of, in addition to a TWEAKR antagonist, one or more chemotherapeutic agents, including various soluble forms thereof, selected from the group consisting of Flt3 ligand, CD40 ligand, interleukin-2, interleukin-12, 4-1BB ligand, anti-4-1BB antibodies, TNF antagonists and TNF receptor antagonists, TRAIL, VEGF antagonists, VEGF receptor (including VEGF-R1 and VEGF-R2, also known as Flt1 and Flk1 or KDR) antagonists, Tek antagonists, and CD148 (also referred to as DEP-1, ECRTP, and PTPRJ, see Takahashi et al., J. Am. Soc. Nephrol. 10:2135-45, 1999) agonists. In some preferred embodiments the TWEAKR antagonists of the invention are used as a

component of, or in combination with, "metronomic therapy," such as that described by Browder et al. and Klement et al. (Cancer Research 60:1878, 2000: J. Clin. Invest. 105(8):R15, 2000; see also Barinaga. Science 288:245, 2000).

The polypeptides, compositions, and methods of the present invention may be used as a first line treatment, for the treatment of residual disease following primary therapy, or as an adjunct to other therapies including chemotherapy, surgery, radiation, and other therapeutic methods known in the art.

When the nucleic acid sequences of the present invention are delivered according to the methods disclosed herein, it is advantageous to use a delivery mechanism so that the sequences will be incorporated into a cell for expression. Delivery systems that may advantageously be employed in the contemplated methods include the use of, for example, viral delivery systems such as retroviral and adenoviral vectors, as well as non-viral delivery systems. Such delivery systems are well known by those skilled in the art.

G. Methods of Screening

The TWEAK receptor as described herein may be used in a variety of methods of screening to isolate, for example, TWEAKR agonists and antagonists. TWEAKR agonists are compounds that promote the biological activity of TWEAKR and TWEAKR antagonists are compounds that inhibit the biological activity of TWEAKR. Compounds identified via the following screening assays can be used in compositions and methods for modulating angiogenesis to treat a variety of disease states. The present invention provides methods of screening for compounds that (1) modulate TWEAK receptor or ligand gene expression in a target tissue or cell, (2) modulate the TWEAK receptor-ligand interaction to regulate angiogenesis; (3) bind to the TWEAK receptor or ligand to influence angiogenesis; or (4) interfere with or regulate the bound TWEAK receptor-ligand complex's influence on downstream events such as angiogenesis.

The present invention contemplates the use of assays that are designed to identify compounds that modulate the activity of a TWEAK receptor or ligand gene (i.e., modulate the level of TWEAK gene expression and/or modulate the level of TWEAK gene product activity). Assays may additionally be utilized that identify compounds that bind to TWEAK gene regulatory sequences (e.g., promoter sequences; see e.g., Platt, 1994, J. Biol. Chem. 269, 28558-28562), and that may modulate the level of TWEAK gene expression.

Such an assay may involve, for example, the use of a control system, in which transcription and translation of the TWEAK receptor or ligand gene occurs, in comparison to a system including a test compounds suspected of influencing normal transcription or translation of a TWEAK gene. For example, one could determine the rate of TWEAK receptor RNA produced by cardiac cells, and use this to determine if a test compound influences that rate. To assess the influence of a test compound suspected to influence this normal rate of transcription, one would first determine the rate of TWEAK receptor RNA production in a cardiac cell culture by, for example, Northern Blotting. One could then administer the test compound to a cardiac cell culture under otherwise identical conditions as the control culture. Then the rate of TWEAK receptor RNA in the culture treated with the test compound could be determined by, for example, Northern Blotting, and compared to the rate of TWEAK receptor RNA produced by the control culture cells. An

increase in the TWEAK receptor RNA in the cells contacted with the test compound relative to control cells is indicative of a stimulator of TWEAK receptor gene transcription and/or translation in cardiac cells. while a decrease is indicative of an inhibitor of TWEAK receptor gene transcription and/or translation in cardiac cells.

There are a variety of other methods that can be used to determine the level of TWEAK receptor or ligand gene expression as well. and may further be used in assays to determine the influence of a test compound on the level of TWEAK receptor or ligand gene expression. For example, RNA from a cell type or tissue known, or suspected. to express the TWEAK receptor or ligand gene. such as cardiac. may be isolated and tested utilizing hybridization or PCR techniques. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or. alternatively. to test the effect of compounds on the expression of the TWEAK receptor or ligand gene. Such analyses may reveal both quantitative and qualitative aspects of the expression pattern of the TWEAK receptor or ligand gene, including activation or inactivation of TWEAK receptor or ligand gene expression.

In one embodiment of such a detection scheme. a cDNA molecule is synthesized from an RNA molecule of interest (e.g., by reverse transcription of the RNA molecule into cDNA). A sequence within the cDNA is then used as the template for a nucleic acid amplification reaction. such as a PCR amplification reaction. or the like. The nucleic acid reagents used as synthesis initiation reagents (e.g., primers) in the reverse transcription and nucleic acid amplification steps of this method are chosen from among the TWEAK receptor or ligand gene nucleic acid segments described above. The preferred lengths of such nucleic acid reagents are at least 9-30 nucleotides. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product may be made such that the product may be visualized by standard ethidium bromide staining or by utilizing any other suitable nucleic acid staining method.

Additionally, it is possible to perform such TWEAK receptor or ligand gene expression assays "in situ", i.e., directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. TWEAK receptor or ligand gene nucleic acid segments described above can be used as probes and/or primers for such in situ procedures (see. for example, Nuovo, G. J., 1992, "PCR In Situ Hybridization: Protocols And Applications". Raven Press. NY).

Compounds identified via assays such as those described herein may be useful. for example, in modulating angiogenesis influenced by the TWEAK receptor-ligand interaction. Such methods of stimulating or inhibiting TWEAK-influenced angiogenesis are discussed herein.

Alternatively. assay systems may be designed to identify compounds capable of binding the TWEAK receptor or ligand polypeptide of the invention and thereby influencing angiogenesis resulting from this interaction. Compounds identified may be useful. for example. in modulating the vascularization of target tissues or cells. may be utilized in screens for identifying compounds that disrupt normal TWEAK receptor-ligand interactions. or may in themselves disrupt such interactions.

The principle of the assays used to identify compounds that bind to the TWEAK receptor or ligand involves preparing a reaction mixture of the TWEAK receptor or ligand and the test compound under

conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex that can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways. For example, one method to conduct such an assay screening for compounds that bind to the TWEAK receptor, would involve anchoring the TWEAK receptor or the test substance onto a solid phase and detecting TWEAK receptor/test compound complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the TWEAK receptor may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly. Alternatively, these same methods could be used to screen for test compounds that bind to the TWEAK ligand rather than receptor.

In practice, microtiter plates may conveniently be utilized as the solid phase. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein to be immobilized may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the non-immobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously non-immobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the previously non-immobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for the TWEAK receptor or ligand or the test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

Those compounds identified as binding agents for either the TWEAK receptor or the TWEAK ligand may further be assessed for their ability to interfere with TWEAK receptor-ligand interaction, as described below, and thereby suppress or promote angiogenesis resulting from TWEAK receptor-ligand interaction. Such compounds may then be used therapeutically to stimulate or inhibit angiogenesis.

The TWEAK receptor and ligand polypeptides of the present invention may also be used in a screening assay to identify compounds and small molecules which specifically interact with the disclosed TWEAK receptor or ligand to either inhibit (antagonize) or enhance (agonize) interaction between these molecules. Thus, for example, polypeptides of the invention may be used to identify antagonists and agonists from cells, cell-free preparations, chemical libraries, and natural product mixtures. The antagonists and agonists may be natural or modified substrates, ligands, enzymes, receptors, *etc.* of the polypeptides of the instant invention, or may be structural or functional mimetics of the polypeptides. Potential antagonists of the TWEAK receptor-ligand interaction of the instant invention may include small molecules, peptides,

and antibodies that bind to and occupy a binding site of the polypeptides, causing them to be unavailable to interact and therefore preventing their normal ability to modulate angiogenesis. Other potential antagonists are antisense molecules which may hybridize to mRNA *in vivo* and block translation of the mRNA into the polypeptides of the instant invention. Potential agonists include small molecules, peptides and antibodies which bind to the instant TWEAK polypeptides and influence angiogenesis as caused by the disclosed interactions of the TWEAK polypeptides of the instant invention.

Small molecule agonists and antagonists are usually less than 10K molecular weight and may possess a number of physiochemical and pharmacological properties that enhance cell penetration, resist degradation and prolong their physiological half-lives. (Gibbs, "Pharmaceutical Research in Molecular Oncology," *Cell*, Vol. 79, (1994).) Antibodies, which include intact molecules as well as fragments such as Fab and F(ab')2 fragments, may be used to bind to and inhibit the polypeptides of the instant invention by blocking the commencement of a signaling cascade. It is preferable that the antibodies are humanized, and more preferable that the antibodies are human. The antibodies of the present invention may be prepared by any of a variety of well-known methods.

Specific screening methods are known in the art and many are extensively incorporated in high throughput test systems so that large numbers of test compounds can be screened within a short amount of time. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays, cell based assays, etc. These assay formats are well known in the art. The screening assays of the present invention are amenable to screening of chemical libraries and are suitable for the identification of small molecule drug candidates, antibodies, peptides and other antagonists and agonists.

One embodiment of a method for identifying molecules which antagonize or inhibit TWEAK receptor-ligand interaction involves adding a candidate molecule to a medium which contains cells that express the polypeptides of the instant invention; changing the conditions of said medium so that, but for the presence of the candidate molecule, the polypeptides would interact; and observing the binding and inhibition of angiogenesis. Binding of the TWEAK receptor and ligand can be determined according to competitive binding assays outlined above, and well known in the art. The angiogenic effect of this binding can be determined *via* cell proliferation assays such as, for example, cell density assays, or other cell proliferation assays that are also well-known in the art. The activity of the cells contacted with the candidate molecule may then be compared with the identical cells which were not contacted and agonists and antagonists of the TWEAK polypeptide interactions of the instant invention may be identified. The measurement of biological activity may be performed by a number of well-known methods such as measuring the amount of protein present (*e.g.* an ELISA) or of the protein's activity. A decrease in biological stimulation or activation would indicate an antagonist. An increase would indicate an agonist.

Screening assays can further be designed to find molecules that mimic the biological activity resulting from the TWEAK polypeptide interactions of the instant invention. Molecules which mimic the biological activity of a polypeptide may be useful for enhancing the biological activity of the polypeptide. To identify compounds for therapeutically active agents that mimic the biological activity of a polypeptide, it must first be determined whether a candidate molecule binds to the polypeptide. A binding candidate

molecule is then added to a biological assay to determine its biological effects. The biological effects of the candidate molecule are then compared to those of the polypeptide.

Additionally, complex formation within reaction mixtures containing the test compound and normal TWEAK receptor or ligand gene protein may also be compared to complex formation within reaction mixtures containing the test compound and a mutant TWEAK receptor or ligand gene protein. This comparison may be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal TWEAK receptor or ligand gene proteins.

The assay for compounds that interfere with the interaction of the TWEAK receptor or ligand gene products and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the TWEAK receptor or ligand gene product or the binding partner onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the TWEAK receptor or ligand gene products and the binding partners, e.g., by competition, can be identified by conducting the reaction in the presence of the test substance; i.e., by adding the test substance to the reaction mixture prior to or simultaneously with the TWEAK receptor and ligand gene products. Alternatively, test compounds that disrupt preformed complexes, e.g., compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the TWEAK receptor or ligand gene product, is anchored onto a solid surface, while the non-anchored species is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the TWEAK receptor or ligand gene product and drying. Alternatively, an immobilized antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g., by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds that inhibit complex formation or that disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using

an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds that inhibit complex or that disrupt preformed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the TWEAK receptor or ligand gene product is prepared in which either the TWEAK receptor or ligand gene product or its binding partners is labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Pat. No. *4,109,496* by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances that disrupt TWEAK receptor or ligand gene product interaction can be identified.

In a particular embodiment, the TWEAK receptor or ligand gene product can be prepared for immobilization using recombinant DNA techniques. For example, the TWEAK receptor or ligand coding region can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive binding partner can be purified and used to raise a monoclonal antibody, using methods routinely practiced in the art. This antibody can be labeled with the radioactive isotope <125> I, for example, by methods routinely practiced in the art. In a heterogeneous assay, e.g., the GST-TWEAK receptor or ligand fusion protein can be anchored to glutathione-agarose beads. The TWEAK receptor or ligand gene product can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the TWEAK receptor and ligand gene products can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

Alternatively, a GST-TWEAK receptor gene fusion protein and TWEAK ligand gene product (or *vice versa*) can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the TWEAK receptor-ligand gene product interaction can be detected by adding the labeled antibody and measuring the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of the TWEAK receptor and/or ligand protein, in place of one or both of the full length proteins. Any number of methods routinely practiced in the art can be used to identify and isolate the binding sites. These methods include, but are not limited to, mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the gene encoding the second species in the complex can then be selected. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a

solid surface using methods described in this Section above, and allowed to interact with and bind to its labeled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labeled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the segments can be engineered to express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesized.

For example, and not by way of limitation, a TWEAK receptor or ligand gene product can be anchored to a solid material as described, above, in this Section by making a GST-TWEAK receptor or ligand fusion protein and allowing it to bind to glutathione agarose beads. The interactive binding partner obtained can be labeled with a radioactive isotope, such as <35> S, and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-TWEAK receptor fusion protein or TWEAK ligand fusion protein and allowed to bind. After washing away unbound peptides, labeled bound material, representing the binding partner binding domain, can be eluted, purified, and analyzed for amino acid sequence by well-known methods. Peptides so identified can be produced synthetically or fused to appropriate facilitative proteins using recombinant DNA technology.

The TWEAK receptor-ligand interactions of the invention, *in vivo*, initiate a cascade of events that either stimulate or suppress angiogenesis in a target group of cell or tissue. Molecules, such as nucleic acid molecules, proteins, or small molecules may, in turn, influence this cascade. Compounds that disrupt the TWEAK receptor-ligand interaction effects in this way may be useful in regulating angiogenesis.

The basic principle of the assay systems used to identify compounds that interfere with the angiogenic or anti-angiogenic effect of TWEAK receptor-ligand interaction involves preparing a reaction mixture containing the TWEAK receptor and ligand under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity of the effect of this interaction, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of the TWEAK receptor-ligand complex. Control reaction mixtures are incubated without the test compound or with a placebo. The inhibition or potentiation of any effect of the TWEAK complex on vascularization is then detected. Normal angiogenic response in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the cascade of events initiated by the TWEAK receptor-ligand interaction. Enhanced angiogenesis in the test compounds-containing culture indicates a stimulator of the TWEAK receptor-ligand complex effect.

Throughout the description and the claims of this specification the word "comprise" and variations of the word, such as "comprising" and "comprises" is not intended to exclude other additives, components, integers or steps.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

EXAMPLES

The following examples are intended to illustrate particular embodiments and not to limit the scope of the invention.

EXAMPLE 1
Identification of the TWEAK Receptor

A. Expression Cloning of TWEAK Receptor cDNA

To clone TWEAK Receptor cDNA, an expression vector encoding a growth hormone leader, a leucine zipper multimerization domain, and the C-terminal extracellular domain of human TWEAK (see Chicheportiche et al., J. Biol. Chem. 272(51):32401, 1997) was constructed. This expression vector, which was named pDC409-LZ-TWEAK, comprised the DNA sequence SEQ ID NO:1 and encoded the polypeptide SEQ ID NO:2. pDC409-LZ-TWEAK conditioned supernatants were produced by transient transfection into CV1-EBNA cells. These supernatants were incubated with magnetic beads coated with polyclonal goat anti-mouse antibody that had previously been incubated with a mouse monoclonal antibody against the leucine zipper. Control beads were produced by mixing the coated beads with supernatants from cells transfected with empty vector.

A monolayer of COS cells grown in a T175 flask was transfected with 15 µg of DNA pools of complexity of 100,000 from a HUVEC cDNA expression library. After 2 days these cells were lifted from the flask, and incubated in 1.5 mls of binding media plus 5% non-fat dried milk for 3 hours at 4 degrees C on a rotator wheel. Cells were pre-cleared by adding control beads and rotated at 4 degrees C for an additional 45 minutes after which bead bound cells were removed with a magnet. Pre-clearing was repeated 2-3 times, then TWEAK coated beads were added to the cells and rotated 30 minutes at 4 degrees C. Cells binding the TWEAK beads were separated by use of a magnet and washed 4x in PBS. Plasmid DNA was extracted from these cells by lysing in 0.1% SDS, and electroporating the supernatants in DH101B cells. Colonies were grown overnight on ampicilin selective media. Transformants were pooled and used as a source of plasmid DNA for a further round of panning. After 2 rounds of panning, positive clones were picked from the resulting pool based on their ability to bind TWEAK using a slide binding protocol like that described in Part B, below.

The human TWEAK receptor (also called TWEAKR) cDNA was determined to have the sequence SEQ ID NO:3, which encodes a 129 residue polypeptide (SEQ ID NO:4). Examination of the sequence predicts a polypeptide having an approximately 78 amino acid extracellular domain (residues 1-78 of SEQ ID NO:4, including the signal peptide), an approximately 23 amino acid transmembrane domain (residues 79-101 of SEQ ID NO:4), and an approximately 28 amino acid intracellular domain (residues 102-129 of SEQ ID NO:4). TWEAKR is the smallest known TNF receptor family member. It has a single cysteine-rich repeat region in the extracellular domain, as compared to the 3-4 repeats of other TNF receptor family members. The TWEAKR polypeptide was previously described as a transmembrane protein encoded by a human liver cDNA clone (WO 98/55508, see also WO 99/61471), but had not been identified as the TWEAK receptor. A murine homolog, the FGF-inducible Fn14 (Meighan-Mantha et al., J. Biol. Chem. 274(46):33166, 1999), is approximately 82% identical to the human protein, as shown by the alignment in Figure 1.

The newly identified TWEAK receptor was tested side by side with DR3 (which had been identified as the TWEAK receptor by Marsters et al., Current Biology 8:525, 1998) for the ability to bind to TWEAK.

B. The TWEAK Receptor Binds to TWEAK

Slides of COS cells were transfected with expression vectors containing TWEAKR. DR3, or vector without insert (control). After two days the cells were incubated with concentrated supernatants from CV-1 cells transfected with a vector encoding the leucine zipper TWEAK extracellular domain fusion protein. One hour later the cells were washed and probed with an I-125 labeled antibody against the leucine-zipper domain. The slides were washed, fixed, and autoradiography was performed using x-ray film. The TWEAKR transfected cells bound significant amounts of TWEAK. TWEAK did not bind to the cells transfected with DR3 or the control cells. This experiment confirmed that the TWEAKR polypeptide identified in part A above, rather than DR3, is the major receptor for TWEAK. After discovery of the functional TWEAK receptor, other investigators also reported that DR3 is not the major receptor for TWEAK (Kaptein et al., FEBS Lett., 485(2-3):135. 2000. The TWEAK-TWEAKR binding interaction was further characterized by Scatchard analysis.

CV-1 cells were transfected with human full length TWEAK and mixed 1:30 with Raji cells, which do not express TWEAK. The cells were incubated with serial dilutions of 125-I labeled human TWEAK receptor-Fc for 2 hours at 4 degrees Celsius. Free and bound probe was separated by microfuging the samples through a phalate oil mixture in plastic tubes. Supernatants and pellets were gamma-counted. Scatchard analyses of TWEAK ligand binding the TWEAK receptor showed a binding affinity constant (Ka) of approximately $4.5 \times 10^8 M^{-1}$.

C. The TWEAK Receptor is Strongly Expressed in Cardiac Tissue

To determine the expression pattern of the TWEAK receptor, Northern blot analyses were performed. Human multiple tissue northern blots were purchased from Clontech (Palo Alto, CA) and probed with P-32 labeled random primed DNA from the TWEAK receptor coding region. The blots were washed and autoradiography was performed using x-ray film. Results showed that in the adult TWEAKR is strongly expressed in heart, placenta, and some skeletal muscle samples. Strong expression in heart tissue further supports the utility of TWEAKR in the diagnosis and treatment of cardiac disease. In contrast to the adult, the fetal tissues expressed TWEAKR more ubiquitously; TWEAKR transcripts were seen in the lung and liver.

EXAMPLE 2
Preparation of TWEAKR Antagonists and Agonists

Because TWEAK induces angiogenesis, TWEAKR agonists (such as agonistic antibodies) may be used to promote angiogenesis and TWEAKR antagonists (such as soluble receptors and antagonistic antibodies) may be used to inhibit angiogenesis.

A. Recombinant Production of Soluble TWEAK Receptor-Fc (TWEAKR-Fc) Fusion Polypeptides

To construct a nucleic acid encoding the TWEAKR extracellular domain fused to Fc, a nucleic acid encoding the N-terminal 79 amino acids from TWEAKR, including the leader (signal peptide), was joined to a nucleic acid encoding an Fc portion from human IgG1. Sequences for this construct are shown as SEQ ID

NO:6 (nucleic acid) and SEQ ID NO:7 (amino acid). In SEQ ID NO:7, residues 1-27 are the predicted signal peptide (predicted to be cleaved upon secretion from the cell; the actual cleavage site was identified by N-terminal sequence analysis, see below), residues 28-79 are from the cysteine-rich TWEAKR extracellular domain, residues 80-81 are from a BglII cloning site, and the remainder is the Fc portion. Upon insertion into a mammalian expression vector, and expression in and secretion from a mammalian host cells, this construct produced a polypeptide designated TWEAKR-Fc. N-terminal sequence analysis determined that the secreted polypeptide designated TWEAKR-Fc had an N-terminus corresponding to residue 28 (Glu) of SEQ ID NO:7. Anti-angiogenic activity of TWEAKR-Fc was demonstrated using assays such as those described in the following examples. An analogous Fc-fusion construct was prepared using the murine TWEAKR extracellular domain.

B. Production of Antibodies that Bind the TWEAKR Extracellular Domain

BALB/c mice are immunized with TWEAKR extracellular domain and spleen cells are collected and used to prepare hybridomas using standard procedures. Hybridoma supernatants are screened, using ELISA, for the ability to bind TWEAKR. Positives are cloned two times, to insure monoclonality, then isotyped and reassayed for reactivity to TWEAKR. Antibodies and antibody derivatives are also prepared using transgenic mice that express human immunoglobulins and through the use of phage display. The resulting antibodies are tested in assays such as those described in the examples below, to characterize their ability to modulate the TWEAK-TWEAKR interaction, TWEAKR signaling, angiogenesis, and other downstream biological activities.

Agonistic antibodies are used to promote TWEAK-induced biological activities such as angiogenesis, and antagonistic antibodies are used to inhibit TWEAK-induced biological activities such as angiogenesis. For some applications, the activity of antagonistic antibodies is augmented by conjugation to a radioisotope, to a plant-, fungus-, or bacterial-derived cytotoxin such as ricin A or diptheria toxin, or to another chemical poison. And because of the restricted tissue distribution of TWEAKR, antibodies that bind to TWEAKR are particularly useful as targeting agents for imaging or delivering therapeutics to the vasculature. Antibodies that bind TWEAKR can be used, for example, to target a detectable label or chemotherapeutic to the mural cells (pericytes and vascular smooth muscle cells). Detectable labels may include radioisotopes, chemiluminescent and fluorescent compounds, and enzymes. These techniques are useful, for example, in the diagnosis, staging, and treatment of neoplasms.

EXAMPLE 3
Activity of TWEAKR-Fc In a Wound Closure Assay

A planar endothelial cell migration (wound closure) assay was used to quantitate the inhibition of angiogenesis by TWEAKR-Fc in vitro. In this assay, endothelial cell migration is measured as the rate of closure of a circular wound in a cultured cell monolayer. The rate of wound closure is linear, and is dynamically regulated by agents that stimulate and inhibit angiogenesis in vivo.

Primary human renal microvascular endothelial cells, HRMEC, were isolated, cultured, and used at the third passage after thawing, as described in Martin et al., In Vitro Cell Dev Biol 33:261, 1997. Replicate

circular lesions, "wounds," (600-800 micron diameter) were generated in confluent HRMEC monolayers using a silicon-tipped drill press. At the time of wounding the medium (DMEM + 1% BSA) was supplemented with 20 ng/ml PMA (phorbol-12-myristate-13-acetate), EGF (4 ng/ml), and 0.150 to 5 μg/ml TWEAKR-Fc, or a combination of 40 ng/ml EGF and 0.150 to 5 μg/ml TWEAKR-Fc. The residual wound area was measured as a function of time (0-12 hours) using a microscope and image analysis software (Bioquant, Nashville, TN). The relative migration rate was calculated for each agent and combination of agents by linear regression of residual wound area plotted over time. The results are shown in Figures 2-3.

Compared to huIgG or media+BSA, TWEAKR-Fc inhibited PMA-induced endothelial migration in a dose responsive manner, reducing the rate of migration to unstimulated levels at 5 μg/ml (Figure 2). Neither huIgG nor TWEAKR-Fc inhibited basal (uninduced) migration. When HRMEC migration was induced by EGF, TWEAKR-Fc inhibited endothelial migration in a dose-dependent manner, reducing the rate of migration to unstimulated levels at 5 μg/ml (Figure 3).

EXAMPLE 4
Activity of TWEAKR-Fc In a Corneal Pocket Assay

A mouse corneal pocket assay was used to quantitate the inhibition of angiogenesis by TWEAKR-Fc in vivo. In this assay, agents to be tested for angiogenic or anti-angiogenic activity are immobilized in a slow release form in a hydron pellet, which is implanted into micropockets created in the corneal epithelium of anesthetized mice. Vascularization is measured as the appearance, density, and extent of vessel ingrowth from the vascularized corneal limbus into the normally avascular cornea.

Hydron pellets, as described in Kenyon et al., Invest Opthamol. & Visual Science 37:1625, 1996, incorporated sucralfate with bFGF (90 ng/pellet), bFGF and IgG (14 μg/pellet, control), or bFGF and TWEAKR-Fc (14 μg). The pellets were surgically implanted into corneal stromal micropockets created by micro-dissection 1mm medial to the lateral corneal limbus of 6-8 week old male C57BL mice. After five days, at the peak of neovascular response to bFGF, the corneas were photographed, using a Zeiss slit lamp, at an incipient angle of 35-50° from the polar axis in the meridian containing the pellet. Images were digitized and processed by subtractive color filters (Adobe Photoshop 4.0) to delineate established microvessels by hemoglobin content. Image analysis software (Bioquant, Nashville, TN) was used to calculate the fraction of the corneal image that was vascularized, the vessel density within the vascularized area, and the vessel density within the total cornea.

As shown in Table 1, TWEAKR-Fc (100 pmol) inhibited bFGF (3 pmol)-induced corneal angiogenesis, reducing the vascular density to 50% of that induced by FGF alone or FGF+IgG.

Table 1
Effect of TWEAKR-Fc on FGF-induced Angiogenesis in the Mouse Corneal Pocket Assay

Treatment	Greater than 50% Reduction in Number and Length of Vessels n/total n (%)
FGF alone	0/2 (0%)
FGF+IgG	0/2 (0%)
FGF+TWEAKR-Fc	6/9 (67%)

EXAMPLE 5
Qualitative TRAF Binding to the TWEAK Receptor (TWEAKR) Cytoplasmic Domain

Members of the TRAF family are intra-cellular signaling molecules. Several members of the TRAF family are known to associate with members of the TNF receptor family in order to initiate a signaling cascade that activates the NF-kappa-B pathway, resulting in cell activation and proliferation. A qualitative in vitro binding assay was performed to test whether members of the TRAF family of intra-cellular signaling molecules bind to the cytoplasmic domain of TWEAKR and to learn, therefore, whether the small cytoplasmic domain of TWEAKR is capable of mediating a signal into the cell via the TRAF pathway.

A GST fusion vector consisting of the C-terminal 29 amino acids of TWEAKR fused to glutathione S-transferase was created by sub-cloning the appropriate insert into the pGEX-4T (Amersham Pharmacia Biotech) vector at the BamHI and NotI sites. The product from this vector was expressed in E.coli and bound to sepharose beads as described by Galibert et al., J. Biol. Chem. 273(51):34120, 1998. Similarly constructed beads coated with RANK cytoplasmic domain-GST fusion proteins were used as a positive control, and beads coated with GST alone were used as a negative control. [35S]methionine/cysteine labeled TRAF proteins were produced in reticulocyte lysates (TNT-coupled Reticulocyte Lysate Systems, Promega) according to the manufacturer's protocol. Reticulocyte lysates containing the labeled TRAF molecules were first pre-cleared using the control beads followed incubation with the indicated fusion protein coated beads in binding buffer (50 mM HEPES [pH 7.4], 250 mM NaCl, 0.25% (v/v) Nonidet P-40, 10% glycerol, 2 mM EDTA) at 4 degrees Celsius for 2 hours. After washing 4x with binding buffer bound TRAF molecules eluted from the beads in SDS-loading buffer, separated by SDS-PAGE, dried and exposed to X-ray film.

Binding above background levels was seen with TRAFS 1,2 and 3. No binding above background levels was seen with TRAFS 4,5, and 6. The ability of TWEAKR to bind to TRAFs 1,2, and 3 demonstrates that TWEAKR is capable of inducing a signal to the cell via the TRAF pathway, and therefore transmitting a proliferative signal into the host cell. This experiment provides further evidence that TWEAKR is the functional receptor for TWEAK. It also illustrates a further means by which signaling can be inhibited: by disrupting the TRAF-TWEAKR interaction with a small molecule, or by use of a dominant negative variant of the TRAF molecule.

EXAMPLE 6
Activity of TWEAKR-Fc in an Endothelial Cell Proliferation Assay

An endothelial cell proliferation assay was used to quantitate the inhibition of bFGF or TWEAK induced-proliferation by TWEAKR-Fc in vitro. In this assay, endothelial cell proliferation is measured after 4 days of cell growth in microtiter wells using a cell labeling molecule called calcein AM. Esterases expressed by the cells cleave the calcein and cause it to fluoresce when excited at 485 nm. Uncleaved calcein does not fluoresce. The amount of fluorescence is directly related to the number of endothelial cells in the culture well. Endothelial cell proliferation is often regulated by agents that stimulate and/or inhibit angiogenesis in vivo.

Primary HUVEC (human umbilical vein endothelial cells) were obtained from a commercial source (Clonetics, Walkersville, MD), cultured, and used at passage 2 to 7. Replicate cultures were set up by adding 3000 HUVEC to each microtiter well in endothelial cell basal media (EBM, an endothelial cell basal media that contains no growth factors or serum and is based on the media formulations developed by Dr. Richard Ham at the University of Colorado, Clonetics) plus 0.05% FBS (fetal bovine serum). At the time of culture initiation FGF-2 (fibroblast growth factor-2, 10 ng/ml) or human TWEAK (100 ng/ml) was added to the cultures in the presence of human IgG (huIgG, control) or human TWEAKR-Fc at concentrations ranging from 0.08 μg/ml to 20 μg/ml (0.25 to 20 μg/ml for TWEAK-induced and 0.08 to 6.7 μg/ml for FGF-2-induced). The HUVEC containing cultures were incubated for 4 days at 37 degrees C. 5% CO_2. On the fourth day of culture 4 μM calcein-AM was added to the cultures and 2 hours later the wells were evaluated for fluorescence. The results, expressed as the average fluorescence (485-530 nm) counts for replicate wells plus or minus the SEM, are shown in Figures 4 and 5.

TWEAKR-Fc specifically inhibited TWEAK-induced HUVEC proliferation in a dose-dependent manner when compared to huIgG which did not effect TWEAK-induced proliferation (Figure 4). In addition, TWEAKR-Fc inhibited the basal proliferation of HUVEC observed during culture in EBM plus 0.05% FBS, as compared to huIgG which did not. Interestingly, TWEAKR-Fc also inhibited FGF-2 mediated HUVEC proliferation at concentrations of greater than 2 μg/ml, as compared to huIgG which did not effect the FGF-2 induced HUVEC proliferative response (Figure 5). These results show that TWEAKR-Fc inhibits HUVEC proliferation induced by the addition of exogenous recombinant human TWEAK. That TWEAKR-Fc partially inhibits serum -induced HUVEC-proliferation indicates HUVEC produce endogenous TWEAK that promotes growth/survival of the EC (endothelial cell) via the TWEAKR. TWEAKR-Fc attenuation of FGF-2 induced proliferation indicates that at least part of the EC response to FGF-2 is dependent on endogenous TWEAK/TWEAKR interaction.

EXAMPLE 7
Inhibition of Neovascularization by TWEAKR Antagonists in a Murine Cardiac Ischemia/Engraftment Model

Survival of heterotopically transplanted cardiac tissue from one mouse donor to the ear skin of another genetically similar mouse requires adequate neovascularization by the transplanted heart and the surrounding tissue, to promote survival and energy for cardiac muscle function. Inadequate vasculature at the site of transplant causes excessive ischemia to the heart, tissue damage, and failure of the tissue to engraft. Agents that antagonize factors involved in endothelial cell migration and vessel formation can decrease angiogenesis at the site of transplant, thereby limiting graft tissue function and ultimately engraftment itself. A murine heterotopic cardiac isograft model is used to demonstrate the effects of TWEAKR antagonists, including antibodies and TWEAKR-Fc, on neovascularization.

Female BALB/c (≈12 weeks of age) recipients are given neonatal heart grafts from donor mice of the same strain. The donor heart tissue is grafted into the left ear pinnae of the recipient on day 0 and the mice are divided into two groups. The control group receives human IgG (Hu IgG) while the other group receives the TWEAKR antagonist, both intraperitoneally. The treatments are continued for five consecutive

days. The functionality of the grafts is determined by monitoring visible pulsatile activity on days 7 and 14 post-engraftment. The inhibition of functional engraftment, as a function of the dose of TWEAKR antagonist, is determined. The histology of the transplanted hearts is examined is order to visualize the effects of the TWEAKR antagonist on edema at the site of transplant and host and donor tissue vasculature (using, e.g., Factor VIII staining).

EXAMPLE 6
Treatment of Tumors With TWEAKR Antagonists

TWEAKR antagonists, including antibodies and TWEAKR-Fc, are tested in animal models of solid tumors. The effect of the TWEAKR antagonists is determined by measuring tumor frequency and tumor growth.

The relevant disclosures of publications cited herein are specifically incorporated by reference. The examples presented above are not intended to be exhaustive or to limit the scope of the invention. The skilled artisan will understand that variations and modifications and variations are possible in light of the above teachings, and such modifications and variations are intended to be within the scope of the invention.

EDITORIAL NOTE

APPLICATION NUMBER – 12140/01

The following Sequence Listing pages 1-8 is part of the description. The claims pages follow on pages "32" to "35".

SEQUENCE LISTING

<110> WILEY, Steven R.
IMMUNEX CORPORATION

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<130> 2968-WO

<140> to be assigned
<141> 2000-12-19

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RECTIFIED SHEET (RULE 91)

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225                 230                 235                 240

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            260                 265                 270

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RECTIFIED SHEET (RULE 91)

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of modulating angiogenesis in a mammal in need of such treatment comprising administering a therapeutically-effective amount of a composition comprising a TWEAK receptor antagonist or TWEAK receptor agonist, wherein the TWEAK receptor comprises a sequence of amino acids from about 28-79 of SEQ ID NO:7 or a naturally occurring variant thereof, and wherein the TWEAK receptor agonist is an agonistic TWEAK receptor antibody, a TWEAK receptor-binding compound that is not TWEAK, or a small molecule that binds to the TWEAK receptor.

2. The method of claim 1 wherein the composition further comprises a pharmaceutically acceptable carrier.

3. The method of claim 1 or claim 2, wherein the mammal is a human.

4. A method of inhibiting angiogenesis according to one of claims 1-3, wherein the composition comprises a TWEAK receptor antagonist.

5. The method of claim 4, wherein the TWEAK receptor antagonist is selected from the group consisting of a soluble TWEAK receptor fragment, an antibody that binds to TWEAK receptor and blocks the interaction of a TWEAK receptor with its ligand, an antisense nucleic acid that inhibits TWEAK receptor expression, a triple helix forming nucleic acid that inhibits TWEAK receptor expression, a peptide that binds TWEAK receptor, and a small molecule that binds TWEAK receptor.

6. The method of claim 5, wherein the TWEAK receptor antagonist comprises a soluble TWEAK receptor fragment.

7. The method of claim 6, wherein the TWEAK receptor antagonist further comprises an Fc polypeptide, a peptide linker, or a leucine zipper domain.

8. The method of claim 7 wherein the TWEAK receptor antagonist comprises an Fc polypeptide fused to: (a) a TWEAK receptor extracellular domain; or (b) a fragment or variant of (a) that is capable of binding TWEAK.

9. The method of claim 8, wherein the TWEAK receptor extracellular domain comprises amino acids 28-79 of SEQ ID NO : 7.

10. The method of claim 9, wherein the TWEAK receptor antagonist comprises amino acids 28-309 of SEQ ID NO : 7.

11. The method of claim 5, wherein the TWEAK receptor antagonist comprises an antibody that binds specifically a TWEAK receptor extracellular domain.

12. The method claim 11, wherein the antibody is selected from the group consisting of a monoclonal antibody, a humanized antibody, a transgenic antibody, and a human antibody.

13. The method of claim 11 or 12, wherein the antibody is conjugated to a radioisotope; to a plant-, a fungus-, or a bacterial-derived toxin such as ricin A or diptheria toxin; or to another chemical poison.

14. The method of claim 5 wherein the TWEAK receptor antogonist disrupts the interaction between the TWEAK receptor and a TRAF molecule.

15. The method of one of claims 4-14, wherein the mammal has a disease or condition mediated by angiogenesis.

16. The method of claim 15 wherein the disease or condition is characterized by ocular neovascularization.

17. The method of claim 15, wherein the disease or condition is a solid tumor.

18. The method of one of claims 15-17, wherein the method further comprises treating the mammal with radiation.

19. The method of one of claims 15-18, wherein the method further comprises treating the mammal with a second chemotherapeutic agent.

20. The method of claim 19, wherein the second chemotherapeutic agent is selected from the group consisting of alkylating agents, antimetabolites, vinca alkaloids and other plant-derived chemotherapeutics, nitrosoureas, antitumor antibiotics, antitumor enzymes, topoisomerase inhibitors, platinum analogs, adrenocortical suppressants, hormones, hormone agonists, hormone antagonists, antibodies, immunotherapeutics, blood cell factors, radiotherapeutics, and biological response modifiers.

21. The method of claim 19, wherein the second chemotherapeutic agent is selected from the group consisting of cisplatin, cyclophosphamide, mechloretamine, melphalan, bleomycin, carboplatin, fluorouracil, 5-fluorodeoxyuridine, methotrexate, taxol, asparaginase, vincristine, and vinblastine, lymphokines and cytokines such as interleukins, interferons (including alpha, beta, or delta), and TNF, chlorambucil, busulfan, carmustine, lomustine, semustine, streptozocin, dacarbazine, cytarabine, mercaptopurine, thioguanine, vindesine, etoposide, teniposide, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, mitomycin, L-asparaginase, hydroxyurea, methylhydrazine, mitotane, tamoxifen, and fluoxymesterone.

22. The method of claim 19, wherein the second chemotherapeutic agent is selected from the group consisting of Flt3 ligand, CD40 ligand, interleukin-2, interleukin-12, 4-1BB ligand, anti-4-1BB antibodies, TNF antagonists and TNF receptor antagonists, TRAIL, CD148 agonists, VEGF antagonists, VEGF receptor antagonists, and Tek antagonists.

23. A method of promoting angiogenesis according to one of claims 1-3, wherein the composition comprises a TWEAK receptor agonist.

24. The method of claim 23, wherein the TWEAK receptor agonist is an agonistic antibody that binds specifically to a TWEAK receptor extracellular domain.

25. The method of claim 24, wherein the antibody is selected from the group consisting of a monoclonal antibody, a humanized antibody, a transgenic antibody, and a human antibody.

26. The method of one of claims 23-25, wherein the agonist is administered:

(a) to treat a vascularization deficiency in cardiac or peripheral tissue, including coronary artery disease, myocardial ischemia, myocardial infarction, angina pectoris, peripheral circulation deficits, limb ischemia/reperfusion injury;

(b) to enhance wound healing, organ transplantation, reconnection of severed digits or limbs, or vascular or skin grafting; or

(c) in conjunction with bypass surgery or angioplasty.

27. An antagonist comprising a soluble TWEAK receptor fragment for use in medicine.

28. The antagonist of claim 27, further comprising an Fc polypeptide, a peptide linker, or a leucine zipper domain.

29. The antagonist of claim 28, wherein the antagonist comprises an Fc polypeptide fused to: (a) a TWEAK receptor extracellular domain; or (b) a fragment or variant of (a) that is capable of binding TWEAK.

30. The antagonist of claim 29, wherein the TWEAK receptor extracellular domain comprises amino acids 28-79 of SEQ ID NO:7.

31. The antagonist of claim 30, wherein the antagonist comprises amino acids 28-309 of SEQ ID NO:7.

32. A nucleic acid encoding an antagonist according to one of claims 27-31.

33. The nucleic acid of claim 32, comprising a sequence as set forth in SEQ ID NO:6.

34. An expression vector comprising the nucleic acid of claim 32 or 33.

35. A recombinant host cell comprising the nucleic acid of claim 32 or 33.

36. A method of producing a TWEAK receptor antagonist comprising culturing the host cell of claim 35 under conditions promoting expression of the TWEAK receptor antagonist.

37. The use of a composition comprising a TWEAK receptor antagonist or TWEAK receptor agonist for the preparation of a medicament for modulating angiogenesis in a mammal in need of such treatment, wherein the TWEAK receptor comprises a sequence of amino acids from about 28-79 of SEQ ID NO:7 or a naturally occurring variant thereof.

38. A method of identifying a compound that is capable of modulating angiogenesis comprising:

identifying a test compound that binds to a TWEAK receptor extracellular domain wherein the TWEAK receptor extracellular domain comprises a sequence selected from the group consisting of (a) amino acids 28-78 of SEQ ID NO:4, and (b) fragments (a) that bind TWEAK, wherein the test compound is not TWEAK.

39. A method of identifying a compound that is capable of modulating angiogenesis comprising identifying a test compound that affects the interaction between a TWEAK and a TWEAK receptor, wherein the TWEAK receptor comprises a sequence selected from the group consisting of (a) amino acids 28-78 of SEQ ID NO:4, and (b) fragments (a) that bind TWEAK.

40. A method of identifying a compound that is capable of modulating angiogenesis comprising identifying a test compound that modulates the interaction between a TWEAK receptor and a TRAF, wherein the TWEAK receptor comprises a sequence selected from (a) amino acids 102-129 of SEQ ID NO:4, or (b) a fragment (a) that binds the TRAF.

41. The method of one of claims 38-40 further comprising determining the ability of the test compound to modulate endothelial cell proliferation and/or endothelial cell migration and/or angiogenesis.

42. The method of one of claims 38-41 wherein the modulation is stimulatory.

43. The method of one of claims 38-41 wherein the modulation is inhibitory.

44. A compound identified according to the method of one of claims 38-43, wherein the compound is not TWEAK.

45. A method of modulating the binding of TWEAK to a TWEAK receptor in a mammal in need of such treatment, comprising administering to the mammal an inhibition-effective amount of a composition comprising a TWEAK receptor antagonist selected from the group consisting of: (a) a soluble TWEAK receptor extracellular domain; and (b) an antibody that binds to the TWEAK receptor extracellular domain.

46. A method for targeting a detectable label or chemotherapeutic to vascular tissue comprising contacting vascular tissue with an antibody that binds TWEAK receptor.

47. The method of claim 46 wherein the antibody is conjugated to a radioisotope, chemiluminescent or fluorescent compound, or enzyme.

48. The method of claim 46 wherein the antibody is conjugated to a cytotoxin.

49. A method according to claim 1, substantially as hereinbefore described with reference to the Examples.

50. An antagonist according to claim 27, substantially as hereinbefore described with reference to the Examples.

51. The use according to claim 37, substantially as hereinbefore described with reference to the Examples.

52. A method according to any one of claims 38, 39, 40, 45 or 46, substantially as hereinbefore described with reference to the Examples.

Dated: 18 June 2002

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Attorneys for

IMMUNEX CORPORATION

```
  1 MAPGWPRSLPQILVLGFGLVLMRAAAGEQAPGTSPCSSGSSWSADLDKCM 50
    ||  |   | |  .:||||   | |:|. ||||||||.||| ||||||||||
  1 MARGSLRRLLRLLVLGLWLALLRSVAGEQAPGTAPCSRGSSWSADLDKCM 50
              .          .          .          .          .
 51 DCASCPARPHSDFCLGCAAAPPAHFRLLWPILGGALSLVLVLALVSSFLV 100
    |||||  |||||||||||||||| ||||||||||||||   || |.| |||
 51 DCASCRARPHSDFCLGCAAAPPAPFRLLWPILGGALSLTFVLGLLSGFLV 100
              .          .
101 WRRCRRREKFTTPIEETGGEGCPGVALIQ 129
    |||||||||||||||||||||| |||||
101 WRRCRRREKFTTPIEETGGEGCPAVALIQ 129
```

Fig. 1

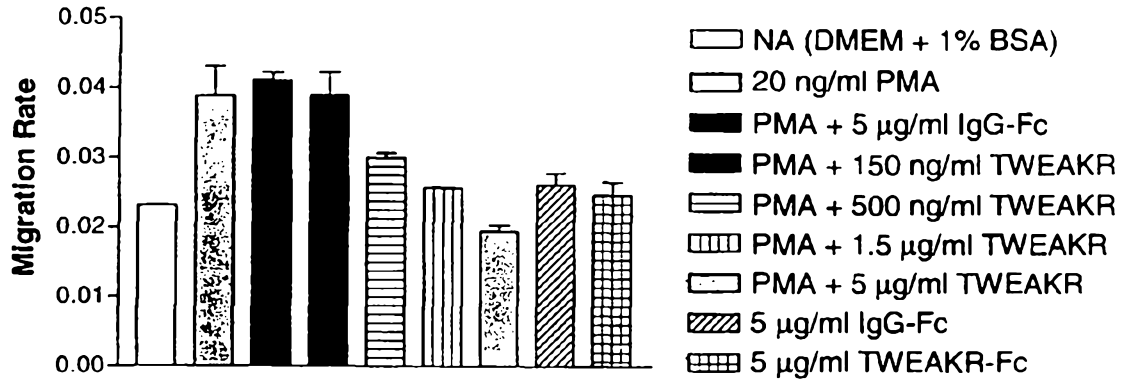
Migration Rate
0.05
0.04
0.03
0.02
0.01
0.00
NA (DMEM + 1% BSA)
20 ng/ml PMA
PMA + 5 µg/ml IgG-Fc
PMA + 150 ng/ml TWEAKR
PMA + 500 ng/ml TWEAKR
PMA + 1.5 µg/ml TWEAKR
PMA + 5 µg/ml TWEAKR
5 µg/ml IgG-Fc
5 µg/ml TWEAKR-Fc

Fig. 2

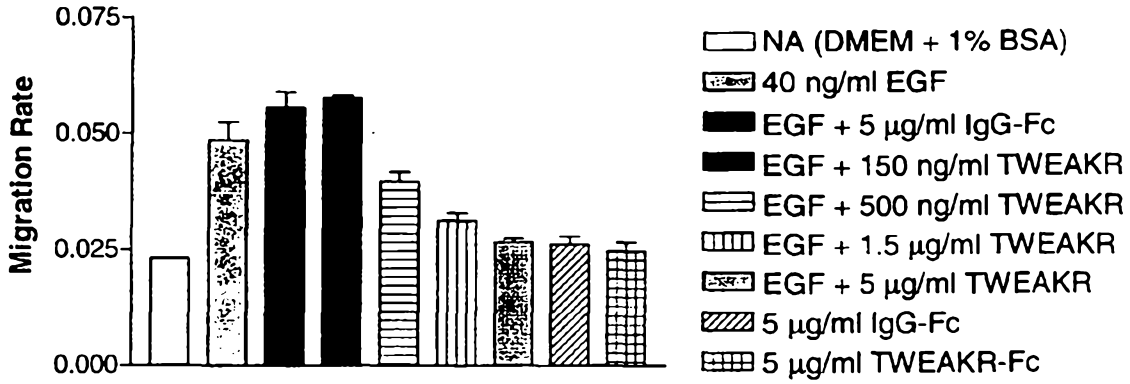
Migration Rate
0.075
0.050
0.025
0.000
NA (DMEM + 1% BSA)
40 ng/ml EGF
EGF + 5 µg/ml IgG-Fc
EGF + 150 ng/ml TWEAKR
EGF + 500 ng/ml TWEAKR
EGF + 1.5 µg/ml TWEAKR
EGF + 5 µg/ml TWEAKR
5 µg/ml IgG-Fc
5 µg/ml TWEAKR-Fc

Fig. 3

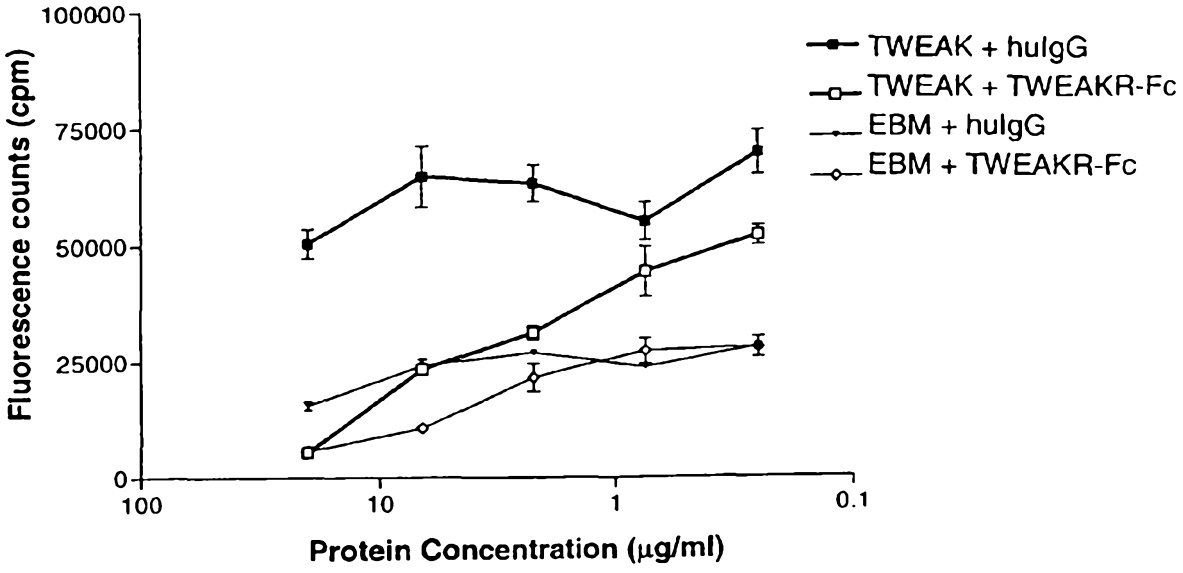
100000
75000
50000
25000
0
Fluorescence counts (cpm)
100
10
1
0.1
Protein Concentration (μg/ml)
TWEAK + hulgG
TWEAK + TWEAKR-Fc
EBM + hulgG
EBM + TWEAKR-Fc

Fig. 4

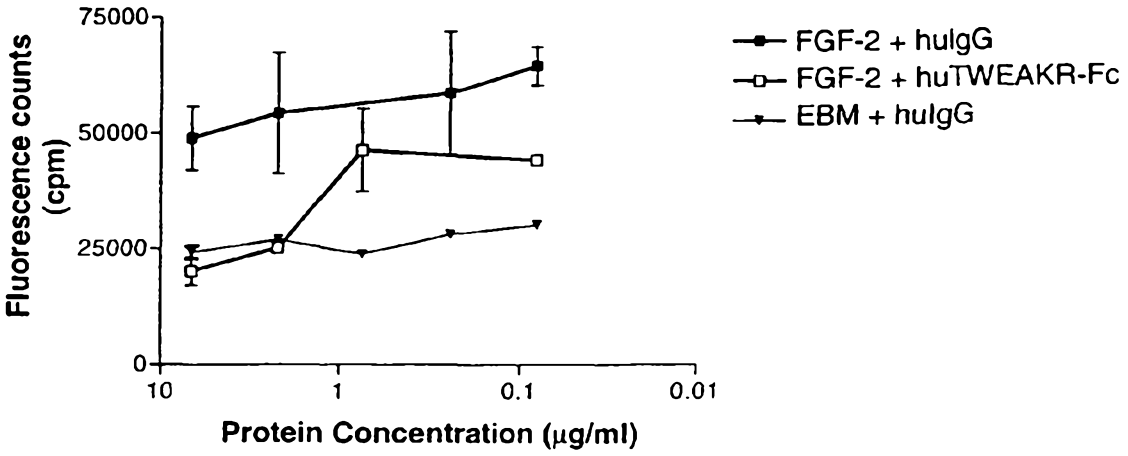
75000
50000
25000
0
Fluorescence counts (cpm)
10
1
0.1
0.01
Protein Concentration (μg/ml)
FGF-2 + hulgG
FGF-2 + huTWEAKR-Fc
EBM + hulgG

Fig. 5