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(54) **COMPOSITIONS A BASE D'OLIGOMERES PROTEINIQUES RENFERMANT LA PROTEINE ENDOSTATINE ET PROCEDES D'UTILISATION**

(54) **PROTEIN OLIGOMER COMPOSITIONS COMPRISING ENDOSTATIN PROTEIN AND METHODS OF USING THE SAME**

(57) L'invention concerne des compositions à base d'oligomères protéiniques renfermant la protéine endostatine, ainsi que des procédés relatifs à l'utilisation desdites compositions, pour interrompre la formation de tubules et inhiber la tumorigenèse. Ces compositions représentent une nouvelle classe de facteurs de dispersion et comprennent spécifiquement les dimères et les trimères de la protéine endostatine, s'étendant éventuellement à des ions métalliques du type zinc.

(57) Protein oligomer compositions comprising endostatin protein are provided as well as methods of using the protein oligomer compositions to disrupt tubule formation and inhibit tumorigenesis. The compositions of the present invention constitute a new class of scatter factors and specifically include endostatin protein dimers and trimers, and optionally include metal ions such as zinc.

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(54) Title: PROTEIN OLIGOMER COMPOSITIONS COMPRISING ENDOSTATIN PROTEIN AND METHODS OF USING THE SAME

(57) Abstract

Protein oligomer compositions comprising endostatin protein are provided as well as methods of using the protein oligomer compositions to disrupt tubule formation and inhibit tumorigenesis. The compositions of the present invention constitute a new class of scatter factors and specifically include endostatin protein dimers and trimers, and optionally include metal ions such as zinc.

PROTEIN OLIGOMER COMPOSITIONS COMPRISING ENDOSTATIN PROTEIN AND METHODS OF USING THE SAME

Cross Reference to Related Applications

This application claims priority to provisional application Serial No. 60/087,890 filed June 3, 1998; provisional application Serial No. 60/092,393 filed July 10, 1998; and provisional application Serial No. 60/098,790 filed September 1, 1998. Each of the above-referenced applications is incorporated herein in its entirety.

Field of the Invention

The present invention relates to the fields of oncology, angiogenesis and morphogenesis and more particularly to novel protein oligomers comprising endostatin protein. The endostatin oligomers are useful for inhibiting endothelial cell tubule formation, regulating cellular morphogenesis, and treating metastatic cancers and angiogenesis-dependent diseases.

Background of the Invention

Several lines of direct evidence now suggest that the growth and persistence of solid tumors as well as their metastases to distant organs is critically dependent upon angiogenesis, or the recruitment of new blood vessels (Folkman, 1989; Hori et al.,

1991; Kim et al., 1993; Millauer et al., 1994). Angiogenesis not only provides the increased nutrients and pathways for the removal of waste needed for the expansion of the tumor, but it also facilitates tumor metastasis by providing a route for tumor cells to leave the primary site and enter the bloodstream (Zetter, 1998). In particular, angiogenesis increases the entry of tumor cells into the bloodstream by providing an increased density of immature, highly permeable blood vessels that have thinner basement membranes and fewer intracellular junction complexes than normal mature vessels (Zetter, 1998).

It is postulated that the angiogenic phenotype is the result of a net balance between both positive and negative regulators of neovascularization (Good et al., 1990; O'Reilly et al., 1994; Parangi et al., 1996; Rastinejad et al., 1989). Tumors themselves, along with other accessory host cells such as macrophages, mast cells and lymphocytes, stimulate angiogenesis by up-regulating their production of a variety of angiogenic factors, including the fibroblast growth factor (FGF). (Kandel et al., 1991) and vascular endothelial cell growth factor/vascular permeability factor (VEGF/VPF) (Zetter, 1998). However, many malignant tumors also generate inhibitors of angiogenesis, including angiostatin protein and thrombospondin (Chen et al., 1995; Good et al., 1990; O'Reilly et al., 1994; U.S. Patent No. 5,639,725).

Several angiogenic and anti-angiogenic proteins are stored as inactive precursors in the blood or basement membranes (Hanahan, 1996). One example of such an inactive precursor is endostatin protein. Endostatin protein is an approximately 20 kDa C-terminal globular domain of the collagen-like protein, collagen XVIII (c18) which is localized in the basement membrane around blood vessels (Oh et al., 1994). Endostatin is stored *in vivo* as the C-terminal portion of the c18 protein (Rehn et al., 1994; Muragaki et al., 1995) and fragments of c18 longer than endostatin are believed not to inhibit endothelial cell proliferation (O'Reilly et al., 1997). Endostatin was first isolated from a hemangioendothelioma cell line for its ability to inhibit the

proliferation of capillary endothelial cells as described in U.S. Patent Number 5,854,205, which is hereby incorporated by reference.

Although it is known that endostatin is stored as an inactive precursor, it is not known how endostatin is activated. Previous x-ray crystallographic studies indicated that endostatin does not contain the characteristic Ca^{2+} binding sites that are active in selectins, a similar group of molecules (Hohenester et al., 1998). These same studies indicated that no metal ions were associated with human endostatin.

Metal ions are often involved in the biologic activity of proteins. In particular, the zinc cation is a critical component of many proteins and plays a key role in a host of biological processes (Coleman, 1992; O'Reilly et al, 1996, Vallee et al. 1990). In most cases, zinc is directly involved in the catalytic activity, however structural roles for zinc have also been described. For example, zinc is involved in the dimerization of human growth hormone and increases the affinity of human growth hormone for the prolactin receptor by approximately 8,000 fold (Cunningham et al., 1990; Cunningham et al., 1991).

Summary of the Invention

The present invention includes protein oligomers comprising more than one endostatin protein monomers, wherein the oligomer has scatter factor activity. Endostatin protein is a carboxy-terminal region fragment of collagen XVIII having a molecular weight of approximately 20 kDa as determined by reducing gel electrophoresis and 18 kDa as determined by non-reducing gel electrophoresis. In one embodiment of the present invention, the protein oligomer comprises a dimer of endostatin monomers. In an alternate embodiment, the protein oligomer comprises more than one NC1 region fragment of collagen XVIII having a molecular weight of approximately 38 kDa under reducing gel electrophoresis such that each NC1 fragment contains an endostatin monomer. In a preferred embodiment, the

protein oligomer comprising more than one NC1 region fragment is a trimer. The protein oligomers of the present invention may optionally comprise a metal component, which is preferably zinc.

The novel protein oligomers described herein constitute a novel class of scatter factors. The protein oligomers of the present invention have anti-tubulogenic effects and induce reorganization of the actin cytoskeleton, destruction of tubular lumens, and elongation of cells. The novel protein oligomers are also anti-tumorigenic and anti-angiogenic.

Also included in the present invention are methods of using the protein oligomers comprising endostatin protein. The protein oligomers described herein may be used to inhibit tubulogenesis and tumorigenesis.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

Brief Description of the Figures

Figure 1. *Structure of Human Endostatin*

ß strands are labeled in sequential order A-P, α helices are denoted, and zinc is shown as a sphere.

Figure 2. *The N-terminal Loop and Zinc Binding Site of Human Endostatin*

The zinc (black circle) ligands histidines 1, 3, and 11 and aspartic acid 76, as well as the second shell of interactions that position the zinc ligands, glutamic acid 175, residue 11 carbonyl oxygen, and arginine 4 from the N-terminal loop of an adjacent molecule in a dimer are shown as ball and stick models.

Figure 3A-B. *A Zinc Dependent Dimer in Human Endostatin Crystals*

Figure 3A. The zinc (black sphere) site N-terminal loops of two monomers contact across a central dyad axis. Glutamine 7, phenylalanine 6, and arginine 5 of the loop project from one monomer to the next. Also shown are two phenylalanine rings, residues 31 and 34, that project from an endostatin α helix and form another dimeric contact in the crystal.

Figure 3B. Contacts in the interface of the dimer seen in crystals of human endostatin. Zinc (black sphere) ligands have open bonds, interface residues have solid bonds. The path of the polypeptide chains of the two monomers are shown as tubes. The solvent accessible surface buried in this dimer interface is 403 $Å^2$ (probe size = 1.4 Å) per monomer.

Figure 4. *Oligomeric Endostatins Exhibit Scatter Factor Activity*

HUVEC tubules on matrigel were pre-formed for 16 hours and treated with human endostatin monomer, human Fc-endostatin dimer, human Fc dimers, human Fc-endostatin(C7) dimers, human endostatin(C7) dimers, human endostatin/NC1 trimers, human Fc collagen 15 dimers, or Fc angiostatin dimers. Only endostatin dimers and trimers exhibited scatter activity and inhibited tubule formation.

Detailed Description

The present invention provides a new class of scatter factors comprising endostatin oligomers. Oligomers in this new class of scatter factors act as "anti-matrix" scatter factors and are capable of inhibiting endothelial tubule assembly evoked by the presence of extracellular matrix proteins. The endostatin oligomers are additionally anti-angiogenic and anti-tumorigenic. Although the endostatin oligomers have been defined functionally, it is to be understood that this functional definition in no way limits the bioactivity of the endostatin oligomers.

The protein oligomers of one embodiment of the present invention comprise a plurality of endostatin monomers, wherein the endostatin monomers are approximately 20 kDa proteins as determined under reduced gel electrophoresis or approximately 18 kDa as determined under non-reduced gel electrophoresis, and are characterized by their ability to inhibit proliferating cultured endothelial cells. The endostatin oligomers are carboxy-region fragments of collagen-like molecules such as collagen XVIII, and may be derived from any mammal. In a preferred embodiment, the endostatin monomer begins at approximately amino acid position 1132 of murine collagen XVIII, and correlates to the human endostatin fragment of SEQ ID NO:1 shown below.

HSHRDFQPVLHLVALNSPLSGGMRGIRGADFQCFQQARAVG
LAGTFRAFLSSRLQDLYSIVRRADRAAVPIVNLKDELLFPSW
EALFSGSEGPLKPGARIFSFDGKDVLRHPTWPQKSVWHGSD
PNGRRLTESYCETWRTEAPSATGQASSLLGGRLLGQSAASC
HHAYIVLCIENSFMTAS

As described below, amino acid substitutions may occur in the sequence of endostatin which still yield a functional endostatin protein. For example, when the above gene sequence is recombinantly expressed, an observable doublet of protein results, both versions of which are functional endostatin proteins. In addition to the above endostatin protein, the following endostatin variant occurs, which is the former protein minus the first four amino acids. This demonstrates the variability of functional endostatin protein molecules. Therefore, in an alternate embodiment, the endostatin monomer correlates to the human endostatin fragment of SEQ ID NO:2 shown below.

DFQPVLHLVALNSPLSGGMRGIRGADFQCFQQARAVGLAG
TFRAFLSSRLQDLYSIVRRADRAAVPIVNLKDELLFPSWEAL
FSGSEGPLKPGARIFSFDGKDVLRHPTWPQKSVWHGSDPNG

RRLTESYCETWRTEAPSATGQASSLLGGRLLGQSAASCHHA YIVLCIENSFMTAS

The terms "endostatin" and "endostatin monomer" are synonymous and include naturally occurring, recombinant, or synthetic endostatin proteins that contain conservative, or "silent" amino acid substitutions, deletions and additions, yet upon oligomerization retain scatter factor activity. The term "scatter factor activity" refers to the disruption of endothelial tubule formation as determined by a matrigel tube-formation assay. In a preferred embodiment, the endostatin monomers are modified at the seventh amino acid such that glutamine is replaced with cysteine. Replacement of glutamine with cysteine facilitates dimerization or oligomerization between endostatin monomers. The term "endostatin monomer" also includes shortened proteins wherein one or more amino acid is removed from either or both ends of an endostatin monomer, or from an internal region of the protein, yet upon oligomerization retain scatter factor activity. The term "endostatin monomer" also includes lengthened proteins or peptides wherein one or more amino acids is added to either or both ends of an endostatin monomer, or to an internal location, yet upon oligomerization retain scatter factor activity. One example of such a modification is the addition of tyrosine to the first position. Tyrosine labeled molecules may be further labeled with ^{125}iodine for use in assays. Labeling with other radioisotopes or chemicals such as ricin may also be useful in providing a molecular tool for destroying the target cells containing endostatin oligomer receptors. Endostatin monomers can also be recombinantly fused to other proteins or peptides, such as a Fc portion of an antibody as described in the Examples below.

Additionally, silent substitutions of amino acids, are well known in the art and are intended to fall within the scope of the appended claims. Silent substitutions occur when the replacement of an amino acid with a structurally or chemically similar amino acid does not significantly alter the structure, conformation or

activity of the protein. Also included in the definition of the term "endostatin monomer" are modifications of the protein, its subunits and peptide fragments. Such modifications include substitutions of naturally occurring amino acids at specific sites with other molecules, including but not limited to naturally and non-naturally occurring amino acids. Such substitutions may modify the bioactivity of endostatin oligomers, such as by increasing or decreasing the scatter factor activity, and produce biological or pharmacological agonists or antagonists.

In one embodiment, the protein oligomers are endostatin dimers. The present invention includes a novel class of scatter factors that includes endostatin dimers. The endostatin dimers of the present invention progressively disperse established tubes into constituent cells as early as 2-3 hours and without obvious toxicity. During the endostatin dimer induced scatter, dramatic reorganization of the cytoskeleton can be observed along with destruction of tubular lumens and cellular elongation. The endostatin oligomers differ from the previously described Hepatocyte Growth Factor/ Scatter Factor (HGF/SF) class of scatter factors. In contrast to the HGF/SF class of scatter factors that promote tube formation, the endostatin oligomers of the present invention have anti-tubulogenic effects. The endostatin oligomers also affect different types of cells as compared to the HGF/SF class of scatter factors. In addition to scatter activity, the endostatin dimers of the present invention are also capable of anti-tumorigenic and anti-angiogenic activity when bound to a metal such as zinc.

In an alternate embodiment, the invention provides protein oligomers comprising a plurality of endostatin/NC1 proteins. The endostatin/NC1 proteins of the present invention are approximately 38 kDa C-terminal region fragments of collagen XVIII that each contain the approximately 20 kDa endostatin proteins described above. The present invention describes for the first time that endostatin/NC1 trimers are included in the new class of scatter factors described above. Endostatin/NC1 trimers

induce scattering of endothelial cell tubular structures as well as other cellular morphogenic changes in a manner similar to endostatin dimers. The anti-tubulogenic activity of the endostatin/NC1 trimers can also be inhibited by endostatin monomers.

In another embodiment, the protein oligomers described above comprise endostatin monomers that are fusion proteins. The endostatin fusion proteins may comprise endostatin and anti-angiogenic molecules, angiogenic molecules, and/or molecules that facilitate dimerization of the endostatin monomers. In a preferred embodiment, the endostatin fusion proteins comprise endostatin and the Fc portion of an antibody, wherein the Fc portion of the antibody promotes dimerization. The Fc portion may be derived from the IgG, IgE, IgA or IgM isotype, however, the preferred isotype is IgG.

In a further embodiment, the protein oligomers, dimers and monomers are bound to a metal ion, preferably a zinc ion. The invention provides for the first time that endostatin contains a zinc binding site and requires metal binding for activity. While prior attempts to study crystallized endostatin resulted in disordered residues and inaccurate results, the inventors have expressed endostatin monomers in such a manner that the residues are not disordered and have identified a zinc binding domain on endostatin. In the present invention, human endostatin has been expressed as a secreted protein in a murine myeloma cell line as a chimera with the Fc domain of IgG-1 and released from the Fc portion by enterokinase digestion (as more specifically described below). Additionally human endostatin crystals were grown at a higher pH where the N-terminal histidines would not be charged. These modifications of prior art methods have allowed the inventors to accurately identify endostatin as a zinc binding protein.

Using the above methods, the inventors discovered that the zinc binding site of human endostatin is tetrahedral with three zinc ligands from the N-terminal loop, histidines 1, 3, and 11, and

a fourth ligand, aspartic acid 76, from the loop between the E and F ß-strands (Fig. 1 and Fig. 2). The inventors also describe for the first time zinc dependent dimeric contacts between endostatin monomers in human endostatin crystals. Based upon these findings, the present invention provides novel endostatin compositions bound to zinc wherein zinc is essential to the anti-tumorigenic activity of the endostatin compositions, and methods of using these compositions. In a preferred embodiment, the Fc-endostatin dimers are bound to zinc. In another preferred embodiment, NC1/endostatin trimers are bound to zinc.

The present invention also encompasses methods of activating endostatin *in vitro* or *in vivo* comprising the binding of one or more metal ions to endostatin oligomers. In a preferred embodiment, the metal ion is zinc, and in a further preferred embodiment, one zinc molecule is bound per endostatin monomer. Activation of endostatin includes increased anti-angiogenic activity, increased tumorigenic activity and/or increased scatter factor activity. In one embodiment, zinc binding causes dimerization of endostatin monomers and consequently activates endostatin. Also included in the invention are methods of forming endostatin oligomers by the addition of a metal ion, preferably a zinc ion.

Typically, the isolated, endostatin oligomers of the invention are at least about 80% pure, usually at least about 90%, and preferably at least about 95% as measured by band intensity on a silver stained gel. The phrases "isolated", "biologically pure" or “substantially pure” refer to material which is essentially free from at least some of the components which normally accompany it as found in its native state. Other important terms that are used herein are defined as follows. The terms "a", "an" and "the" as used herein are defined to mean one or more and include the plural unless the context is inappropriate. “Anti-tumor activity” and “anti-tumorigenic” refer to the ability of an endostatin oligomer to regress a tumor. "Regression" refers to the reduction of tumor mass or size. The term “anti-angiogenic” refers to

inhibition of vascularization. Additionally, "substantial sequence homology" means at least approximately 70% homology between a known amino acid residue sequence in an endostatin oligomer, preferably at least approximately 80% homology, more preferably at least approximately 90% homology. Homology can be determined by sequence identity, or by using a well-known computer program, such as DNA Star or GeneJockey, on default setting parameters.

The endostatin and NC1 monomers that constitute portions of the protein oligomers described above may be isolated from body fluids and tissues including, but not limited to, serum, urine and ascites fluid. Endostatin monomers and NC1 may also be produced from recombinant sources, genetically altered cells implanted into animals, tumors, cell cultures and other sources. Recombinant techniques include gene amplification from DNA sources using amplification techniques such as the polymerase chain reaction (PCR), and gene amplification from RNA sources using amplification techniques such as reverse transcriptase/PCR. The endostatin monomers may be produced by polypeptide synthesis, or derived by *in vitro* enzymatic catalysis of larger, encompassing polypeptides such as collagen XVIII to yield active endostatin monomers. In a preferred embodiment, the endostatin monomers of the present invention are produced from a recombinant expression system, wherein the cell is *Pichia pastoris*.

The endostatin oligomers of the present invention have several uses. The endostatin oligomers are particularly useful for inhibiting endothelial cell tubule formation and for the study of morphogenesis. Endostatin oligomers, and active fragments thereof, are also useful for treating metastatic cancers and tumors as well as angiogenesis-related cancers and diseases.

For example, the endostatin oligomers may be employed to treat metastatic and angiogenesis-dependent cancers and other angiogenesis-related diseases. One example of metastatic disease is metastatic cancer. Angiogenesis-related diseases include, but are

not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation. The endostatin oligomers of the present invention are useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. They are also useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochele minalia quintosa*) and ulcers (*Helobacter pylori*).

The endostatin oligomers may also be used to develop affinity columns for isolating antibodies directed toward the endostatin oligomers. Those antibodies may be isolated and purified, followed by amino acid sequencing. Also, polypeptides that bind to endostatin oligomers with high specificity and avidity may be labeled with a label or reporter group and employed for visualization and quantitation in the assays described herein using detection techniques such as autoradiographic and membrane binding techniques. The reporter group or label is commonly a fluorescent or radioactive group or an enzyme. Such applications provide important diagnostic and research tools.

Endostatin oligomers can also be employed to develop affinity columns for isolation of endostatin oligomer receptors. Isolation and purification of such receptors may be followed by amino acid sequencing. Using this information, the gene or genes coding for the receptors can be identified and isolated. Next,

cloned nucleic acid sequences may be developed for insertion into vectors capable of expressing the receptors.

Applicants' invention also encompasses recombinant expression systems wherein nucleic acids encoding the protein constituents of the endostatin oligomers described herein are contained in a single cell. The nucleic acids encoding the oligomeric constituents may be in a single vector or multiple vectors. In a preferred embodiment, the recombinant expression system is *Pichia pastoris*. The biologically active endostatin oligomers and nucleic acid sequences corresponding to the proteins are useful for modulating endothelial processes *in vivo*, and for diagnosing and treating endothelial cell-related diseases, for example by gene therapy.

Nucleic acid sequences that correspond to, and code for, the protein constituents of endostatin oligomers can be prepared based upon the knowledge of the amino acid sequence, and the art recognized correspondence between codons (sequences of three nucleic acid bases), and amino acids. Because of the degeneracy of the genetic code, wherein the third base in a codon may vary, yet still code for the same amino acid, many different possible coding nucleic acid sequences are derivable for any particular protein or peptide fragment. Alternatively, the nucleic acid sequence may be derived from a gene bank using oligonucleotides probes designed based on the N-terminal amino acid sequence and well known techniques for cloning genetic material. Nucleic acid sequences are synthesized using automated systems well known in the art. Either the entire sequence may be synthesized or a series of smaller oligonucleotides are made and subsequently ligated together to yield the full length sequence. The genes encoding constituents of endostatin oligomers may also be isolated from cells or tissue that express high levels of endostatin monomers or NC1 by (1) isolating messenger RNA from the tissue, (2) using reverse transcriptase to generate the corresponding DNA sequence and then (3) using PCR with the appropriate primers to amplify the DNA sequence coding for the active sequence.

The isolated, recombinant or synthetic endostatin oligomers described herein are useful for generating antibodies specific for the endostatin oligomers. These antibodies that specifically bind to the endostatin oligomers can be used in diagnostic methods and kits that are well known to those of ordinary skill in the art to detect or quantify the endostatin oligomers in a body fluid or tissue. Results from these tests can be used to diagnose or predict the occurrence or recurrence of a cancer and other angiogenesis mediated diseases. Additionally, passive antibody therapy using antibodies that specifically bind endostatin oligomers can be employed to modulate morphogenic processes such as metastatic cancer as well as angiogenesis-dependent processes such as reproduction, development, wound healing, tissue repair, and angiogenesis-dependent diseases. Also, antisera directed to the Fab regions of endostatin oligomer antibodies can be administered to block the ability of endogenous endostatin oligomer antisera to bind endostatin oligomers.

Antibodies specific for endostatin oligomers may be either polyclonal or monoclonal, and are made according to techniques and protocols well known in the art. The preferred method of making monoclonal antibodies is a modified version of the method of Kearney et al. (1979), which is incorporated by reference herein. The monoclonal antibodies are utilized in well known immunoassay formats, such as competitive and non-competitive immunoassays, including ELISA, sandwich immunoassays and radioimmunoassays (RIAs), to determine the presence or absence of the endostatin oligomers of the present invention in body fluids and tissues. Examples of body fluids include, but are not limited to, blood, serum, synovial fluid, peritoneal fluid, pleural fluid, cerebrospinal fluid, uterine fluid, saliva, mucus and vitreous humor.

Polyclonal antisera are also raised using established techniques known to those skilled in the art. For example, polyclonal antisera may be raised in mice, rabbits, rats, goats, sheep, guinea pigs, chickens, and other animals, most preferably

mice and rabbits by administering the antigen to the animals. Isolated, recombinant or synthetic endostatin oligomers conjugated to a carrier molecule such as bovine serum albumin, may be combined with an adjuvant mixture, emulsified and injected subcutaneously at multiple sites on the back, neck, flanks, and sometimes in the footpads of the animals. Booster injections are made at regular intervals, such as every two to four weeks. Blood samples are obtained by venipuncture, for example using the marginal ear veins after dilation, approximately seven to ten days after each injection. The blood samples are allowed to clot and are centrifuged, and the serum removed, aliquoted, and stored under refrigeration for immediate use or frozen for subsequent analysis.

Techniques for the production of single chain antibodies are known to those skilled in the art and described in U.S. Patent No. 4,946,778 and can be used to produce single chain antibodies to the endostatin oligomers described herein. Bispecific antibodies have two antigen binding domains wherein each domain is directed against a different epitope. Phage display technology may be used to select antibody genes having binding activities for endostatin oligomers from PCR-amplified v genes of lymphocytes from naive libraries or humans screened for having antibodies to the endostatin oligomers.

When labeled with a detectable biomolecule or chemical, the endostatin oligomers and antibodies described above are useful for purposes such as *in vivo* and *in vitro* diagnostics and laboratory research using the methods and assays described below. Various types of labels and methods of conjugating the labels to the endostatin oligomers and antibodies are well known to those skilled in the art. Several specific labels are set forth below.

For example, the endostatin oligomers and antibodies are conjugated to a radiolabel such as, but not restricted to, ^{32}P, ^{3}H, ^{14}C, ^{35}S, ^{125}I, or ^{131}I. Detection of a label can be by methods

such as scintillation counting, gamma ray spectrometry or autoradiography.

Bioluminescent labels, such as derivatives of firefly luciferin, are also useful. The bioluminescent substance is covalently bound to the endostatin oligomer or antibody by conventional methods, and the labeled endostatin oligomer or antibody is detected when an enzyme, such as luciferase, catalyzes a reaction with ATP causing the bioluminescent molecule to emit photons of light.

Fluorogens may also be used as labels. Examples of fluorogens include fluorescein and derivatives, phycoerythrin, allo-phycocyanin, phycocyanin, rhodamine, and Texas Red. The fluorogens are generally detected by a fluorescence detector.

The endostatin oligomers and antibodies can alternatively be labeled with a chromogen to provide an enzyme or affinity label. For example, the endostatin oligomer or antibody can be biotinylated so that it can be utilized in a biotin-avidin reaction, which may also be coupled to a label such as an enzyme or fluorogen. Alternatively, the endostatin oligomer or antibody can be labeled with peroxidase, alkaline phosphatase or other enzymes giving a chromogenic or fluorogenic reaction upon addition of substrate. Additives such as 5-amino-2,3-dihydro-1,4-phthalazinedione (also known as Luminol™) (Sigma Chemical Company, St. Louis, MO) and rate enhancers such as p-hydroxybiphenyl (also known as p-phenylphenol) (Sigma Chemical Company, St. Louis, MO) can be used to amplify enzymes such as horseradish peroxidase through a luminescent reaction; and luminogenic or fluorogenic dioxetane derivatives of enzyme substrates can also be used. Such labels can be detected using enzyme-linked immunoassays (ELISA) or by detecting a color change with the aid of a spectrophotometer. In addition, endostatin oligomers or antibodies may be labeled with colloidal gold for use in immunoelectron microscopy in accordance with methods well known to those skilled in the art.

The proteins and antibodies of the present invention are useful for diagnosing and treating metastatic and angiogenesis-related diseases. One example of metastatic disease is metastatic cancer. Angiogenesis-related diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation. The Endostatin oligomers of the present invention are useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. They are also useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochele minalia quintosa*) and ulcers (*Helobacter pylori*).

According to the present invention, endostatin oligomers may be used in combination with other compositions and procedures for the treatment of the above described diseases and conditions. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy combined with endostatin oligomers and subsequently endostatin oligomers may be administered to the patient to extend the dormancy of micrometastases and to stabilize any residual primary tumor.

The endostatin oligomers described above can be provided as isolated and substantially purified proteins and protein fragments in pharmaceutically acceptable formulations using formulation methods known to those of ordinary skill in the art. These formulations can be administered by standard routes. In

general, the combinations may be administered by the topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g., intravenous, intraspinal, subcutaneous or intramuscular) route. In addition, the endostatin oligomers may be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the endostatin oligomer is slowly released systemically. Osmotic minipumps may also be used to provide controlled delivery of high concentrations of endostatin oligomers through cannulae to the site of interest, such as directly into a metastatic growth or into the vascular supply to that tumor. The biodegradable polymers and their use are described, for example, in detail in Brem et al. (1991), which is hereby incorporated by reference in its entirety.

The dosage of an endostatin oligomer of the present invention will depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating humans or animals, between approximately 0.5 mg/kilogram to 500 mg/kilogram of the endostatin oligomer and peptide can be administered. A more preferable range is 1 mg/kilogram to 100 mg/kilogram with the most preferable range being from 2 mg/kilogram to 50 mg/kilogram. Depending upon the half-life of the endostatin oligomer and peptide in the particular animal or human, it can be administered between several times per day to once a week. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

The endostatin oligomer formulations include those suitable for oral, rectal, ophthalmic (including intravitreal or

intracameral), nasal, topical (including buccal and sublingual), intrauterine, vaginal or parenteral (including subcutaneous, intraperitoneal, intramuscular, intravenous, intradermal, intracranial, intratracheal, and epidural) administration. The endostatin oligomer formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose, as herein above recited, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients, particularly mentioned above, the formulations of the present invention may include other agents conventional in the art having regard to the type of formulation in question.

In addition to methods of treatment, the present invention also relates to methods of using endostatin oligomers, and active

fragments thereof, and antibodies that bind specifically to endostatin oligomers and their peptides, to diagnose endothelial cell-related diseases and disorders. Diagnosis is accomplished by detection of endostatin oligomers, or antibodies thereto, in body fluids or tissues. Detection may be accompanied by comparison of the detected levels of endostatin oligomers, or antibodies thereto, to normal levels of endostatin oligomers, or antibodies thereto.

Kits for measurement of endostatin oligomers are also contemplated as part of the present invention. Antisera that possess the highest titer and specificity and can detect endostatin oligomers in extracts of plasma, urine, tissues, and in cell culture media are further examined to establish easy to use kits for rapid, reliable, sensitive, and specific measurement and localization of endostatin oligomers. These assay kits include but are not limited to the following techniques; competitive and non-competitive assays, radioimmunoassay, bioluminescence and chemiluminescence assays, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including ELISA, microtiter plates, antibody coated strips or dipsticks for rapid monitoring of urine or blood, and immunocytochemistry. For each kit, the range, sensitivity, precision, reliability, specificity and reproducibility of the assay is established. Intra-assay and inter-assay variation is established at 20%, 50% and 80% points on the standard curves of displacement or activity.

The assay kit provides instructions, antiserum, endostatin oligomers and possibly radiolabeled endostatin oligomers or peptides and/or reagents for precipitation of bound antibody complexes. The kit is useful for the measurement of endostatin oligomers in biological fluids and tissue extracts of animals and humans with and without tumors or angiogenesis-dependent diseases.

Also included in the present invention are kits for the localization of endostatin oligomers or peptides in tissues and cells. This endostatin oligomer immunohistochemistry kit

provides instructions, endostatin oligomer antiserum, and possibly blocking serum and secondary antiserum linked to a fluorescent molecule such as fluorescein isothiocyanate, or to some other reagent used to visualize the primary antiserum. Immunohistochemistry techniques are well known to those skilled in the art. This endostatin oligomer and peptide immunohistochemistry kit permits localization of endostatin oligomers in tissue sections and cultured cells using both light and electron microscopy. It is used for both research and clinical purposes. For example, tumors are biopsied or collected and tissue sections cut with a microtome to examine sites of hyaluronic acid binding link module production. Such information is useful for diagnostic and possibly therapeutic purposes in the detection and treatment of cancer.

The invention further encompasses a method for identifying and quantitating endostatin oligomer-specific receptors. Labeling of the endostatin oligomers with short lived isotopes enables visualization of receptor binding sites *in vivo* using positron emission tomography or other modern radiographic techniques. These methods are important for the study of angiogenesis and metastasis in cancer and other angiogenesis-related diseases.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

Example 1

Production of Endostatin Monomers and Dimers and Oligomers

To produce endostatin monomers, the polymerase chain reaction (PCR) was used to adapt the endostatin cDNA for

expression in the pdCs-Fc(D4K) vector, which contains the enterokinase recognition site Asp4-Lys at the junction of the fusion protein. The forward primer was 5'-C AAG CTT CAC AGC CAC CGC GAC TTC C (SEQ ID NO:3), containing a Hind III site followed by a sequence encoding the N-terminus of endostatin (HSHRDFQPVLHL, SEQ ID NO:4). The reverse primer was 5'-C CTC GAG CTA CTT GGA GGC AGT CAT G, (SEQ ID NO:5) which was designed to put a translation STOP codon (anti-codon, CTA) immediately after the C-terminus of endostatin, and this was followed by a Xho 1 site (CTCGAG).

The PCR product was cloned and sequenced, and the Hind III-Xho 1 fragment encoding endostatin was ligated to the pdCsFc(D4K) vector. Stable clones expressing Fc(D4K)-endostatin were obtained by electroporation of NS/O cells followed by selection in growth medium containing 100 nM methotrexate. Protein expression levels were assayed by anti-human Fc ELISA as described in Lo, K. M. et al. (1998) *Protein Engineering* 11:495-500 and confirmed by SDS-PAGE. The best producing clone was sub-cloned by limiting dilutions.

The Fc-human endostatin fusion protein was purified on Protein A sepharose using 100mM citric acid, pH=3.0 as elution buffer. Enterokinase and trypsin digestions were carried out resulting in two forms of cleaved endostatin. For trypsin, the ratio of enzyme to protein (w/w) was 1:200, 18 hours room temperature resulting in an endostatin molecule with four amino acids, HSHR, cleaved from N-terminus. Enterokinase digestion led to a product with one additional amino acid, leucine, at the N-terminus of endostatin. Both cleaved products were purified further using heparin Sepharose and SP sepharose (Pharmacia, Bridgewater, New Jersey).

Disulfide-bonded human endostatin dimer was produced by replacing the restriction fragment encoding the N-terminal sequence of human endostatin in pdCs-Fc(D4K)-hu Endo with a double-stranded oligonucleotide duplex encoding the same sequence but with the Q7 codon changed to C to generate pd

Cs-Fc(D4K)-hu Endo (C7). This plasmid was transfected into myeloma cells and the Fc-endostatin dimer was purified from conditioned media utilizing protein A affinity chromatography as described in Lo, K.M. et al., (1998) *Protein Engineering* 11:495-500. After the Fc-endostatin dimer was cleaved with enterokinase the endostatin dimer resolved from the Fc portion by gel filtration over S-200.

The endostatin-NC1 trimer was produced as follows. The NC1 protein was produced using two primers, 5' GAT CGG CCC AGC CGG CCC ATC ATC ACC ATC ACC ATG ACG ATG ACG ATA AGT CAG GGC AGG TGA GGC TCG CTA CAC GCA GG 3' (SEQ ID NO:6) and 5'GAT CGG ATC CCT ACT TGG AGG CAG TCA TGA AGG 3' (SEQ ID NO:7). These primers were used to amplify from the SGQVRLWATRQ amino acid sequence (SEQ ID NO:8) through the C terminus of human collagen 18 using PCR. The PCR products were cloned in the Sfil-BamHl site in pSecTag2a (Invitrogen, San Diego, California). Stable 293T cell transfectants were selected in zeocin and conditioned media eluted from Ni-agarose (QIAGEN, Valencia, California) with 250 mM imidazole/PBS followed by dialysis into PBS.

Example 2

Zinc-Dependent Dimers Observed in Crystals of Human Endostatin

The X-ray structure was determined from crystals obtained at 4° C by hanging drop vapour diffusion, with equal volumes of 10 mg/ml protein in 20 mM Tris-HCl pH 8.5, 150 mM NaCl and 50 mM Tris-HCl pH 8.5, 6% PEG 8K, 150 mM NaCl and 2 mM MgC1 mixed at room temperature in the drops, equilibrated against 0.5 ml of the later buffer. Crystals (C2 a=92.76 Å, b=74.27 Å, c=137.80 Å, ß=102.56 have 4 monomers in the asymmetric unit. Crystals were transferred to 15% PEG 6K, 20% glycerol, 50 mM Tris-HC1 pH 8.5 and 150 mM NaCl for about 20 seconds before flash cooling in a stream of cold nitrogen. Data

were collected at the CHESS A1 station (λ=0.92A) with the 80 mm 2K CCD (bined mode) (0.5 degree oscillations). Data were integrated and scaled with DENZO and SCALEPACK (HKL Research, Charlottesville, Virginia). Most of the subsequent processing used the CCP4 programs (T. A. Jones (1992)*"Molecular Replacement"* CCP4, 92-105; G. J. Kleywegt and T. A. Jones (1994)*"From First Map to Final Model"* CCP4, 59-66).

Following the above methods it was determined that the zinc binding site of human endostatin is tetrahedral with three zinc ligands from the N-terminal loop, histidines 1, 3, and 11, and a fourth ligand, aspartic acid 76, from the loop between the E and F ß-strands (Fig. 1 and Fig. 2). The zinc binding site most closely resembles that from zinc proteases. Atomic absorption spectroscopy confirmed that zinc is a constituent of the Fc-endostatin chimeras described above of both human and murine endostatin in solution as shown below in Table I.

Table I

Protein	N-terminus sequence	Endostatin Concentration (mg/mL)	Zinc Concentration (micromolar)	Zinc per Endostatin Monomer
hE-Ek	LHSHRD	4	176	0.9
hE-Tryp	D	3.8	14	0.1
hFc-hE		0.4	18	0.9
mFc-mE		2	79	0.9

The first line of Table I also demonstrates that enterokinase treated Fc-human endostatin, which yields full length endostatin, also showed approximately one atom of zinc per endostatin monomer. Trypsin cleaved Fc-human endostatin, which results in loss of the first four residues of endostatin, contains no detectable zinc (second line of Table I). These results are consistent with the

human endostatin crystal structure that shows histidines at residues 1 and 3 to be two of the zinc ligands.

A number of two-fold symmetric dimers are evident in the packing of the four endostatin monomers in the asymmetric unit of the human endostatin crystals. One dimer interface is formed by the contact of two solvent exposed phenylalanines on the α 1 α helix from each monomer, a prominent non-polar patch suggested as a potential inter-domain interaction site in murine endostatin (residues F31 and F34 in Fig. 3). Another dimeric contact, that would be zinc dependent, is formed between the projecting N-terminal loops of two monomers; each loop ordered around a zinc ion (Center in Fig. 3a). This contact is formed primarily by three residues that project from the rim of the N-terminal loop: arginine 4, phenylalanine 6, and glutamine 7 (Fig. 3b). The glutamines at residue 7 of each monomer contact each other forming a hydrogen bond at the center of the interface. Each ring of the phenylalanines at residue 7 contacts a non-polar patch containing leucine 78 and valine 72 of the adjacent monomer. Arginine 4 from each monomer forms hydrogen bonds with two main chain carbonyl oxygens on the loop containing the aspartic acid zinc ligand at residue 76 (carbonyls at residue 75 and 76) (Fig. 3b). This dimeric interaction could only occur if the N-terminal loops were ordered as the result of binding zinc, and in turn, the zinc binding would probably be stabilized by the dimer interface.

Example 3

Zinc Binding of Endostatin is Essential for Anti-Angiogenic Activity

As described in Example 2, it was determined that histidines 1, 3, 11 and aspartic acid 76 coordinate the zinc ion. Based upon this discovery, point mutations were introduced into the endostatin protein to change the Zn^{2+} ligands to alanines. One double (H1/3A) and two single (H11 A and D76A) mutants were created using the QuikChange site-directed mutagenesis kit

(Stratagene, La Jolla, California) in an *E. coli* expression vector. The mutations were confirmed by sequence analysis.

Wild-type endostatin and the three mutants were purified in parallel and tested in the Lewis lung carcinoma model as previously described in O'Reilly, M. S. et al., Cell 88:277 (1997) and T. Boehm et al. Nature 390:404 (1997). The different endostatin preparations were compared on a sodium dodecyl sulfate (SDS) gel under reducing and non-reducing conditions and no significant difference in the purity and cross-linking pattern was observed. SDS-PAGE of endostatin purified from *E. coli* showed a protein ladder including monomers, dimers, trimers and higher molecular weight complexes caused by cross-linking of endostatin to other endostatin molecules via disulfide bond formation.

Unlike the wild-type endostatin, none of the three mutants of endostatin regressed Lewis lung carcinoma (data not shown). Although the H1/3A endostatin mutant was weakly active, the weak activity might be due to either a distorted conformation caused by using two histidines from the His.-tag to coordinate the Zn^{2+} ion, or by the loss of the ability to bind zinc.

Example 4

Scatter of Pre-formed Endothelial Structures by Endostatin Dimers

In order to determine the ability of endostatin monomers and dimers to influence morphogenesis, a matrigel tube-formation assay was used. The assay utilizes endothelial cells plated on extracellular matrix proteins that spontaneously aggregate and assemble into densely multi-cellular capillary-like tubular structures. The assay was performed as follows.

HUVEC (human umbilical vein endothelial cells), passage <10, isolated from single donor (Clonetics, San Diego, California), were cultured in EBM media (Clonetics, San Diego, California) supplemented with EGM-2-MV growth factors including 5% FCS, hVEGF, hFGF, (10 ng/ml), hydrocortisone (1

μg/ml), and GA-1000 (0.1%) at 37° C, 5% $C0_2$, Cells were plated in 1 ml at 40,000 cells/well into 24 well plates containing complete media as above. These wells had been pre-coated at 4° C with matrigel, a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma (Becton Dickinson/Collaborative, Franklin Lakes, New Jersey) at 300 μl/well. Human endostatin derivatives in PBS (10-2000 nM) were added either at the time of addition of the cells to the matrigel-coated plates, or after 16 hours of tube formation. Recombinant hHGF (R&D Systems, Minneapolis, Minnesota) was added at 50 ng/ml.

For inhibition of endostatin oligomers by endostatin monomers, pre-formed tubes were pre-incubated with endostatin monomer (2000 nM) for 30 minutes prior to oligomer treatment. Wells were subsequently incubated at 37° C, 5% CO_2, and then photographed under phase contrast microscopy at 40-200x. Identical results and procedures have been obtained with human microvascular, pulmonary and umbilical artery primary endothelial cultures.

An antibody Fc-endostatin fusion protein containing endostatin at the C-terminus potently inhibited tube formation when included at the time of plating with cells actually becoming more dispersed over time rather than coalescing into tubular structures (data not shown). Identical results were observed with primary cultures of human microvascular, umbilical vein, umbilical artery and pulmonary artery endothelial cells (data not shown). The rapidity of this effect argued against a primary effect on cell growth, and cell counts, BrdU staining and ^3H incorporation did not reveal significant stimulation of proliferation versus untreated cells during this time interval (data not shown).

Intriguingly, after enterokinase cleavage of the Fc-endostatin fusion into Fc and endostatin, neither Fc nor endostatin singly, nor the combination of Fc and endostatin demonstrated inhibitory activity (Figure 4). Recombinant endostatin from

baculovirus and yeast un-fused to Fc were also ineffective (data not shown). However, an artificially dimerized endostatin created by utilizing a close contact between adjacent glutamine-7 residues predicted by the dimeric crystal structure of endostatin, was found to exhibit scatter activity. Mutation of endostatin glutamine-7 to cysteine and fusion to antibody Fc allowed formation of a novel intermolecular cysteine bond at cysteine-7, with enterokinase cleavage liberating the free endostatin dimer migrating at 40 kDa and 20 kDa under non-reducing and reducing conditions respectively (data not shown). Both mouse and human endostatin dimers potently reproduced the inhibitory activity of the Fc-endostatin fusion protein on tube formation with IC_{50} of approximately 10 nM (Figure 4) and were inactive against bovine capillary endothelial cell or HUVEC proliferation (data not shown).

During endostatin dimer-induced scattering, rapidly demarginating cells appeared as early as 1-2 hours with prominent lamellipod and pseudopod formation (data not shown). Confocal microscopy with phalloidin-FITC revealed a dramatic reorganization of the actin cytoskeleton within two hours with prominent stress fiber formation (data not shown), consistent with a signaling pathway linked to rapid actin polymerization. As visualized by electron microscopy, endostatin oligomer-induced scattering was accompanied by destruction of tubular lumens and by cellular elongation (data not shown).

The scatter factor activity of the endostatin oligomers described herein is specific. Although collagen XV (c15) and collagen XVIII, from which endostatin monomers are derived, exhibit 60% amino acid identity in the endostatin domain, and share the NC1 trimerization domain, collagen XV oligomers did not act as scatter factors (Figure 4). Fc fusion protein containing the c15 endostatin-like domain was unable to scatter tubules, in contrast to Fc endostatin dimers, and also could not antagonize the effects of Fc endostatin dimers (data not shown). Fc-angiostatin or monomeric angiostatins also did not inhibit tube formation,

consistent with the complete lack of structural homology between endostatin and angiostatin (data not shown). Overall, only oligomeric endostatin compositions displayed scatter activity.

Additionally, pretreatment of established tubules with excess of endostatin monomer (1000 nM) strongly blocked the ability of endostatin dimers (20-50 nM) to scatter cells (data not shown). This is potentially consistent with endostatin monomers acting in a dominant negative fashion to block endostatin oligomers, for example, by non-productively occupying a receptor that requires oligomer induced cross-linking for activity.

Example 5

Scatter of Pre-formed Endothelial Structures by Endostatin-NC1 Trimers

Human and murine NC1 proteins were isolated from transfected 293T cell supernatants as described above in Example 1. Trimerization was confirmed by cross-linking the trimerized complexes and resolving the products by non-reduced denaturing polyacrylamide gel electrophoresis (PAGE). Cross-linking was carried out in the presence of 5mM Ethylene Glycol-bis(Succinimidyisuccinate) (EGS), at room temperature for 20 minutes. The EGS was dissolved in DMSO and was diluted ten fold in PBS. The reaction was stopped by adding Tris at a final concentration of 100mM. The resulting gel produced an approximately 120 kDa species upon EGS cross-linking (data not shown).

The murine and human NC1-endostatin trimers identically inhibited endothelial tube formation with an IC_{50} of approximately 10 nM (Figure 4), again consistent with dependence of activity on endostatin domain oligomerization. The NCI-endostatin trimer was slightly less effective than the endostatin dimers at higher concentrations, possibly reflecting decreased potency from non-covalent versus covalent oligomerization.

Scattering results obtained with the endostatin/NC1 trimer were similar to the endostatin dimer. The endostatin/NC1 trimer induced scattering, rapidly demarginating cells appeared as early as 1-2 hours with prominent lamellipod and pseudopod formation (data not shown). Confocal microscopy with phalloidin-FITC revealed a dramatic reorganization of the actin cytoskeleton within two hours with prominent stress fiber formation, consistent with a signaling pathway linked to rapid actin polymerization. As visualized by electron microscopy, endostatin oligomer-induced scattering was accompanied by destruction of tubular lumens and by cellular elongation (data not shown).

Pretreatment of established tubules with excess of endostatin monomer (1000 nM) strongly blocked the ability of the NCI-endostatin trimer (20-50 nM) to scatter cells (data not shown). As stated above, this is potentially consistent with endostatin monomers acting in a dominant negative fashion to block endostatin oligomers, for example, by non-productively occupying a receptor that requires oligomer induced cross-linking for activity.

Example 6

Endostatin Oligomers Comprise a New Class of Scatter Factors

The oligomeric endostatin-induced cell scattering and cytoskeletal reorganization were reminiscent of morphogenic changes evoked by Hepatocyte Growth Factor/Scatter Factor (HGF/SF), which with the related Macrophage Stimulatory Protein (MSP) comprises a scatter factor family regulating cell motility, dispersion, proliferation and apoptosis. Although Human Umbilical Vein Endothelial Cells (HUVECs) abundantly express the HGF receptor c-met 16, in contrast to endostatin oligomers, HGF was completely unable to scatter HUVEC tubules (data not shown). Conversely, while HGF potently dis-aggregated and scattered MDCK and HepG2 cell aggregates cultured on plastic, oligomeric endostatin derivatives were inactive (data not shown). MSP was also unable to elicit HUVEC scatter (data not shown).

Notably, it is well established that HGF actually promotes tube formation in collagen matrices as opposed to the anti-tubulogenic effects of endostatin described here. Endostatin oligomers therefore define a novel scatter factor class, both structurally and functionally distinct from the previously unique HGF/MSP family.

Modifications and variations of the present method will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

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Claims

We Claim:

1. An isolated protein oligomer, comprising more than one endostatin monomer, wherein the oligomer has scatter factor activity.

2. The isolated protein oligomer of Claim 1, wherein the endostatin monomer is a carboxy-terminal region fragment of collagen XVIII having a molecular weight of approximately 20 kDa as determined by reducing gel electrophoresis and 18 kDa as determined by non-reducing gel electrophoresis.

3. The isolated protein oligomer of Claim 1, wherein the oligomer is a dimer of endostatin monomers.

4. The isolated protein oligomer of Claim 3, further comprising a metal component.

5. The isolated protein oligomer of Claim 4, wherein the metal component is zinc.

6. The isolated protein oligomer of Claim 5, wherein the oligomer has anti-tumorigenic activity.

7. The protein oligomer of Claim 1, wherein the protein oligomer comprises more than one NC1 region fragment of collagen XVIII having a molecular weight of approximately 38 kDa under reducing gel electrophoresis such that each NC1 fragment contains an endostatin monomer.

8. The isolated protein oligomer of Claim 7, wherein the oligomer is a trimer.

9. The isolated protein oligomer of Claim 8, further comprising a metal component.

10. The isolated protein oligomer of Claim 9, wherein the metal component is zinc.

11. The isolated protein oligomer of Claim 10, wherein the oligomer has anti-tumor activity.

12. The protein oligomer of Claim 1, wherein the endostatin monomers are fusion proteins.

13. The protein oligomer of Claim 12, wherein the endostatin monomers contain endostatin and the Fc portion of an antibody.

14. A method of inhibiting tubulogenesis comprising, administering to an endothelial cell a tubulogenesis inhibiting amount of a protein oligomer comprising more than one endostatin monomer, wherein the oligomer has scatter factor activity.

19. The method of Claim 18, wherein the protein oligomer is a dimer of endostatin monomers.

20. The method of Claim 19, wherein the protein oligomer further comprises a metal component.

21. The method of Claim 20, wherein the metal component is zinc.

22. The method of Claim 18, wherein the protein oligomer comprises more than one NC1 region fragment of collagen XVIII having a molecular weight of approximately 38 kDa under reducing gel electrophoresis such that each NC1 fragment contains an endostatin monomer.

23. The method of Claim 22, wherein the oligomer is a trimer.

24. The method of Claim 23, wherein the protein oligomer further comprises a metal dimerizing component.

25. The method of Claim 24, wherein the metal dimerizing component is zinc.

26. A method of inhibiting tumorigenesis comprising, administering to an endothelial cell a tumorigenesis inhibiting amount of a protein oligomer comprising more than one endostatin monomer, wherein the oligomer has scatter factor activity.

27. The method of Claim 26, wherein the protein oligomer is a dimer of endostatin monomers.

28. The method of Claim 27, wherein the protein oligomer further comprises a metal component.

29. The method of Claim 28, wherein the metal component is zinc.

30. The method of Claim 26, wherein the protein oligomer comprises more than one NC1 region fragment of collagen XVIII having a molecular weight of approximately 38 kDa under reducing gel electrophoresis such that each NC1 fragment contains an endostatin monomer.

31. The method of Claim 30, wherein the oligomer is a trimer.

32. The method of Claim 31, wherein the protein oligomer further comprises a metal dimerizing component.

33. The method of Claim 32, wherein the metal dimerizing component is zinc.

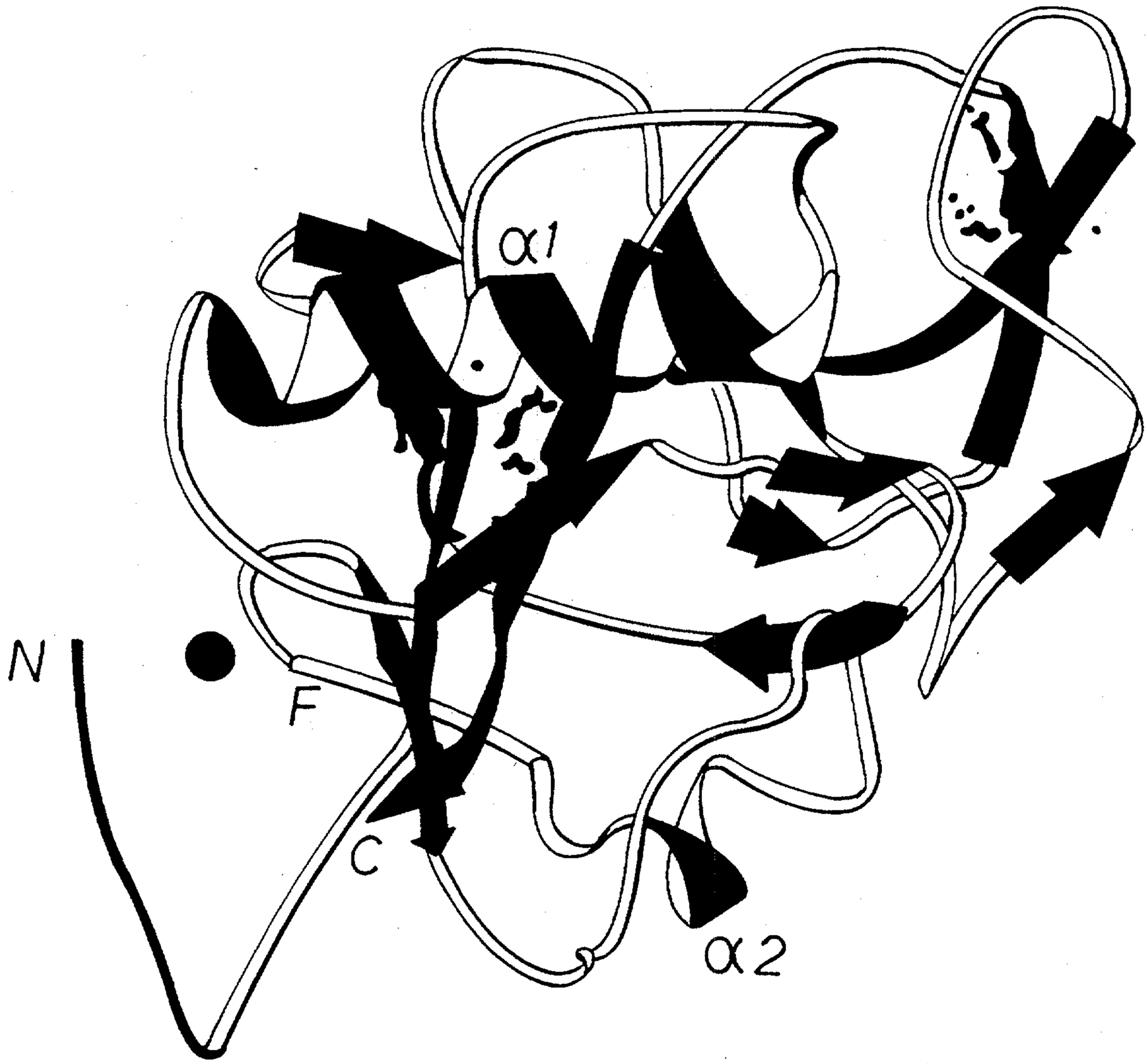


Fig_1

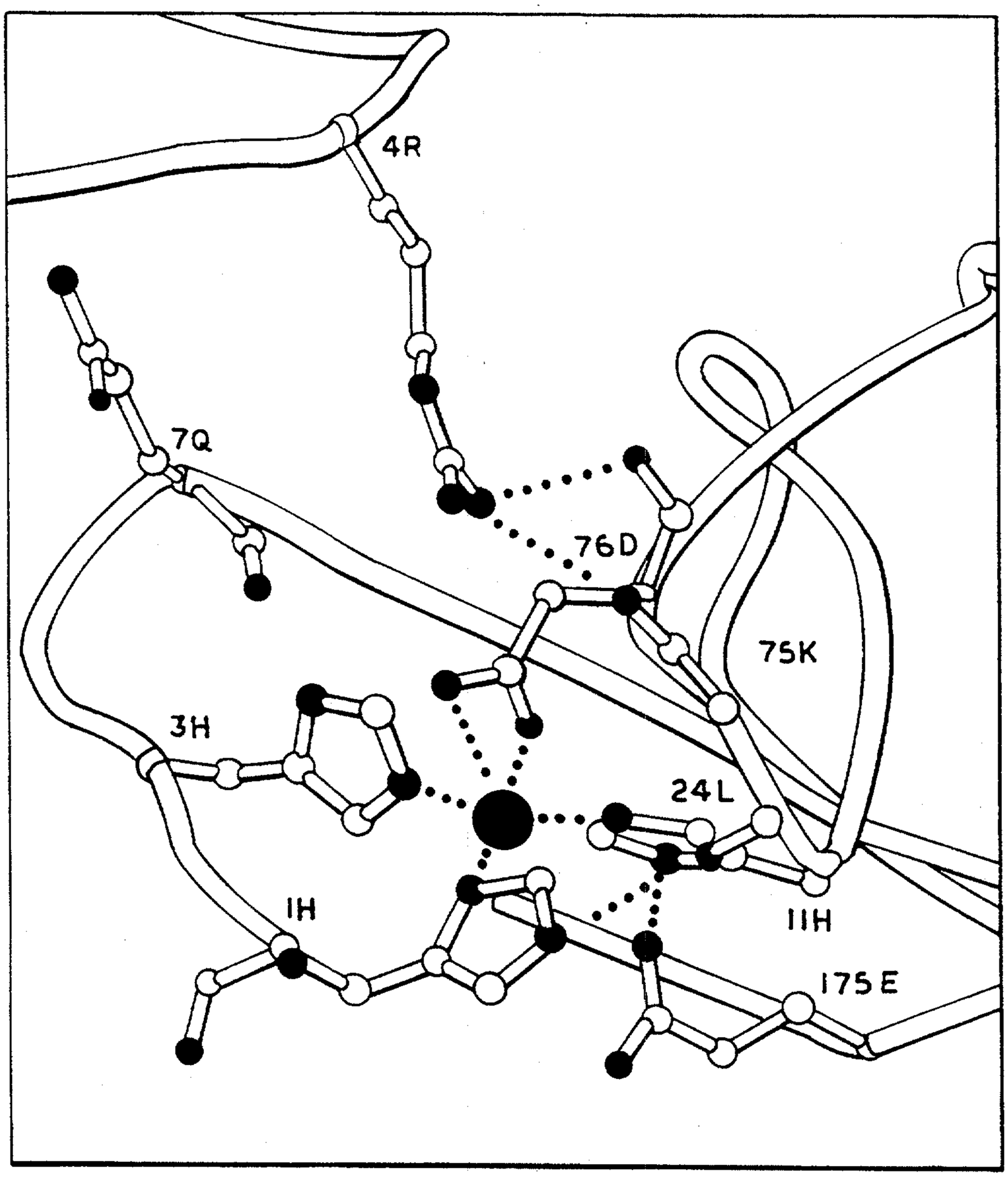


Fig_2

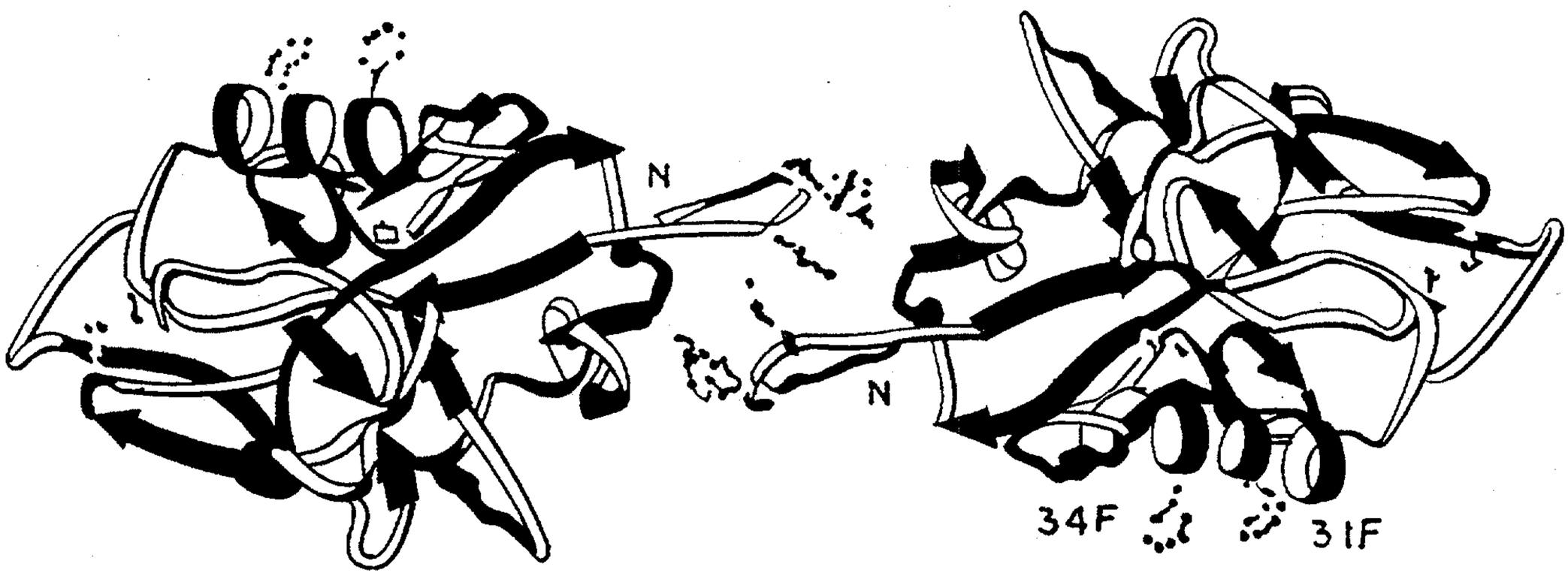


Fig_3A

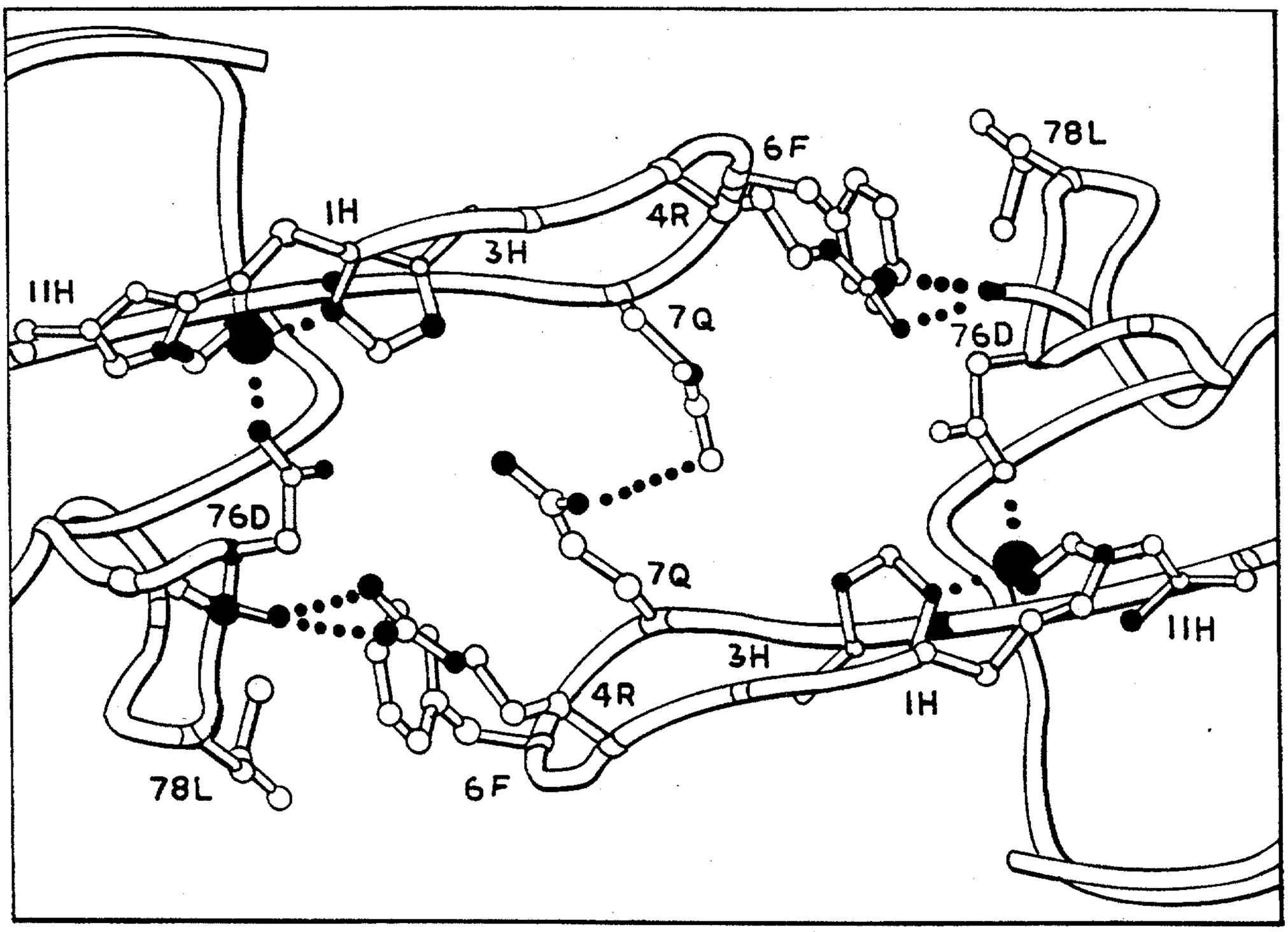


Fig_3B

SUMMARY OF DIFFERENT ENDOSTATIN DERIVATIVES IN MATRIGEL TUBE FORMATION ASSAY

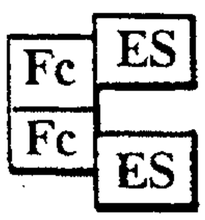
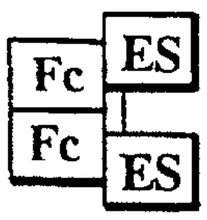
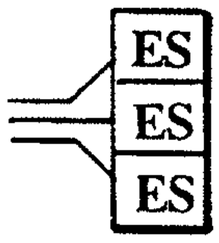
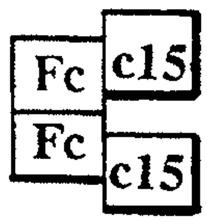
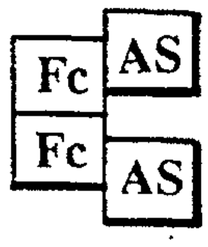
	OLIGOMERIZATION STATE OF ES	INHIBITION OF TUBE FORMATION	SCATTER ACTIVITY
	MOMO	—	—
	DIMER	+	+
	—	—	—
	MONO	—	—
	DIMER	+	+
	DIMER	+	+
	TRIMER	+	+
	—	—	—
	—	—	—

Fig. 4