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(57) Abstract

This invention relates to novel, biologically active molecules which bind to and inhibit the thrombin receptor. The molecules of this invention are characterized by a portion which binds to the "hirudin-like" domain of the thrombin receptor (amino acids 52-69). These thrombin receptor antagonists are further characterized by their ability to inhibit thrombin-induced platelet aggregation without affecting the catalytic activity of thrombin. More specifically, the thrombin receptor antagonist of this invention comprises the formula: $Y\text{-}A_1\text{-}W\text{-}X_1\text{-}X_2\text{-}X_3\text{-}X_4\text{-}A_2\text{-}Z$; wherein Y is hydrogen, a peptide of from 1 to 10 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 30 backbone atoms; each of A_1 and A_2, either the same or different, are selected from the group consisting of a positively charged amino acid and an acyl or alkyl chain comprising from 1 to 10 backbone atoms and a positively charged side group; W and each X is any amino acid, either the same or different; and Z is OH, a peptide of from 1 to 11 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 33 backbone atoms, with the proviso that at least one of W, X_1 and X_2 is not a basic amino acid; and wherein said antagonist is optionally cyclized by a covalent bond between Y and Z. This invention also relates to compositions and methods which employ these molecules for therapeutic and prophylactic purposes.

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THROMBIN RECEPTOR ANTAGONISTS

TECHNICAL FIELD OF THE INVENTION

This invention relates to novel, biologically active molecules which bind to and inhibit the thrombin receptor. The molecules of this invention are characterized by a portion which binds to the "hirudin-like" domain of the thrombin receptor (amino acids 52-69). These thrombin receptor antagonists are further characterized by their ability to inhibit thrombin-induced platelet aggregation without affecting the catalytic activity of thrombin. More specifically, the thrombin receptor antagonist of this invention comprises the formula: $Y-A_1-W-X_1-X_2-X_3-X_4-A_2-Z$; wherein Y is hydrogen, a peptide of from 1 to 10 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 30 backbone atoms; each of A_1 and A_2, either the same or different, are selected from the group consisting of a positively charged amino acid and an acyl or alkyl chain comprising from 1 to 10 backbone atoms and a positively charged side group; W and each X is any amino acid, either the same or different; and Z is OH, a peptide of from 1 to 11 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 33 backbone atoms, and wherein said antagonist is optionally cyclized by a covalent bond between Y and Z; with the proviso that at least one of W, X_1 and X_2 is not a basic amino acid. This invention also relates to compositions and methods which employ these molecules for therapeutic and prophylactic purposes.

BACKGROUND OF THE INVENTION

Thrombin is a naturally occurring protein which has several bioregulatory roles [J. W. Fenton, II, "Thrombin Bioregulatory Functions", Adv. Clin. Enzymol., 6, pp. 186-93 (1988)]. In addition to being intimately involved in blood coagulation, thrombin is also known to play a role in platelet and endothelial cell activation, smooth muscle cell and neuroblast proliferation and bone resorption. Thrombin exerts its various biological effects through one of two mechanisms. The first is purely enzymatic, involving only the catalytic activity of thrombin. This is the mechanism by which thrombin converts fibrinogen to fibrin, the final step in the coagulation cascade.

The second mechanism of thrombin action is mediated through a cell surface receptor. This receptor has recently been cloned [T.-K. H. Vu et al., "Molecular Cloning of a Functional Thrombin Receptor Reveals a Novel Proteolytic Mechanism of Receptor Activation", Cell, 64, pp. 1057-68 (1991)]. It is believed that thrombin activates cells via its receptor by first associating with the hirudin-like extracellular domain of the receptor (receptor amino acids 52-69) [L.-W. Liu et al., "The Region of the Thrombin Receptor Resembling Hirudin Binds to Thrombin and Alters Enzyme Specificity", J. Biol. Chem., 266, pp. 16977-80 (1991)]. Thrombin then cleaves off a 41 amino acid peptide from its receptor's extracellularly located N-terminus, exposing a new N-terminus on the receptor. The newly exposed N-terminus then interacts with another, distant part of the receptor, resulting in activation. It is believed that activation may ultimately involve a secondary messenger, such as a kinase or cAMP, which is triggered by the cleaved receptor.

It has previously been shown that a peptide corresponding to the first 14 amino acids of the newly exposed N-terminus of the thrombin receptor (referred to as "the tethered ligand peptide") can activate the thrombin receptor in the absence of thrombin. This activation does not require the presence of an extracellular anionic domain in the receptor [T.-K. H. Vu et al., supra].

The receptor-driven mechanism is responsible for thrombin-induced platelet aggregation and release reactions. Such reactions initiate arterial clot formation. In addition, the receptor mechanism is thought to be responsible for thrombin-induced endothelial cell activation. This activation stimulates the synthesis of platelet activating factor (PAF) in these cells [S. Prescott et al., "Human Endothelial Cells in Culture Produce Platelet-Activating Factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) When Stimulated With Thrombin", Proc. Natl. Acad. Sci. USA, 81, pp. 3534-38 (1984)]. PAF is exposed on the surface of endothelial cells and serves as a ligand for neutrophil adhesion and subsequent degranulation, which results in inflammation [G. M. Vercolletti et al., "Platelet-Activating Factor Primes Neutrophil Responses to Agonists: Role in Promoting Neutrophil-Mediated Endothelial Damage", Blood, 71, pp. 1100-07 (1988)]. Alternatively, thrombin may promote inflammation by altering endothelial cells to produce increased vascular permeability which can lead to edema [P. J. Del Vecchio et al., "Endothelial Monolayer Permeability To Macromolecules", Fed. Proc., 46, pp. 2511-15 (1987)], a process that also may involve the thrombin receptor.

Smooth muscle cells are also known to express thrombin receptors on their surface [D. T. Hung et al., "Thrombin-induced Events in Non-Platelet Cells Are

Mediated by the Unique Proteolytic Mechanism Established for the Cloned Platelet Thrombin Receptor, J. Cell Biol., 116, pp. 827-32 (1992)]. When thrombin interacts with these cells, a proliferative response is generated. This proliferation of smooth muscle cells may contribute to restenosis following balloon angioplasty and subsequent coronary failure [A. J. B. Brady et al., "Angioplasty and Restenosis", Brit. Med. J., 303, pp. 729-30 (1991)]. Other cells which have thrombin receptors are fibroblasts, neuroblasts and osteoclasts. For each of these cells, thrombin has been shown to be responsible for some bioregulatory function.

For example, thrombin has been implicated in neurodegenerative diseases because of its ability to cause neurite retraction [D. Gurwitz et al., "Thrombin Modulates and Reverses Neuroblastoma Neurite Outgrowth", Proc. Natl. Acad. Sci. USA, 85, pp. 3440-44 (1988)]. And thrombin has been hypothesized to play a role in osteoporosis due to its ability to stimulate bone resorption by osteoclasts [D. N. Tatakis et al., "Thrombin Effects On Osteoblastic Cells -II. Structure-Function Relationships", Biochem. Biophys. Res. Comm., 174, pp. 181-88 (1991)].

Therefore, the ability to regulate the in vivo activity of thrombin has many significant clinical implications. More importantly, the ability to selectively inhibit thrombin's receptor-driven functions without affecting fibrin formation is highly desirable so that bleeding will be reduced or eliminated as a potential side effect of treatment.

Compounds which inhibit thrombin directly may inhibit some or all of thrombin's functions to one degree or another. All active site inhibitors of thrombin inhibit both enzymatic and receptor-mediated functions of the molecule. Surprisingly, other

thrombin inhibitors which do not bind to the active site can also inhibit both mechanisms. For example, peptides modelled on the C-terminal amino acid sequence of hirudin, a naturally occurring anticoagulant isolated from leeches, inhibit both fibrinogen cleavage, as well as thrombin-induced platelet and endothelial cell activation [copending United States patent application Serial No. 677,609].

Other compounds are capable of selectively inhibiting thrombin's activities, but these compounds do not inhibit thrombin or its receptor directly and therefore are less specific. For example, heparin, which complexes with and activates antithrombin III, affects fibrin clot formation without affecting platelet-dependent thrombosis. But certain patients cannot be treated with heparin because of circulating antibodies against this compound. Also, heparin is known to cause bleeding complications. Arg-Gly-Asp-containing peptides inhibit platelet activation, without inhibiting fibrin formation. However, these peptides are general antiplatelet agents and are not specific for thrombin-induced platelet activation. These peptides work by competitively binding to the platelet surface protein, glycoprotein IIb/IIIa.

Thus, there is a great need for a direct and selective antagonist of thrombin's functions. Specifically, a compound which can inhibit thrombin's receptor-mediated functions without affecting fibrin-mediated clotting would have great utility. Such a compound would be particularly useful in preventing restenosis following angioplasty -- a phenomenon linked to thrombin-induced smooth muscle cell proliferation [J. N. Wilcox, "Thrombin and Other Potential Mechanisms Underlying Restenosis", Circulation, 84, pp. 432-35 (1991)]. Thrombin receptor antagonists would also be useful in treating or preventing arterial thrombosis

without blocking fibrin formation.

SUMMARY OF THE INVENTION

The present invention solves the problems set forth above by providing novel molecules which competitively bind to and inhibit the thrombin receptor without affecting thrombin's activities toward fibrinogen and other proteins involved in coagulation. These novel antagonists are characterized by a portion which binds to the "hirudin-like" anionic domain of the thrombin receptor (amino acids 52-69). The antagonists of this invention are preferably modelled on the anion binding exosite portion of thrombin (amino acids 65-83), or a portion thereof, which is known to associate with the hirudin-like domain of its receptor.

The thrombin receptor antagonists of this invention may be utilized in compositions and methods for controlling certain thrombin-mediated functions without the risk of bleeding. This is because the molecules of this invention are specific for inhibiting thrombin receptor-mediated functions, while exerting no effect on the ability of thrombin to form fibrin clots. Pharmaceutical compositions comprising the antagonists of this invention are useful in inhibiting platelets; treating or preventing inflammation; treating or preventing bone resorption (a process prevalent in osteoporosis); treating or preventing neurodegenerative disease; and inhibiting smooth muscle cell proliferation. This last utility is particularly important following coronary angioplasty, where restenosis, which is believed to be caused by smooth muscle cell growth at the initial vessel blockage site, occurs with relatively high frequency.

Due to the fact that the antagonist molecules of the present invention may be prepared by chemical synthesis techniques, commercially feasible amounts may

be produced inexpensively. Moreover, because the antagonist molecules of the present invention are significantly smaller than the thrombin inhibitors presently employed in medical treatment, they are less likely to stimulate an undesirable immune response in patients treated with them.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the inhibition of platelet aggregation by varying concentrations of TRIP-1 in the presence of varying concentrations of thrombin.

Figure 2 depicts the lack of antiplatelet effect of TRIP-1 on platelets treated with the tethered ligand peptide of the thrombin receptor.

Figure 3 depicts the efficacy of TRIP-1 in the inhibition of platelet aggregation induced by various agonists.

Figure 4 depicts the comparative efficacies of TRIP-1, TRIP-4 (both linear and cyclized) and TRIP-5 (both linear and cyclized) in inhibiting thrombin-induced platelet aggregation.

Figure 5 depicts the relative thrombin receptor inhibitory activity of single position alanine and glycine substitutions in TRIP-6 compared to the unsubstituted antagonist.

Figure 6 depicts the inhibition of thrombin-induced smooth muscle cell proliferation by TRIP-6 and TRIP-20, as measured by ^{3}H-thymidine uptake.

Figure 7 depicts the nucleotide sequence of a plasmid which encodes an erabutoxinA-TRIP fusion protein.

Figure 8 depicts the ability of various erabutoxinA-TRIP fusion proteins expressed in phage to bind to an immobilized thrombin receptor peptide.

DETAILED DESCRIPTION OF THE INVENTION

The following standard abbreviations are used throughout the specification and in the claims:

Orn - ornithine	Gly - glycine
Ala - alanine	Val - valine
Leu - leucine	Ile - isoleucine
Pro - proline	Phe - phenylalanine
Trp - tryptophan	Met - methionine
Ser - serine	Thr - threonine
Cys - cysteine	Tyr - tyrosine
Asn - asparagine	Gln - glutamine
Asp - aspartic acid	Glu - glutamic acid
Lys - lysine	Arg - arginine
His - histidine	Nle - norleucine
Ac - acetyl	Suc - succinyl
BOC - tertButoxycarbonyl	Tos - paraToluenesulfonyl
Sar - sarcosine	CBZ - carbobenzyloxy
HArg - homoarginine	MPR - mercaptopropionic acid
GBA - guanidinobenzoic acid	PAM - phenylacetamido-methyl
Mts - mesitylenesulfonyl	Bzl - benzyl
OBzl - O-benzyl	MeBzl - methylbenzyl
Tyr(I_2) - diiodotyrosine	Alk-Cys - alkylated cysteine

The term "any amino acid" as used herein includes the L-isomers of the naturally occurring amino acids, as well as other "non-protein" α-amino acids commonly utilized by those in the peptide chemistry arts when preparing synthetic analogues of naturally occurring peptides. The naturally occurring amino acids are glycine, alanine, valine, leucine, isoleucine, serine, methionine, threonine, phenylalanine, tyrosine, tryptophan, cysteine, proline, histidine, aspartic acid, asparagine, glutamic acid, glutamine, γ-carboxyglutamic acid, arginine, ornithine

and lysine. Examples of "non-protein" α-amino acids include norleucine, norvaline, alloisoleucine, homoarginine, thioproline, dehydroproline, hydroxyproline (Hyp), isonipecotic acid (Inp), homoserine, cyclohexylglycine (Chg), α-amino-n-butyric acid (Aba), cyclohexylalanine (Cha), aminophenylbutyric acid (Pba), phenylalanines substituted at the ortho, meta, or para position of the phenyl moiety with one or two of the following: a (C_1-C_4) alkyl, a (C_1-C_4) alkoxy, halogen or nitro groups or substituted with a methylenedioxy group; ß-2- and 3-thienylal-alanine, ß-2- and 3-furanylalanine, ß-2-, 3- and 4-pyridylalanine, ß-(benzothienyl-2- and 3-yl)alanine, ß-(1- and 2-naphthyl)alanine, O-alkylated derivatives of serine, threonine or tyrosine, S-alkylated cysteine, S-alkylated homocysteine, O-sulfate, O-phosphate and O-carboxylate esters of tyrosine, 3-sulfo-tyrosine, 3-carboxy-tyrosine, 3-phospho-tyrosine, 4-methane sulfonic acid ester of tyrosine, 4-methane phosphonic acid ester of tyrosine, 3,5-diiodotyrosine, 3-nitrotyrosine, ε-alkyl lysine, delta-alkyl ornithine, and the D-isomers of any of the above amino acids. Unless specifically indicated, all amino acids referred to in this application are in the L-form.

The term "positively charged amino acid" as used in this application includes any naturally occurring or non-naturally occurring amino acid having a positively charged side chain. Examples of positively charged amino acids are arginine, lysine, histidine, homoarginine, ornithine and delta-alkyl ornithine.

The term "amino acid containing an aryl side chain" as used herein means any amino acid having an aromatic group. Tyrosine, phenylalanine, tryptophan, O-sulfate esters of tyrosine and 5-nitrotyrosine exemplify such amino acids.

The term "polar amino acid" means any amino acid having an uncharged side chain which is relatively soluble in water. Examples include glutamine, asparagine, glycine, serine, hydroxyproline and homoserine.

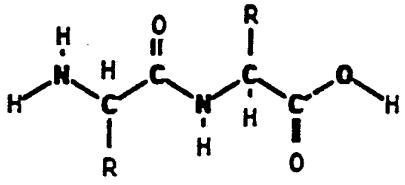
The term "hydrophobic amino acid" means any amino acid having an uncharged side chain which is relatively insoluble in water. This group includes leucine, valine, tryptophan, norleucine, norvaline, alloisoleucine, thioproline, dehydroproline, cyclohexylalanine and cyclohexylglycine.

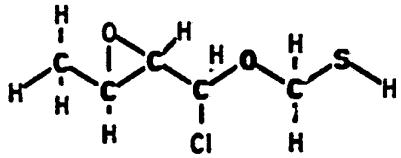
The term "acidic amino acid" refers to any amino acid having a negatively charged side chain. Examples include aspartic acid and glutamic acid.

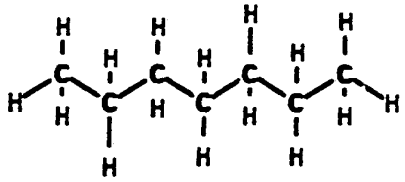
The term "patient" as used in this application refers to any mammal, especially humans.

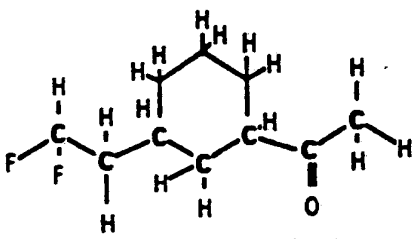
The term "backbone chain", as used herein, refers to the portion of a chemical structure that defines the smallest number of consecutive bonds that can be traced from one end of that chemical structure to the other. The atomic components that make up a backbone chain may comprise any atoms that are capable of forming bonds with at least two other atoms.

For example, each of the following chemical structures is characterized by a backbone chain of 7 atoms (the atoms which comprise the backbone chain are indicated in boldface):









The present invention relates to molecules that compete with thrombin for binding to its cell surface receptor. This property allows the molecules of this invention to inhibit those functions of thrombin that are mediated through its receptor.

Applicant has previously shown that peptides corresponding to the C-terminal amino acid sequence of hirudin inhibit thrombin-induced platelet activation (PCT publication No. WO 90/03391). These C-terminal hirudin peptides were known to associate with the anion binding exosite of thrombin. This work indicated that the anion binding exosite domain of thrombin was involved in platelet activation.

The recent cloning and sequencing of the platelet thrombin receptor confirmed this indication [T.-K. H. Vu et al., "Domains Specifying Thrombin-Receptor Interaction", Nature, 353, pp. 674-77 (1991)]. The deduced amino acid sequence of the thrombin receptor revealed the presence of a hirudin-like anionic region in an extracellular domain at amino acids 52-69. The importance of this region for thrombin's association with its receptor was demonstrated by comparing a wild-type receptor with a deletion mutant lacking that anionic region. Thrombin was 100-fold less potent in activating the mutant receptor as compared to the wild type [T.-K. H. Vu et al., supra].

By utilizing the three-dimensional X-ray crystallographic structure of a thrombin-C-terminal hirudin peptide complex [E. Skrzypczak-Jankun et al., "Structure of the Hirugen and Hirulog 1 Complexes of α-Thrombin", J. Mol. Biol., 221, pp. 1379-93 (1991)], applicant has identified some of the structural features necessary for thrombin to bind to an anionic domain. In particular, amino acids 65-83 of the thrombin ß-chain form a loop at the end of two

antiparallel ß-strands. Many of the interactions between thrombin and the C-terminus of hirudin are mediated by side chain constituents of the loop.

By analogy, applicant believes that these side chains, which include numerous positively charged amino acids, form electrostatic complimentarity with the hirudin-like, anionic domain of the thrombin receptor. Peptides and other molecules which comprise such features will bind to the thrombin receptor and inhibit its activation by thrombin in vivo and in vitro. These peptides and other molecules, as well as compositions and methods which employ them, make up the present invention.

According to one embodiment, the thrombin receptor antagonists of the present invention are characterized by a region that binds to the "hirudin-like" domain of the thrombin receptor. Preferably, that region is modelled on the thrombin anion binding exosite (thrombin amino acids 65-83; SEQ ID NO:1: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile-Ser), or a portion thereof. The antagonists of this invention are further characterized by: (1) the ability to inhibit thrombin-induced platelet aggregation in a simple in vitro assay, and (2) the inability to inhibit thrombin's catalytic activity.

In designing an antagonist molecule according to this invention, two important considerations must be taken into account. First, the molecule must be able to physically associate with the thrombin receptor. The present theory of thrombin receptor activation suggests that the initial step in activation requires, at a minimum, an ionic interaction between the receptor and thrombin. Specifically, thrombin utilizes its anion binding exosite region, i.e., a cluster of positive charges, to bind to the anionic domain of the

receptor. It is also probable that other molecular interactions, such as hydrogen bonding and hydrophobic interactions, are important for this association. Therefore, the identification and maintenance of these interactions are critical in designing a potent thrombin receptor antagonist.

The second consideration in designing the antagonists of this invention is secondary and tertiary structure. While certain portions of the antagonist do not directly participate in molecular interactions with the receptor, they may play a role in the overall conformation of the antagonist. This, in turn, can have a dramatic effect on potency. If the antagonist cannot assume the proper conformation, the molecular interactions required for association with the receptor cannot be achieved, even if the components capable of forming such interactions are present in the molecule. Accordingly, an antagonist of this invention must be designed so that it assumes a conformation which allows it to associate with the receptor. Conformational requirements may be in the nature of overall three-dimensional structure and orientation of the antagonist, or merely the spacing between two sites on the antagonist which directly interact with the receptor.

In order to identify and define the important structural and conformational attributes necessary to the thrombin receptor antagonists of this invention, one of ordinary skill in the art begins with a peptide consisting of amino acids 65-83 of thrombin. This peptide itself is a thrombin receptor antagonist of the present invention.

To test which of the 19 amino acids in this peptide are responsible for crucial molecular interactions with the thrombin receptor, both an alanine- and a glycine-scanning procedure are carried

out. In these procedures, a series of peptides, each having a single alanine or glycine substitution at a different residue, is synthesized. The peptides are then assayed to determine if they inhibit thrombin-induced platelet aggregation, without affecting thrombin catalyzed fibrin formation.

Those alanine- or glycine-substituted peptides which retain a platelet inhibitory activity similar to that of thrombin amino acids 65-83, indicate portions of the antagonist that do not directly interact with the receptor and which do not have side chains which play a critical role in the folding of the molecule. Such peptides are preferred antagonists of the present invention. Conversely, those peptides which lack or have greatly reduced antiplatelet activity point up areas of the antagonist that are important for activity. These latter peptides suggest the nature of an important inter-or intramolecular interaction based upon the amino acid substituted for. For example, an arginine-to-alanine or arginine-to-glycine substitution which resulted in reduced activity suggests the location of an important positive charge -- either an ionic interaction with the receptor or an intramolecular ionic interaction within the antagonist -- which is required to maintain optimal conformation. A serine-to-alanine or serine-to-glycine substitution which had a negative effect on activity indicates the location of an important hydrogen bond. Again, the hydrogen bond may be between the antagonist and the thrombin receptor, or it may be an intramolecular hydrogen bond that plays an important role in the conformation of the antagonist.

Some of the above single substitution peptides will differ in activity depending upon whether the substitution at any given position is alanine or glycine. The important differences between glycine and

alanine are: alanine is optically active, it is more bulky and more hydrophobic. Thus, when an alanine-substituted peptide retains its inhibitory activity while the corresponding glycine-substituted peptide does not, one of skill in the art will know that the substituted position requires one of the three above-described characteristics at the substituted position to be active.

Those of skill in the art will realize that distinguishing between whether a structural feature is important for an inter- or an intramolecular interaction can only be achieved by examining an X-ray crystal structure of the antagonist-receptor complex. However, that distinction is of little import in designing the antagonists of this invention. Once the nature of the interaction is determined, i.e., electrostatic, hydrophobic, ionic, the choice of potential substitutes at that position becomes clear.

To further ascertain those sites that are important for proper folding and orientation of the thrombin receptor antagonists of this invention, a single position deletion analysis is performed. In this procedure, a series of peptides containing single deletions at positions which do not affect inhibitory activity (as determined above) are synthesized and assayed for antiplatelet activity. The peptides from this series that retain significant antiplatelet activity indicate areas of the antagonist that are not essential for proper conformation. Such peptides are also within the scope of this invention.

Deletion peptides from this series which have significantly lower platelet inhibitory activity indicate the location of components which provide critical spacing in the antagonist. This may be verified by replacing the deleted amino acid with a different, yet analogous structure. For example,

substitution of any conformationally important amino acid with a three carbon alkyl chain without a significant loss of activity confirms that spacing is critical at that part of the molecule.

Additional information about important structural and conformational features necessary for designing a potent thrombin receptor antagonist of this invention may be obtained through 3-dimensional X-ray crystallographic procedures coupled with computer modelling. Specifically, one of ordinary skill in the art may analyze a thrombin receptor/thrombin amino acid 65-83 peptide complex using such a method. Alternatively, one could use the X-ray crystallographic data on the analogous C-terminal hirudin peptide-thrombin complex [E. Skrzypczak-Jankun et al., "Structure of the Hirugen and Hirulog 1 Complexes of α-Thrombin", J. Mol. Biol., 221, pp. 1379-93 (1991)]. Finally, one of average skill in the art could also employ multiple alanine substitutions or multiple deletions to identify important intramolecular interactions in the antagonist itself. It will also be apparent that each new thrombin receptor antagonist designed and tested will, itself, provide additional information about structural features important for thrombin receptor inhibition.

Once the critical residues in the thrombin 65-83 peptide have been located and characterized, other thrombin receptor antagonists of this invention may be designed and synthesized. This is achieved by substituting the identified key residues of the thrombin peptide with other components having similar features. These substitutions will initially be conservative, i.e., the replacement component will have approximately the same size, shape, hydrophobicity and charge as the key residue. Those of ordinary skill in the art are well aware of appropriate replacements for

a given amino acid [Dayhoff et al., in Atlas of Protein Sequence and Structure No. 5, 1978 and Argos et al., EMBO J., 8, pp. 779-85 (1989)]. Typical conservative substitutions for an amino acid are other amino acids with similar charges. For example, aspartic acid for glutamic acid, arginine for lysine, asparagine for glutamine, hydroxyproline for proline and vice versa. Substitutions with non-natural amino acids may also be performed to reduce the peptidic nature of the antagonist. Some examples are cyclohexylalanine for tyrosine, sarcosine for glycine, statine for threonine and homoarginine for arginine. These modifications may increase the biological stability of the antagonist, in addition to increasing its potency.

After the molecule containing the substitute component is shown to be an effective thrombin receptor antagonist, less conservative replacements may be made at the same position. These substitutions typically involve the introduction of non-amino acid components which contain the important feature imparted by the amino acid at that position. Such substitutes are well-known in the art. For example, the sequence Leu-Val-Arg (corresponding to amino acids 65-67 of thrombin) can be replaced by p-guanidinobenzoic acid. This substitution maintains the hydrophobicity of Leu-Val, as well as the guanidinium functionality of Arg.

It will be apparent that there is greater freedom in selecting the substitute for a non-essential amino acid in the thrombin 65-83 amino acid sequence. Moreover, a non-essential amino acid may simply be eliminated. Almost any substitute that does not impart a change in conformation may be employed for a non-essential amino acid. These include, but are not limited to, straight chain alkyl and acyl groups. Also, because of the importance of the net positive charge of the antagonist, anionic substitutes should be

avoided. Components which are known in the art to alter conformation should also be avoided. One such component is proline, an amino acid which causes a turn structure in a molecule. Others are well-known in the art [G. D. Rose et al., "Turns in Peptides and Proteins", Adv. Prot. Chem., 37, pp. 1-110 (1985)].

In addition to those antagonists resulting from the substitutions and deletions described above, novel thrombin receptor antagonists according to this invention may be designed by insertions at various sites along the thrombin 65-83 peptide. To determine areas of the thrombin 65-83 peptide where a component may potentially be inserted, a series of peptides having a single alanine insertion at various sites is synthesized. Those peptides from this series which retain antiplatelet activity indicate potential insertion sites.

In choosing a component to be inserted, one should be guided by the same considerations set forth above in selecting a substitute component. Specifically, one must keep in mind how the insertions may potentially affect the molecular interactions between the antagonist and the thrombin receptor and how they affect conformation of the antagonist. For example, the insertion of an anionic component adjacent to a critical cationic amino acid in thrombin 65-83 could interfere with an important ionic interaction and should therefore be avoided. Similarly, the insertion of a component which is known to cause structural perturbations, e.g., a proline, should also be avoided.

Using any or all of the above deletion, substitution and insertion techniques allows those of ordinary skill in the art to design thrombin receptor antagonists according to this invention. Moreover, the potential effect of any of these changes may be theoretically observed prior to synthesizing the

antagonist through the use of computer modelling techniques known in the art. Such modelling allows one to observe the predicted structure of a thrombin receptor complexed with the potential antagonist. If that theoretical structure suggests insufficient interaction between the receptor and the potential antagonist, one need not spend time and resources in synthesizing and testing the molecule. On the other hand, if computer modelling indicates a strong interaction, the molecule may then be synthesized and assayed for antiplatelet activity. In this manner, inoperative molecules may be eliminated before they are synthesized.

Finally, cyclic derivatives of any antagonist designed by the above techniques are also part of the present invention. Cyclization may allow the antagonist to assume a more favorable conformation for association with the thrombin receptor. Cyclization may be achieved by methods well-known to those in the art. One method is the formation of a disulfide bond between two non-adjacent cysteine residues (D- or L-conformation) or any two appropriately spaced components having free sulfhydryl groups. It will be understood that disulfide bonds, as well as other intramolecular covalent bonds, may be formed between a variety of components within the antagonist. The components which form such bonds may be side chains of amino acids, non-amino acid components or a combination of the two.

Based on the data obtained in both the alanine/glycine-substitutions and the deletion series analysis, one may then synthesize truncated peptides (i.e., multiple N- and/or C-terminal deletions) to determine their thrombin receptor antagonist activity. Using the above-described analyses, applicants have identified the minimal portion of thrombin necessary

for receptor inhibition -- Arg-Ile-Gly-Lys-His-Ser-Arg (thrombin amino acids 67-73; amino acids 3-9 of SEQ ID NO:1). Applicants have made further substitutions within this minimal sequence and have developed peptides which have increased inhibitory activity over that thrombin peptide. These substitutions are discussed below and in more details in the specific examples.

The conservative substitutions referred to above characterize a preferred thrombin receptor antagonist of this invention which comprises the formula: $Y-A_1-W-X_1-X_2-X_3-X_4-A_2-Z$; wherein Y is hydrogen, a peptide of from 1 to 10 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 30 backbone atoms; each of A_1 and A_2, either the same or different, are selected from the group consisting of a positively charged amino acid and an acyl or alkyl chain comprising from 1 to 10 backbone atoms and a positively charged side group; W and each X is any amino acid, either the same or different; and Z is OH, a peptide of from 1 to 11 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 33 backbone atoms, and wherein said antagonist is optionally cyclized by a covalent bond between Y and Z; with the proviso that at least one of W, X_1 and X_2 is not a basic amino acid.

More preferred antagonists of the present invention comprise the general formula indicated above, wherein Y has the formula $Y_1-B_n-C_m$, wherein Y_1 is hydrogen, a peptide of from 1 to 6 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 18 backbone atoms; B is a hydrophobic amino acid; C is an acidic amino acid, either the same or different, n is 0 or 1; and m is an integer from 1 and 3. Other preferred embodiments are antagonists wherein A_1 is Arg; antagonists wherein A_2 is

Arg; antagonists wherein W is Ile, D-Cys or Ala; antagonists wherein X_2 is an anionic amino acid; antagonists wherein X_3 is His; and antagonists wherein X_4 is Ser.

The most preferred antagonists of the present invention are referred to as thrombin receptor inhibitory peptides or TRIPs. These antagonists have the formulae:

TRIP-1: SEQ ID NO:2: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu;

TRIP-2: Leu-Val-Arg-Ile-Cys-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-(D-Cys);

TRIP-3: SEQ ID NO:3: Leu-Cys-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile;

TRIP-4: Leu-Val-Arg-(D-Cys)-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Cys-Glu-Lys-Ile; and

TRIP-5: Leu-Val-Arg-(D-Cys)-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-(D-Cys)-Glu-Lys-Ile;

TRIP-6: SEQ ID NO:4: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile;

TRIP-7: SEQ ID NO:5: Leu-Val-Arg-Ile-Gly-Orn-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile;

TRIP-8: SEQ ID NO:6: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Asp-Arg-Asn-Ile-Glu-Lys-Ile;

TRIP-9: SEQ ID NO:7: Leu-Val-HArg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile;

TRIP-10: SEQ ID NO:8: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Cha-Gly;

TRIP-11: SEQ ID NO:9: MPR-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Cys;

TRIP-12: SEQ ID NO:10: MPR-Cha-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Cys;

TRIP-13: SEQ ID NO:11: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Cha-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile;

TRIP-14: SEQ ID NO:12: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr(I_2)-Glu-Arg-Asn-Ile-Glu-Lys-Ile;

TRIP-15: SEQ ID NO:13: GBA-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile;

TRIP-16: Leu-Val-Arg-D-Cys-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn;

TRIP 17: SEQ ID NO:14: Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn;

TRIP 19: SEQ ID NO:15: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg;

TRIP 20: Leu-Val-Arg-D-Cys-Gly-Lys-His-Ser-Arg;

Alk-TRIP 20: Leu-Val-Arg-alkCys-Gly-Lys-His-Ser-Arg;

TRIP 21: Leu-Val-Arg-D-Ser-Gly-Lys-His-Ser-Arg;

TRIP 22: SEQ ID NO:16: Leu-Val-Arg-Ser-Gly-Lys-His-Ser-Arg;

TRIP 23: SEQ ID NO:17: Leu-Val-Arg-Met-Gly-Lys-His-Ser-Arg;

TRIP 24: Leu-Val-Arg-D-Cys-Gly-Lys-Tyr-Ala-Arg;

TRIP 25: Leu-Val-Arg-D-Cys-Gly-Leu-His-Phe-Arg;

TRIP 26: Val-Val-Arg-D-Cys-Gly-Lys-His-Phe-Arg;

TRIP 27: Leu-Val-Arg-D-Cys-Ser-Lys-His-Ser-Arg;

TRIP 28: Leu-Val-Arg-D-Cys-Thr-Lys-His-Ser-Arg;

TRIP 29: Leu-Val-Arg-D-Cys-Tyr-Lys-His-Ser-Arg;

TRIP 30: Leu-Val-Arg-D-Cys-Asn-Tyr-His-Ser-Arg;

TRIP 31: Leu-Val-Arg-D-Cys-Gln-Lys-His-Ser-Arg;

TRIP 32: Leu-Val-Arg-D-Cys-Asp-Lys-His-Ser-Arg;

TRIP 33: Leu-Val-Arg-D-Cys-Glu-Lys-His-Ser-Arg;

TRIP 34: Leu-Val-Arg-D-Cys-Lys-Lys-His-Ser-Arg;

TRIP 35: Leu-Val-Arg-D-Cys-Arg-Lys-His-Ser-Arg;

TRIP 36: Leu-Val-Arg-D-Cys-His-Lys-His-Ser-Arg;

TRIP 37: Leu-Val-Arg-D-Cys-D-Ala-Lys-His-Ser-Arg;

TRIP 38: Leu-Val-Arg-D-Cys-D-Ser-Lys-His-Ser-Arg;

TRIP 39: Leu-Val-Arg-D-Cys-D-Thr-Lys-His-Ser-Arg;

TRIP 40: Leu-Val-Arg-D-Cys-D-Tyr-Lys-His-Ser-Arg;

TRIP 41: Leu-Val-Arg-D-Cys-D-Asn-Lys-His-Ser-Arg;

TRIP 42: Leu-Val-Arg-D-Cys-D-Gln-Lys-His-Ser-Arg;

TRIP 43: Leu-Val-Arg-D-Cys-D-Asp-Lys-His-Ser-Arg;

TRIP 44: Leu-Val-Arg-D-Cys-D-Glu-Lys-His-Ser-Arg;

TRIP 45: Leu-Val-Arg-D-Cys-D-Lys-Lys-His-Ser-Arg;

TRIP 46: Leu-Val-Arg-D-Cys-D-Arg-Lys-His-Ser-Arg;

TRIP 47: Leu-Val-Arg-D-Cys-D-His-Lys-His-Ser-Arg;

TRIP 48: D-Cys-Arg-Leu-Ser-Val-His-Arg-Lys-Gly;

TRIP 49: SEQ ID NO:18: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Lys-Thr-Arg-Tyr-Glu-Lys-Asn;

TRIP 50: SEQ ID NO:19: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-His-Thr-Arg-Tyr-Glu-His-Asn;

TRIP 51: SEQ ID NO:20: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Ala-Thr-Arg-Tyr-Glu-Ala-Asn;

TRIP 52: SEQ ID NO:21: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Cys-Tyr-Glu-Arg-Asn;

TRIP-2, TRIP-4, TRIP-5, TRIP-11 and TRIP-12 each contains an intramolecular disulfide bond and are examples of cyclized thrombin receptor antagonists. In TRIP-2, TRIP-4 and TRIP-5, the disulfide bond is between the cysteine residues. In TRIP-11 and TRIP-12, the disulfide bond is formed between the N-terminal mercaptopropionic acid, a non-amino acid component, and the C-terminal cysteine residue.

It is known that amino acids 65-83 of thrombin form a ß-loop in between two antiparallel ß-strands in the naturally occurring molecule [W. Bode et al., "The Refined 1.9 Å Crystal Structure of Human α-

Thrombin: Interaction With D-Phe-Pro-Arg chloromethylketone and Significance of the Tyr-Pro-Pro-Trp Insertion Segment", EMBO J., 8, pp. 3467-75 (1989)]. Without being bound by theory, applicant believes that antagonists of this invention which can assume a similar conformation will be more structurally stable and therefore more potent. Thus, another embodiment of this invention is a fusion protein which comprises the formula: PP_L-T-PP_R; wherein T is a thrombin receptor antagonist of this invention; and PP_L and PP_R are both portions of a polypeptide of the formula: PP_L-BL-PP_R; wherein BL forms a ß-loop structure in said polypeptide; and each of PP_L and PP_R are characterized by an antiparallel ß-strand structure in said polypeptide.

The design of such a fusion protein initially involves identifying a naturally occurring protein which possesses a ß-loop between two antiparallel ß-strands structure. Several naturally occurring proteins are known to contain such a structure. These include erabutoxinA, both the heavy and light chain immunoglobulins; and serine proteases, such as trypsin. Any protein which has been crystallized can be scanned for such a loop/anti-parallel strand structure using X-ray crystallography.

Once an appropriate protein has been identified, the amino acids which make up the ß-loop portion will be replaced with a peptide shown by the methods described above to act as a thrombin receptor antagonist. This replacement will normally be done at the nucleotide level by recombinant DNA technology. Because of this, the gene or a cDNA encoding the naturally occurring protein must be available. Also, the ß-loop replacement i.e., the thrombin receptor antagonist peptide, must be made up entirely of naturally occurring amino acids and a synthetic gene

encoding that antagonist must be synthesized.

The manipulations required to construct such a fusion protein are commonly employed in the art. Typically, this involves removing the naturally occurring ß-loop coding region via restriction endonuclease digestions, exonuclease digestions or a combination of both. The remainder of the DNA encoding that protein is then isolated and ligated to a synthetic gene encoding the antagonist. Large quantities of the DNA encoding the desired fusion protein may be obtained via PCR (polymerase chain reaction) or by conventional cloning techniques. Alternately, the gene encoding the desired fusion protein may be constructed synthetically by piecing together various oligonucleotides.

The fusion protein is subsequently produced by transformation or transfection of an appropriate host with the above DNA. Those of skill in the art are well aware of the factors necessary to achieve sufficient expression of the fusion protein. These include the operative linkage of the DNA encoding the fusion protein to an appropriate promoter, choice of appropriate host, proper growth conditions, etc.

The antiplatelet activity of the antagonists of this invention may be measured by any of a number of conventional, in vitro platelet assays. Preferably, the assay will measure a change in the degree of aggregation of platelets or a change in the release of a platelet secretory component in the presence of thrombin. The former may be measured in an aggregometer. The latter may be measured using RIA or ELISA techniques specific for the secreted component.

The inability of the antagonists of this invention to inhibit thrombin's catalytic activity may be assayed in vitro utilizing a synthetic peptidyl substrate with a colorimetric marker. An example of

such a substrate is Spectrozyme TH (tosyl-Gly-Pro-Arg-p-nitroanilide; American Diagnostica, New York, NY). Cleavage of that substrate can be quantitated using a spectrophotometer. Inhibition of thrombin's catalytic activity may also be assayed using an in vitro assay of fibrinogen cleavage.

The ability of an antagonist of this invention to bind to the anionic domain of the thrombin receptor is easily determined in a competition assay. For example, incubation of thrombin receptor-containing cells (platelets, smooth muscle cells, endothelial cells, etc.) with a radiolabelled antagonist in the presence or absence of an unlabelled peptide corresponding to the thrombin receptor anionic domain ("thrombin receptor peptide") provides a quantification of such binding. Alternatively, affinity chromatography of the labelled antagonist on a column containing a thrombin receptor peptide cross-linked to an insoluble matrix could be used to assay binding. In this latter method, an antagonist that binds to the anionic domain of the thrombin receptor will specifically bind to the column until eluted with soluble, unlabelled thrombin receptor peptide. The above assays and methods are well-known in the art.

The thrombin receptor antagonists of the present invention may be synthesized by various techniques which are well known in the art. These include enzymatic cleavage of thrombin, recombinant DNA techniques, solid-phase peptide synthesis, solution-phase peptide synthesis, organic chemical synthesis techniques, or a combination of these techniques. The choice of synthesis technique will, of course, depend upon the composition of the particular antagonist. In a preferred embodiment of this invention, the thrombin antagonist is entirely peptidic and is synthesized by solid-phase peptide synthesis techniques,

solution-phase peptide synthesis techniques or a combination thereof which constitute the most cost-efficient procedures for producing commercial quantities of these antagonists.

When "non-protein" amino acids are contained in the thrombin receptor antagonist, they may be either added directly to the growing chain during peptide synthesis or prepared by chemical modification of the complete synthesized peptide, depending on the nature of the desired "non-protein" amino acid. Those of skill in the chemical synthesis art are well aware of which "non-protein" amino acids may be added directly and which must be synthesized by chemically modifying the complete peptide chain following peptide synthesis.

The synthesis of those thrombin antagonists of this invention which contain both non-amino acid and peptidic portions is preferably achieved by a mixed heterologous/solid phase technique. This technique involves the solid-phase synthesis of all or most of the peptide portion of the molecule. This is followed by the addition of the non-amino acid components which are synthesized by solution phase techniques and then coupled to the peptidic portion via solid-phase or solution-phase methods. Any remaining peptidic portions may then be added via solid-phase or solution-phase methods.

The thrombin receptor antagonists of the present invention are useful in compositions and methods for the treatment and prophylaxis of various diseases attributed to thrombin-mediated and thrombin-associated functions and processes. These include myocardial infarction, stroke, pulmonary embolism, peripheral arterial occlusion, restenosis following arterial injury or invasive cardiological procedures, acute or chronic atherosclerosis, edema and inflammation, various cell regulatory processes (e.g.

secretion, shape changes, proliferation), and neurodegenerative diseases.

The thrombin receptor antagonists of the present invention may be formulated using conventional methods to prepare pharmaceutically useful compositions, such as the addition of a pharmaceutically acceptable carrier. These compositions and the methods employing them may be used for treating or preventing platelet activation in a patient. Inhibition of thrombin-induced platelet activation is particularly useful in the treatment and prophylaxis of platelet-dependent (arterial) thrombosis.

Various dosage forms may be employed to administer the compositions of this invention. These include, but are not limited to, parenteral administration, oral administration and topical application. The compositions of this invention may be administered to the patient in any pharmaceutically acceptable dosage form, including those which may be administered to a patient intravenously as bolus or by continued infusion, intramuscularly -- including paravertebrally and periarticularly -- subcutaneously, intracutaneously, intra-articularly, intrasynovially, intrathecally, intra-lesionally, periostally or by oral, nasal, or topical routes. Such compositions are preferably adapted for topical, nasal, oral and parenteral administration, but, most preferably, are formulated for parenteral administration.

Parenteral compositions are most preferably administered as a constant infusion. For parenteral administration, fluid unit dose forms are prepared which contain a thrombin receptor antagonist of the present invention and a sterile vehicle. The antagonist may be either suspended or dissolved, depending on the nature of the vehicle and the nature

of the particular antagonist. Parenteral compositions are normally prepared by dissolving the thrombin receptor antagonist in a vehicle, optionally together with other components, and filter sterilizing before filling into a suitable vial or ampule and sealing. Preferably, adjuvants such as a local anesthetic, preservatives and buffering agents are also dissolved in the vehicle. The composition may then be frozen and lyophilized to enhance stability.

Parenteral suspensions are prepared in substantially the same manner, except that the active component is suspended rather than dissolved in the vehicle. Sterilization of the compositions is preferably achieved by exposure to ethylene oxide before suspension in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of its components.

Tablets and capsules for oral administration may contain conventional excipients, such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, and wetting agents. The tablet may be coated according to methods well known in the art. Suitable fillers which may be employed include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include, but are not limited to, starch, polyvinylpyrrolidone and starch derivatives, such as sodium starch glycolate. Suitable lubricants include, for example, magnesium stearate. Suitable wetting agents include sodium lauryl sulfate.

Oral liquid preparations may be in the form of aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or another suitable vehicle before use. Such liquid preparations may contain conventional additives. These include

suspending agents; such as sorbitol, syrup, methyl cellulose, gelatin, hydroxyethyl-cellulose, carboxy-methylcellulose, aluminum stearate gel or hydrogenated edible fats; emulsifying agents which include lecithin, sorbitan monooleate, polyethylene glycols, or acacia; non-aqueous vehicles, such as almond oil, fractionated coconut oil, and oily esters; and preservatives, such as methyl or propyl p-hydroxybenzoate or sorbic acid.

Compositions formulated for topical administration may, for example, be in aqueous jelly, oily suspension or emulsified ointment form.

The dosage and dose rate of the thrombin receptor antagonist will depend on a variety of factors, such as the size of the patient, the specific pharmaceutical composition used, the object of the treatment, i.e., therapy or prophylaxis, the nature of the condition to be treated, and the judgment of the treating physician. A pharmaceutically effective dosage is defined herein as being between about 0.001 and 100 mg/kg body weight of the patient to be treated. It should, however, be understood that other dosages may be useful based on the factors enumerated above.

Once improvement in the patient's condition has occurred, a maintenance dose of a composition of this invention is administered, if necessary. Subsequently, the dosage or the frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained. When the symptoms have been alleviated to the desired level, treatment should cease. Patients may, however, require intermittent treatment upon any recurrence of disease symptoms.

According to an alternate embodiment of the present invention, the thrombin receptor antagonist may be employed in compositions and methods for inhibiting thrombin-induced smooth muscle cell proliferation in a

patient. The thrombin-induced multiplication of intimal smooth muscle cells which line the outer surface of blood vessels has been implicated in advanced atherosclerosis and restenosis following angioplasty [D. T. Hung et al., J. Cell Biol., 116, pp. 827-32 (1992); A. J. B. Brady et al., Brit. J. Med., 303, pp. 729-30 (1991)]. The current belief is that rupture of atherosclerotic plaques -- precipitated by natural causes or angioplasty -- sets off a chain of events that attract thrombin to the plaque site. Once there, thrombin causes thrombus formation and smooth muscle cell proliferation. These two phenomena result in vessel blockage and potential coronary failure. Therefore, the ability to inhibit smooth muscle cell replication is of clinical importance in the ultimate success of angioplasty.

According to a preferred embodiment of the present invention, the inhibition of smooth muscle cell proliferation in a patient is achieved by inserting into said patient a device through which is conveyed a composition comprising the thrombin receptor antagonist of this invention. To allow site-specific delivery of the thrombin receptor to the intimal smooth muscle cells, a portion of the device (preferably the end) must contain aperture means which will allow the antagonist to exit the device. Such devices are well known in the art and are exemplified by balloon catheters. A preferred embodiment of this method employs a weeping balloon catheter as the device which contains the thrombin receptor antagonist composition [see for example, United States patent 5,049,132 and Wolinsky et al., "Use of a Perforated Balloon Catheter to Deliver Concentrated Heparin Into the Wall of the Normal Canine Artery", J. Amer. Coll. Cardiol., 15, pp. 475-81 (1990), the disclosures of which are hereby incorporated by reference].

According to another embodiment of the present invention, thrombin receptor antagonists may be utilized in methods for treating or preventing bone resorption. Osteoclasts are the cells responsible for bone resorption in the process of turnover and remodelling of bone. These cells are known to be stimulated by thrombin through a receptor on the osteoclast cell surface [D. N. Tatakis et al., Biochem. Biophys. Res. Comm., 174, pp. 181-88 (1991)]. By inhibiting thrombin-induced osteoclast activity, bone resorption is decreased. This may be specifically useful in the treatment of osteoporosis and other conditions characterized by abnormally high levels of bone resorption and demineralization.

This invention also relates to methods which employ the above-described thrombin receptor antagonists to inhibit thrombin-induced inflammation. Thrombin is known to induce the synthesis of platelet activation factor (PAF) by endothelial cells [S. Prescott et al., Proc. Natl. Acad. Sci. USA, 81, pp. 3534-38 (1984)]. It is believed that thrombin exerts this effect by binding to its receptor on the endothelial cell surface. These methods have important applications in the treatment of diseases characterized by thrombin-induced inflammation and edema, which are thought to be mediated be PAF. Such diseases include, but are not limited to, adult respiratory distress syndrome, septic shock, septicemia and reperfusion damage.

The thrombin receptor antagonists of this invention may also be employed in methods for treating neurodegenerative diseases. Thrombin is known to cause neurite retraction, a process suggestive of the rounding in shape changes of brain cells and implicated in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. It is believed that

thrombin acts on neuroblasts through a specific receptor on the surface of those cells [D. Gurwitz et al., Proc. Natl. Acad. Sci. USA, 85, pp. 3440-44 (1988)].

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting this invention in any manner.

EXAMPLE 1

Synthesis Of TRIPs

Unless otherwise indicated, we synthesized all of the TRIPs by conventional solid-phase peptide synthesis using an Applied Biosystems 430 A Peptide Synthesizer (Applied Biosystems, Foster City, CA). The resins employed to synthesize the various TRIPs were dependent upon the C-terminal amino acid of the antagonist. Specifically, the resins used were t-BOC-L-Glu(OBzl)(OCH_2)-PAM, t-BOC-D-Cys(4-MeBzl)(-OCH_2)-PAM, t-BOC-L-Ile(OCH_2)-PAM, t-BOC-Gly(OCH_2)-PAM, t-BOC-L-Cys(4-MeBzl)(-OCH_2)-PAM, t-BOC-L-Arg(Mts) and t-BOC-L-Asn. Resins were purchased from either Applied Biosystems or Peninsula Laboratories (Belmont, CA).

We employed various protected amino acids for peptide synthesis depending upon the sequence of amino acids in the particular TRIP. The protected amino acids used in the syntheses were t-BOC-L-Leu, t-BOC-L-Val, t-BOC-Arg(Mts), t-BOC-L-Ile(1/2 H_2O), t-BOC-Gly, t-BOC-L-Lys(2-Cl-z), t-BOC-L-Ser(Bzl), t-BOC-L-Thr(Bzl), t-BOC-L-Tyr(2-Br-z), t-BOC-L-Glu(OBzl), t-BOC-L-Asp(OBzl), t-BOC-L-Asn and t-BOC-L-Orn(2-Cl-z) from Peninsula Laboratories; and t-BOC-L-His(N-im-CBZ), t-BOC-O-(2,6-dichlorobenzyl)-3,5-diiodo-L-tyrosine and t-BOC-cyclohexyl-L-alanine from Bachem (Torrance, CA).

EXAMPLE 2

Synthesis Of TRIP-1

TRIP-1 (SEQ ID NO:2: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu) was synthesized using t-BOC-L-Glu(OBzl)(OCH_2)-PAM resin and the appropriate protected amino acids selected from those listed in Example 1. After completion of synthesis, the peptide was fully deprotected and uncoupled from the resin by treatment with anhydrous HF: p-cresol: ethylmethyl sulfate (10:1:1, v/v/v). Peptides were then extracted from the resin with 5% acetic acid and lyophilized to dryness.

Crude TRIP-1 was purified by reverse-phase HPLC employing an Applied Biosystems 151A liquid chromatographic system and a Vydac C18 column (2.2 x 25 cm). The column was equilibrated in 0.1% trifluoroacetic acid (TFA) in water and developed with a linear gradient of increasing acetonitrile concentration (0 to 80%) in 0.1% TFA over 45 minutes at a flow rate of 4.0 ml/min. The effluent stream was monitored at 229 nm for absorbance. Fractions were collected manually. Fractions containing TRIP-1 were pooled and lyophilized to dryness. Typically, 20-50 mg of crude TRIP-1 was loaded onto the column and yielded 5-35 mg of purified peptide.

We confirmed the structure of TRIP-1 by several techniques. Amino acid hydrolysates were prepared by treating the peptide with 6N HCl, in vacuo, at 100°C for 24 hours. The hydrolysate was then analyzed with a Beckman 6300 amino acid analyzer (Beckman Instruments, Palo Alto, CA) which employs an ion-exchange separation technique coupled with post-column ninhydrin derivatization.

We performed amino acid sequence analysis using automated Edman degradation on an Applied

Biosystems 470A gas-phase sequencer equipped with a Model 900A data system. Phenylthiohydantoin (PTH) amino acids were analyzed on-line using an Applied Biosystems 120A PTH-Analyzer and a PTH-C18 column (2.1 x 220 mm).

We also had FAB-mass spectroscopy performed on the purified TRIP-1 by M-Scan (West Chester, PA), an external contract laboratory.

All of the subsequent TRIPs described below were synthesized in the same manner as that described for TRIP-1, using the appropriate resin and protected amino acids, unless otherwise indicated. Similarly, all of the below-described TRIPs were uncoupled, deprotected, purified and analyzed in the same manner as that described for TRIP-1.

EXAMPLE 3

Synthesis Of TRIP-2

TRIP-2 (Leu-Val-Arg-Ile-Cys-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-(D-Cys)) was synthesized in the same manner as TRIP-1, using the appropriate resin and protected amino acids. Uncoupling, deprotection, purification and analysis were also performed as described for TRIP-1.

Following purification, we cyclized the antagonist by dissolving 10 mg of the peptide in 5.0 ml of 0.2 M sodium borate buffer, pH 9.0. The solution was allowed to stand at room temperature for 1-5 days. This allowed cyclization to occur by simple air oxidation of the cysteine residues.

We monitored the kinetics of cyclization by taking 50 µl aliquots of the solution at various time points. The aliquots were acidified with 50 µl of 0.1% TFA in water and then chromatographed on an Aquapore C8 column (100 x 4.6 mm) using an Applied Biosystems 150A liquid chromatographic system. The column was

developed with a linear gradient of increasing acetonitrile concentration (0-80%) in 0.1% TFA over 45 minutes. We monitored the effluent stream at 214 nm for absorbance. The presence of cyclized antagonist was indicated by the presence of a new peak of ultraviolet absorbance which eluted prior to the peak representing linear TRIP-2. We also observed additional peaks eluting after the linear TRIP-2 which were attributed to the presence of intermolecular cross-linked TRIP-2 molecules.

Cyclized TRIP-2 was purified by the same reverse-phase HPLC chromatography as described for the purification of TRIP-1. The structure of the cyclic TRIP-2 was confirmed by FAB-mass spectroscopy. The parent ion molecular mass for cyclized TRIP-2 was 2 units lower that the mass for the linear TRIP-2. This indicated that the oxidation of thiols to yield a disulfide had occurred.

All of the other cyclic TRIPs described below (TRIP-4, -5, -11 and -12) are cyclized, purified and analyzed in the same manner, unless otherwise indicated.

EXAMPLE 4

Synthesis Of TRIP-3

TRIP-3 has the formula: SEQ ID NO:3: Leu-Cys-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile and was synthesized as described above.

EXAMPLE 5

Synthesis Of TRIP-4

TRIP-4 has the formula: Leu-Val-Arg-(D-Cys)-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Cys-Glu-Lys-Ile. TRIP-4 was synthesized and was cyclized as described above.

EXAMPLE 6

Synthesis Of TRIP-5

TRIP-5 has the formula: Leu-Val-Arg-(D-Cys)-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-(D-Cys)-Glu-Lys-Ile. TRIP-5 was synthesized and was cyclized as described above.

EXAMPLE 7

Synthesis Of TRIP-6

TRIP-6 has the formula: SEQ ID NO:4: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile and was synthesized as described above.

EXAMPLE 8

Synthesis Of TRIP-7

TRIP-7 has the formula: SEQ ID NO:5: Leu-Val-Arg-Ile-Gly-Orn-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile and was synthesized as described above.

EXAMPLE 9

Synthesis Of TRIP-8

TRIP-8 has the formula: SEQ ID NO:6: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Asp-Arg-Asn-Ile-Glu-Lys-Ile and is synthesized as described above.

EXAMPLE 10

Synthesis Of TRIP-9

TRIP-9 has the formula: SEQ ID NO:7: Leu-Val-HArg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile and was synthesized as described above. Boc-HArg was synthesized by conventional

procedures well known in the protein synthesis art.

EXAMPLE 11

Synthesis Of TRIP-10

TRIP-10 has the formula: SEQ ID NO:8: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Cha-Gly and was synthesized as described above.

EXAMPLE 12

Synthesis Of TRIP-11

TRIP-11 has the formula: SEQ ID NO:9: MPR-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Cys. Residues 2-19 of this antagonist are synthesized by conventional peptide synthesis procedures as described in Example 2. The peptide resin was then reacted with a 100-fold molar excess of mercaptopropionic acid (MPR) using the conventional coupling procedure of the Applied Biosystems peptide synthesizer. The successful coupling of MPR to the antagonist was tested by ninhydrin analysis of an aliquot of the reacted resin. Absence of a blue color in the ninhydrin reaction indicates successful incorporation of MPR. If a positive reaction occurs (blue color), an additional cycle of MPR addition was performed.

Cyclization of TRIP-11 was performed as described in Example 3, following synthesis of the complete molecule.

EXAMPLE 13

Synthesis Of TRIP-12

TRIP-12 has the formula: SEQ ID NO:10: MPR-Cha-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Cys. TRIP-12 was synthesized in the same manner described above for TRIP-11, except

that residues 2-20 are synthesized by conventional solid phase synthesis prior to MPR addition. TRIP-12 was cyclized as described above.

EXAMPLE 14

Synthesis Of TRIP-13

TRIP-13 has the formula: SEQ ID NO:11: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Cha-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile and was synthesized as described above.

EXAMPLE 15

Synthesis Of TRIP-14

TRIP-14 has the formula: SEQ ID NO:12: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr(I_2)-Glu-Arg-Asn-Ile-Glu-Lys-Ile and was synthesized as described above.

EXAMPLE 16

Synthesis Of TRIP-15

TRIP-15 has the formula: SEQ ID NO:13: GBA-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Ile. Residues 2-16 of TRIP-15 are synthesized by the conventional solid phase techniques described in Example 2. The peptide resin was then reacted with a 100-fold molar excess of para-guanidinobenzoic acid (GBA) using the conventional coupling procedure of the Applied Biosystems peptide synthesizer. Successful addition of GBA was confirmed by the ninhydrin test described for TRIP-11 (Example 12).

EXAMPLE 17

Platelet Aggregation Assays

Platelet-rich plasma (PRP) was obtained from healthy human volunteers who had refrained from taking

aspirin for at least one week prior to the taking of samples. Blood was collected into 1/10 volume of 3.8% trisodium citrate using a 21 gauge butterfly cannula. The blood was then centrifuged at room temperature for 15 minutes at 100 x g and the supernatant was collected. The supernatant contained 250,000 - 400,000 platelets/μl. We then gel-filtered the platelets by applying 20-40 ml of PRP onto a Sepharose 2B-300 column (2.5 x 10 cm) equilibrated and eluted with a Tyrode-HEPES buffer (129 mM NaCl, 10.9 mM trisodium citrate, 8.9 mM sodium carbonate, 5 mM HEPES, 2.8 mM KCl, 0.8 mM dibasic potassium phosphate, 0.84 mM $MgCl_2$, 0.24 mM $CaCl_2$), supplemented with 0.56 mM dextrose and 0.35% bovine serum albumin, pH 7.4. The effluent stream was monitored visually for the appearance of platelets (observed as a cloudy solution). Platelets obtained from this procedure were maintained at room temperature and used within 2 hours of preparation.

Platelet aggregation studies were performed with gel-filtered platelets using a 4-channel platelet aggregation profiler (PAP4, Biodata, Hatboro, PA). Specifically, the assay was performed as follows: Varying concentrations of TRIP-1, (0 - 1 mM final) were added to 0.45 ml of a gel-filtered platelet suspension. The mixture was then placed in the platelet aggregometer to initiate warming to 37°C and stirring. After equilibration (approximately 5 minutes) we added human thrombin (0.16-0.48 U/ml final) to the mixture. The aggregation response was monitored for 5 minutes. Inhibition was calculated by the following formula:

$$\% \text{ inhibition} = 1 - \frac{\% \text{ aggregation in TRIP sample}}{\% \text{ aggregation in control sample}} \times 100$$

Figure 1 demonstrates that TRIP-1 inhibited thrombin-induced platelet aggregation in a dose-dependent manner at varying thrombin concentrations. At a concentration of 800 μM, TRIP-1 inhibited

thrombin-induced platelet aggregation by >80% for all concentrations of thrombin tested.

We also analyzed the ability of the thrombin receptor antagonists of this invention to inhibit platelet aggregation induced by the tethered ligand (5 μM), collagen (76 μg/ml), or ADP (4 μM). For assaying inhibition of ADP-induced platelet aggregation, the gel filtered platelet suspension was supplemented with purified human fibrinogen (1 mg/ml). For each of these assays, we used concentrations of antagonist that fully inhibited thrombin-induced aggregation (800 μM for TRIP-1). The results of this experiment are depicted in Figures 2 and 3.

Figure 2 demonstrates that TRIP-1, at a concentration which completely inhibited thrombin-induced platelet aggregation, had no inhibitory effect on platelet aggregation induced by the tethered ligand peptide. Figure 3 shows that TRIP-1 was specific for inhibiting thrombin-induced platelet aggregation and had no effect on platelets induced by ADP or collagen.

EXAMPLE 18

Antagonist-Thrombin Receptor Binding Assays

The ability of an antagonist of this invention to bind to the hirudin-like anionic domain of the thrombin receptor was assayed by first labelling the antagonist with ^{125}I. The labelled antagonist was then incubated with cells containing the thrombin receptor, such as platelets or smooth muscle cells. The thrombin receptor-containing cells may be maintained in either a suspension culture or grown adherent on plastic or another suitable substrate. Binding of the antagonist to the anionic domain of the thrombin receptor was demonstrated by (1) specific binding of the labelled antagonist to the cells; and (2) significantly decreased binding of the labelled

antagonist to the same cells when incubated in the presence of an unlabelled peptide corresponding to the anionic domain of the thrombin receptor (amino acids 49-62; "thrombin receptor peptide").

Alternatively, the thrombin receptor peptide may be cross-linked to an insoluble support, such as CNBr-activated Sepharose. This complex may then serve as an affinity matrix in a column for the binding of an ^{125}I-labelled antagonist. Specific binding of an antagonist of this invention to such a matrix (and therefore to the anionic domain of the thrombin receptor) was confirmed by adsorbance to the column. The antagonist will then be specifically eluted from the column with soluble, unlabelled thrombin receptor peptide.

EXAMPLE 19

Effect Of Cyclization Of TRIP-4 And -5 On Inhibition Of Platelet Aggregation

We determined the effect that cyclization had on the platelet aggregation inhibitory properties of TRIP-4 and -5 in an assay similar to that described in Example 17. For this assay, we used a constant amount of thrombin (0.06 U/ml) and varying concentrations of linear or oxidized (cyclized) TRIP-4 and -5 (0 - 1 mM). The results were then compared to those obtained for TRIP-1 under the same conditions. Figure 4 graphically demonstrates the results of this experiment. The table below gives the estimated IC_{50} (concentration of TRIP at which 50% inhibition of platelet aggregation was achieved) values for each of these antagonists.

Antagonist	Estimated IC_{50} (μM)
TRIP-1	300
TRIP-4 (linear)	8
TRIP-4 (cyclized)	200
TRIP-5 (linear)	20
TRIP-5 (cyclized)	150

This table demonstrates that cyclization of TRIP-4 and -5 resulted, respectively, in a 25-fold and 7.5-fold decrease in inhibitory potency.

EXAMPLE 20

Glycine And Alanine Walks Of TRIP-6

We synthesized a series of peptides containing a single alanine or a single glycine substitution at each position of TRIP-6 by the peptide synthesis methods described above. Following purification of the peptides, we measured the ability of each peptide to inhibit platelet aggregation and compared that inhibition to TRIP-6. The results of these alanine and glycine walks are depicted in Figure 5.

This experiment indicated that the C-terminal 10 amino acids of TRIP-6 could be replaced with either glycine or alanine (or, at some position with both) without decreasing inhibition to less than 50% of that observed for TRIP-6. This suggested that these amino acids could be deleted from the molecule without destroying inhibitory properties. Moreover, smaller peptides are more desirable for therapeutic uses due to decreased antigenicity.

EXAMPLE 21

Synthesis Of TRIP-16

TRIP-16 has the formula: Leu-Val-Arg-D-Cys-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn and was synthesized as described above.

EXAMPLE 22

Synthesis Of TRIP-17

TRIP 17 has the formula: SEQ ID NO:14: Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn and was synthesized as described above.

EXAMPLE 23

Synthesis Of TRIP-19

TRIP 19 has the formula: SEQ ID NO:15: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg and was synthesized as described above.

EXAMPLE 24

Synthesis Of TRIP-20

TRIP 20 has the formula: Leu-Val-Arg-D-Cys-Gly-Lys-His-Ser-Arg and was synthesized as described above.

EXAMPLE 25

Synthesis Of Alk-TRIP-20

Alk-TRIP 20 has the formula: Leu-Val-Arg-alkCys-Gly-Lys-His-Ser-Arg and was synthesized as described in Example 24. We then incubated 1 mM of the peptide with 1.1 mM iodoacetamide for 30 minutes at room temperature. The alkylated product was isolated by reverse-phase C-18 chromatography. The identity of the product was confirmed by mass spectroscopy as described above and by failure to produce a colored product with Ellman's reagent (G. E. Means and R. E. Feeney, "Chemical Modification of Protein", Holden-Day, Inc., San Francisco, CA, pp. 107, 155).

EXAMPLE 26

Synthesis Of TRIP-21

TRIP 21 has the formula: Leu-Val-Arg-D-Ser-Gly-Lys-His-Ser-Arg and was synthesized as described above.

EXAMPLE 27

Synthesis Of TRIP-22

TRIP 22 has the formula: SEQ ID NO:16: Leu-Val-Arg-Ser-Gly-Lys-His-Ser-Arg and was synthesized as

described above.

EXAMPLE 28

Synthesis Of TRIP-23

TRIP 23 has the formula: SEQ ID NO:17: Leu-Val-Arg-Met-Gly-Lys-His-Ser-Arg and was synthesized as described above.

EXAMPLE 29

Synthesis Of TRIP-24

TRIP 24 has the formula: Leu-Val-Arg-D-Cys-Gly-Lys-Tyr-Ala-Arg and is synthesized as described above.

EXAMPLE 30

Synthesis Of TRIP-25

TRIP 25 has the formula: Leu-Val-Arg-D-Cys-Gly-Leu-His-Phe-Arg and is synthesized as described above.

EXAMPLE 31

Synthesis Of TRIP-26

TRIP 26 has the formula: Val-Val-Arg-D-Cys-Gly-Lys-His-Phe-Arg and is synthesized as described above.

EXAMPLE 32

Synthesis Of TRIP-27

TRIP 27 has the formula: Leu-Val-Arg-D-Cys-Ser-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 33

Synthesis Of TRIP-28

TRIP 28 has the formula: Leu-Val-Arg-D-Cys-Thr-Lys-His-Ser-Arg and is synthesized as described

above.

EXAMPLE 34

Synthesis Of TRIP-29

TRIP 29 has the formula: Leu-Val-Arg-D-Cys-Tyr-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 35

Synthesis Of TRIP-30

TRIP 30 has the formula: Leu-Val-Arg-D-Cys-Asn-Tyr-His-Ser-Arg and is synthesized as described above.

EXAMPLE 36

Synthesis Of TRIP-31

TRIP 31 has the formula: Leu-Val-Arg-D-Cys-Gln-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 37

Synthesis Of TRIP-32

TRIP 32 has the formula: Leu-Val-Arg-D-Cys-Asp-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 38

Synthesis Of TRIP-33

TRIP 33 has the formula: Leu-Val-Arg-D-Cys-Glu-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 39

Synthesis Of TRIP-34

TRIP 34 has the formula: Leu-Val-Arg-D-Cys-Lys-Lys-His-Ser-Arg and is synthesized as described

above.

EXAMPLE 40

Synthesis Of TRIP-35

TRIP 35 has the formula: Leu-Val-Arg-D-Cys-Arg-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 41

Synthesis Of TRIP-36

TRIP 36 has the formula: Leu-Val-Arg-D-Cys-His-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 42

Synthesis Of TRIP-37

TRIP 37 has the formula: Leu-Val-Arg-D-Cys-D-Ala-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 43

Synthesis Of TRIP-38

TRIP 38 has the formula: Leu-Val-Arg-D-Cys-D-Ser-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 44

Synthesis Of TRIP-39

TRIP 39 has the formula: Leu-Val-Arg-D-Cys-D-Thr-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 45

Synthesis Of TRIP-40

TRIP 40 has the formula: Leu-Val-Arg-D-Cys-D-Tyr-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 46

Synthesis Of TRIP-41

TRIP 41 has the formula: Leu-Val-Arg-D-Cys-D-Asn-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 47

Synthesis Of TRIP-42

TRIP 42 has the formula: Leu-Val-Arg-D-Cys-D-Gln-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 48

Synthesis Of TRIP-43

TRIP 43 has the formula: Leu-Val-Arg-D-Cys-D-Asp-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 49

Synthesis Of TRIP-44

TRIP 44 has the formula: Leu-Val-Arg-D-Cys-D-Glu-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 50

Synthesis Of TRIP-45

TRIP 45 has the formula: Leu-Val-Arg-D-Cys-D-Lys-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 51

Synthesis Of TRIP-46

TRIP 46 has the formula: Leu-Val-Arg-D-Cys-D-Arg-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 52

Synthesis Of TRIP-47

TRIP 47 has the formula: Leu-Val-Arg-D-Cys-D-His-Lys-His-Ser-Arg and is synthesized as described above.

EXAMPLE 53

Synthesis Of TRIP-48

TRIP 48 has the formula: D-Cys-Arg-Leu-Ser-Val-His-Arg-Lys-Gly and is synthesized as described above.

EXAMPLE 54

Synthesis Of TRIP-49

TRIP 49 has the formula: SEQ ID NO:18: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Lys-Thr-Arg-Tyr-Glu-Lys-Asn and is synthesized as described above.

EXAMPLE 55

Synthesis Of TRIP-50

TRIP 50 has the formula: SEQ ID NO:19: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-His-Thr-Arg-Tyr-Glu-His-Asn and is synthesized as described above.

EXAMPLE 56

Synthesis Of TRIP-51

TRIP 51 has the formula: SEQ ID NO:20: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Ala-Thr-Arg-Tyr-Glu-Ala-Asn and is synthesized as described above.

EXAMPLE 57

Synthesis Of TRIP-52

TRIP 52 has the formula: SEQ ID NO:21: Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Cys-Tyr-Glu-Arg-Asn and is synthesized as described above.

EXAMPLE 58

Platelet Aggregation Assay Of Other TRIPs

We assayed the ability of TRIP-2 through TRIP-21 (excluding TRIP-9) to inhibit thrombin-induced platelet aggregation in the assay described in Example 17. The results of these assays are set forth in the table below.

TRIP	IC_{50} (μM)	TRIP	IC_{50} (μM)	TRIP	IC_{50}(μM)
1	177	7	300	15	>400
2	250	8	150	16	20
2 ox	500	10	500	17	600
3	320	11	300	19	700
4	31	11 ox	>400	20	30
4 ox	200	12	20	alk-20	400
5	20	12 ox	140	21	>700
5 ox	150	13	200		
6	202	14	1500		

This table confirms that cyclization of TRIP-2, -4, -5, -11 and -12 actually decreased inhibitory potency. We believe that this decrease was due to the fact that cyclization of these molecules resulted in less favorable configuration and therefore reduced ability to bind to the thrombin receptor. However, it should be noted that the cyclic TRIPs did retain significant inhibitory activity and are useful as thrombin receptor antagonists. TRIP-19 demonstrates that removal of the 10 C-terminal amino acids of TRIP-6 results in an inhibitor that is only 3-4 fold less potent. Replacement of the Ile residue in TRIP-19 with a D-Cys (TRIP-20) produces a greater than 20-fold

increase in inhibitory activity and results in a molecule that is also 6-fold more potent than the longer TRIP-6. This confirms the relative unimportance of thrombin amino acids 74-83 in binding to the thrombin receptor.

Further investigation into the increased potency of TRIP-20 over TRIP-19 was carried out by alkylation of the D-Cys residue in the former inhibitor with iodoacetamide (alk-TRIP-20; Example 25), or by replacement of D-cysteine with D-serine (TRIP 21; Example 26). Both of these latter molecules demonstrated an IC_{50} greater than one order of magnitude higher than TRIP-20 and equivalent to the potency of TRIP-19.

EXAMPLE 59

TRIPs Do Not Inhibit Thrombin's Catalytic Activity

We assayed the ability of TRIP-6 to inhibit thrombin-catalyzed cleavage of Spectrozyme TH (tosyl-Gly-Pro-Arg-p-nitroanilide; American Diagnostica, New York, NY). Specifically, we measured the initial rate velocities in the presence or absence of TRIP-6 (0, 200 μM, 400 μM) at a substrate concentrations of 2.5 to 25 μM. The thrombin-catalyzed rate was monitored in a Cary 19 spectrophotometer at 405 nm and recorded continuously as a function of time. Kinetics were performed at room temperature (25 ± 1°C) in a 0.05 M Tris, pH 7.5, 0.1 M NaCl buffer.

For a typical enzyme reaction, 1.0 ml of buffer was added to both the sample and reference cuvettes. Thrombin (3.2×10^{-9} M, final concentration) and TRIP-6 ($0 - 4 \times 10^{-8}$ M) or HIRULOG™ (a known inhibitor of thrombin's catalytic activity) were added to the sample cuvette prior to addition of Spectrozyme TH. Immediately following addition of substrate, the contents of the sample cuvette were mixed by use of a

plastic pipette. The reaction was monitored spectrophotometrically for 5 - 15 minutes. Kinetic parameters were calculated from double-reciprocal plots of initial velocity data.

The results of this assay demonstrated that neither concentration of TRIP-6 inhibits thrombin's cleavage of Spectrozyme TH. Other thrombin receptor antagonists of this invention also fail to inhibit thrombin's catalytic activity.

EXAMPLE 60

TRIPs Do Not Inhibit Thrombin Polymerization of Fibrin

The ability of the thrombin receptors of this invention to inhibit thrombin's cleavage of fibrinogen to form fibrin was measured indirectly in a turbidimetric assay for fibrin polymerization. Thrombin (0.11 U/ml) was added to a human fibrinogen solution (1 mg/ml) at 25°C in 50 mM Tris, 100 mM NaCl, pH 8 containing buffer (control), TRIP-6 (100 μM), or HIRULOG™ (9 nM). Onset of change in turbidity ("onset time") was recorded in a 96-well plate reader (Thermomax, Molecular Devices, Menlo Park, CA) at 340 nm. The results are depicted below.

	Onset Time	% of Control
Control	240 ± 42	----
+ Hirulog (9 nM)	758 ± 16	315.8
+ TRIP-6 (100 μM)	217 ± 19	90.4

These results demonstrate that TRIP-6 does not inhibit thrombin's catalytic formation of fibrin. Other thrombin receptor antagonists of this invention demonstrate similar results.

EXAMPLE 61

Inhibition Of Other
Thrombin-Induced Cellular Functions

In general, inhibition of thrombin-induced cellular functions is assayed by pre-incubating target cells with varying concentrations of a thrombin receptor antagonist of this invention (0 - 1 mM) for 5-30 minutes. We then add thrombin to these cells for 5-30 minutes to stimulate a given cellular response. The cells and/or media are then collected and assayed for a known thrombin-induced component. Samples incubated with TRIP-1 are compared to control samples (no inhibitor) to quantify inhibition.

Thrombin is known to induce increased neutrophil adhesiveness in endothelial cells. To determine the inhibitory effect of the antagonist on this thrombin-induced function, platelet-activating factor (PAF) generation and cell surface expression of GMP-140 are analyzed [S. Prescott et al., Proc. Natl. Acad. Sci. USA, 81, pp. 3534-38 (1984)].

Polymorphonuclear leukocytes (PMNs) are prepared by centrifuging citrated whole blood (see Example 17) through a commercially available ficoll gradient (Pharmacia, Piscataway, NJ). Specifically, 30 ml of whole blood is slowly pipetted down the side of the tube containing the gradient, being careful not to disturb the interface. The gradient is centrifuged for 40 minutes at 1600 rpm at 22°C. The pellet containing the PMNs is resuspended in 30 ml of white blood cell media (prepared fresh weekly) containing 25 mM HEPES, pH 7.3, with 0.5% bovine serum albumin in HBSS (without calcium and magnesium). The PMNs are then labelled for 30 minutes at 37°C by the addition of 3.0 ml of BCECF-AM (2′,7′-bis-(2-carboxyethyl)-5(and -6)-carboxyfluorescein acetoxymethyl ester) (Molecular Probes, Inc., Eugene, OR). The cells are then diluted

by the addition of 5 volumes of white blood cell media.

Human umbilical vein endothelial cells (HUVECs) are grown in 48-well plates for 3 days until reaching confluency. The HUVECs are then washed with HBSS, followed by the addition of 0 - 1 mM thrombin receptor antagonist. The cells are then incubated at 37°C for 10-30 minutes. The PMN suspension (0.5 ml) is equilibrated to 37°C, added to HUVEC culture and allowed to settle for 2 minutes. Thrombin (0 - 1 U/ml) is then added to the cell mixture which is allowed to incubate for an additional 10 minutes at 37°C. The wells are washed several times with HBSS to remove non-adherent PMNs. The wells are next treated with 0.3 ml of 1% NP-40 and the contents of each well are transferred to a separate 96-well plate. Fluorescence measurements are carried out on the NP-40-treated material using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Fluorescence is proportional to the number of PMNs that adhere to the HUVEC monolayer. Pre-incubation with thrombin receptor antagonist reduces the mean fluorescence as compared to control wells containing thrombin, but no inhibitor.

PAF generation and cell surface expression of GMP-140 are assayed as described previously [S. Prescott et al., Proc. Natl. Acad. Sci. USA, 81, pp. 3534-38 (1984)].

EXAMPLE 62

TRIPs Inhibit Proliferation Of Smooth Muscle Cells In Culture.

To determine the inhibitory effect of the antagonists of this invention on smooth muscle cells, we cultured rat vascular smooth muscle cells (A10, rat smooth muscle embryonic thoracic aorta; ATCC 1476-CRL. Batch #F9432) overnight in 96 well microtiter plates (20,000 cells/well) in DMEM supplemented with 5% bovine

serum, 4 mM glutamine, and 100 units Penicillin/streptomycin. After 24 hours, we replaced the media with serum free media containing various amounts of TRIP-6 or TRIP-20. Incubation was continued for 1 hour at 37°C. We then added thrombin (1 nM) to the cells. Approximately 40 hours later, the cells were pulsed with ^{3}H-thymidine (21 mCi/mg, 1 μCi/well) and harvested after an additional 8 hours of growth. Figure 6 shows that thrombin receptor antagonists of this invention exhibit a dose-dependent inhibition of ^{3}H-thymidine incorporation into smooth muscle cells. This confirms that these thrombin receptor antagonists inhibit the thrombin receptor on the smooth muscle cell surface.

EXAMPLE 63

Construction of an ErabutoxinA/TRIP Fusion DNA

The erabutoxinA-TRIP fusion protein was designed to contain the following structural features, listed in N- to C-terminal order: the signal peptide from phage M13 PIII protein; amino acids 1-24 of erabutoxin-A; a sequence corresponding to amino acids 3-18 of TRIP-6 (amino acids 3-18 of SEQ ID NO:4); erabutoxin-A amino acids 30-57, a linker of two alanine residues; a sequence recognized by the anti-Cmyc antibody 9E10; a sequence encoding a Factor Xa cleavage site; and the full length sequence of phage M13 PIII protein, starting at the first residue of the mature molecule.

The ErabutoxinA/TRIP fusion gene was constructed as a synthetic gene from oligonucleotide fragments shown below. All oligonucleotides were synthesized using an Applied Biosystems Model 394 oligonucleotide synthesizer (Applied Biosystems, Foster City, CA).

27: [SEQ ID NO:22] CGT ATC TGC TTC AAC CAC CAG TCC

TCC CAG CCG CAG ACC ACC AAA ACC TGC TCC CCG GGT;

28: [SEQ ID NO:23] GAA TCC TCC TGC TAC AAC AAA CAG TGG CGT ATC GGT AAA CAC TCC CGT ACC CGT TAC GAA;

29: [SEQ ID NO:24] CGT AAC ATC GAA AAA ACC ATC ATC GAA CGT GGT TGC GGT TGC CCG ACC GTT AAA CCG GGT;

30: [SEQ ID NO:25] ATC AAA CTG TCC TGC TGC GAA TCC GAA GTT TGC AAC AAC GC;

31: [SEQ ID NO:26] CAG GTT TTG GTG GTC TGC GGC TGG GAG GAC TGG TGG TTG AAG CAG ATA CG;

32: [SEQ ID NO:27] GTA CGG GAG TGT TTA CCG ATG GAC CAC TGT TTG TTG TAG CAG GAG GAT TCA CCC GGG GAG;

33: [SEQ ID NO:28] ACG GTC GGG CAA CCG CAA CCA CGT TCG ATG ATG GTT TTT TCG ATG TTA CGT TCG TAA CGG; and

34: [SEQ ID NO:29] GGC CGC GTT GTT GCA AAC TTC GGA TTC GCA GCA GGA CAG TTT GAT ACC CGG TTT A.

The above oligonucleotides were kinased, annealed, and ligated using standard molecular biological techniques. Oligonucleotides 30 and 34 also introduced a Not1 restriction site into the gene. Oligonucleotides 27 and 31 create blunt ends. Once synthesized, this synthetic gene was introduced into a modified PIII gene construct in the commercially available phagemid vector pTZ19R (Pharmacia) by digesting the PIII-pTZ19R vector with Eco47III and Not1 and ligating to the ErabutoxinA-TRIP syngene. The construct was verified by DNA sequencing. The complete DNA sequence of this vector [SEQ ID NO:30] is shown in Figure 7.

The key features of the final construct, in 5′ to 3′ order, are the PIII signal sequence (nucleotides 2665-2733), the first 24 amino acids of erabutoxinA (nucleotides 2734-2820), the TRIP sequence (nucleotides 2821-2868), amino acids 30-57 of erabutoxinA (nucleotides 2869-2952), an Ala-Ala linker (nucleotides 2953-2958), a region coding for an epitope

recognized by the anti-Cmyc antibody 9E10 (nucleotides 2959-2987), a region coding for a Factor Xa cleavage site (nucleotides 2988-3015) and the PIII protein coding sequence (nucleotides 3016-4233). This vector also contained a HindIII site (nucleotide 2657) and a NotI site (nucleotide 2955).

The sequence of the predicted protein encoded by this construct begins with the PIII signal sequence Leu-Glu-Ile-Phe-Asn and ends with Arg-Asn-Lys-Glu-Ser. When expressed and processed by bacteria, the signal peptide sequence was presumed to be removed, creating a new N-terminus which was residue 1 of erabutoxin-A. As a consequence of the oligonucleotides used, amino acids 25-29 (Ser-Asp-Phe-Arg-Gly) of erabutoxin-A is replaced by the TRIP sequence.

EXAMPLE 64

Expression of the ErabutoxinA/TRIP Fusion Protein on Phage

Competent DH5αF'1q E. coli cells were transformed with the ligated pTZ19R/Erabutoxin-A-TRIP/PIII construct and plated on ampicillin (50 μg/ml) LB plates. After colonies appeared, a single colony was picked and used to inoculate 1.5 ml of 2xYT media containing 100 μg AMP and 2% glucose. The transformant were grown with vigorous shaking for 4 to 6 hours at 37°C until reaching an OD_{600} of about 0.9. The cells were then centrifuged and the cell pellet was resuspended in 6 ml 2xYT together with 1 x 10^{11} colony forming units (CFU) of phage M13KO7 and incubated on ice for 5-10 min. The infected cells were transferee to a 250 ml flask and grown with vigorous shaking at 30°C for 1 hr. Phage production was stimulated by adding IPTG (10 μM), ampicillin (80 μg/ml) and kanamycin (83 μg/ml). Cultures were grown for 14-18 hours at 37°C. We then centrifuged the cells and

titered the supernatant for phage. Phage prepared in this manner may be stored at 4°C.

EXAMPLE 65

Modification of the TRIP Sequence In ErabutoxinA/TRIP Fusion Proteins

The PCR method of splicing by overlap extension [R. M. Horton et al., Gene, 77, pp. 61-68 (1989), the disclosure of which is herein incorporated by reference] was used to generate modifications of the TRIP sequence in the erabutoxinA/TRIP fusion proteins.

The 5' segment of this modified construct was made by amplifying pTZ19R/erabutoxinA-TRIP with oligonucleotides ST11 and SRP24. Three different 3' segments were made using oligonucleotide SRP10A and one of oligonucleotides ST8, ST9 or ST10 to make each of three variants in the TRIP sequence. Following PCR, the 5' and 3' segments were isolated on an agarose gel, and recovered using the Gene Clean Kit (Bio 101, San Diego, CA). These overlapping segments were then joined by the splice overlap extension method using the oligonucleotides SRP24 and SRP10A. The resulting fragments were isolated as described above, then digested with HindIII and Not1 and cloned back into the parent vector. The sequences of the above-described oligonucleotides are:

ST11:[SEQ ID NO:31] GGA GTG TTT ACC GAT ACG CCA CTG TTT GTT;

ST8:[SEQ ID NO:32] AAA CAC TCC GCT ACC CGT TAC GAA GCT AAC;

ST9:[SEQ ID NO:33] AAA CAC TCC CAT ACC CGT TAC GAA CAT ACC;

ST10:[SEQ ID NO:34] AAA CAC TCC AAA ACC CGT TAC GAA AAA AAC;

SRP24:[SEQ ID NO:35] CTC TAA AGC TTT TTT TGG AGA TTT

TCA ACG TGA AAA AAT T; and
SRP10A:[SEQ ID NO:36] CTT TCA ACA GTT TCT GCG GCC CCA CGT CCT TCG ATC CCA TTC AGA TCC TCT TCT GAG AT.

We confirmed the successful construction of the desired products by DNA sequencing. The resulting fusion proteins contained modified TRIP sequences characterized by an Arg-to-Lys, and Arg-to-His or an Arg-to-Ala double substitution at amino acids 9 and 14 of TRIP-6 [SEQ ID NO:4]. In addition to the above modified TRIP sequences, a mutational event also produced an additional Arg-to-Cys substitution at amino acid 11 of the Arg-to-Lys double substituted TRIP sequence.

EXAMPLE 66

Thrombin Receptor Binding By ErabutoxinA/TRIP Fusion Proteins

Phage expressing each of the ErabutoxinA/TRIP fusion proteins described in Example 65 on their surface were prepared as described in Example 64 and then concentrated by adding 1/5 volume of 20% PEG 8000, incubating overnight at 4°C, then centrifuging at 15,000xg for 15 minutes. The phage pellet was resuspended in 100 μl of Tris buffered saline (TBS, 100 mM TRIS, 150 mM NaCl). Titers of the concentrated phage ranged between 2 - 9 x 10^{12} CFU/ml. A peptide, Asn-Asp-Lys-Tyr-Glu-Pro-Phe-Trp-Glu-Asp-Glu-Glu-Lys-Asn-Glu-Ser-Gly-Gly-Gly-Cys [SEQ ID NO:37], corresponding to the hirudin like region of the thrombin receptor was coupled to bovine serum albumin ("BSA") at a ratio of 2 mg peptide:5 mg BSA by first reacting the 5 mg of BSA with 0.25 mg SPDP (Pierce Chemical Company, Rockford Il) for 1 hr at room temperature then adding the peptide and incubating an additional hour.

Plastic microtiter plates were coated with 2

μg/ml of thrombin receptor peptide-BSA conjugate in 0.1 M NaH_2CO_3 pH 9.6 for 1 hr, then blocked with 5% BSA in TBS. After washing the plate three times with 0.1% TWEEN-20 in TBS (TBS-Tween), varied concentrations of phage diluted in TBS-Tween were added. After 1 hour, the plate was washed and polyclonal rabbit anti-M13 antibody (1:10,000) was added. After washing, bound rabbit antibody was visualized and quantitated by reaction with horseradish peroxidase conjugated goat anti-rabbit antibody (1:1000) and subsequent development with the chromogenic substrate O-phenylenediamine.

The results of the above experiment are shown in Figure 8 for varied amounts of each of the recombinant phage, as well as for a control phage. These results demonstrated a dose-dependent increase in phage binding (as indicated by an increase in OD_{490}) for phage expressing the erabutoxinA-TRIP construct with the unsubstituted sequence and for the constructs in which the arginines at positions 9 and 14 have been changed to lysine or histidine. Substitution of arginine at position 11 with cysteine produces a fusion protein that is unable to bind. Likewise, wild type phage without the erabutoxinA/TRIP fusion fail to bind to the thrombin receptor derived peptide.

While we have hereinbefore presented a number of embodiments of this invention, it is apparent that our basic construction can be altered to provide other embodiments which utilize the processes and products of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than the specific embodiments which have been presented hereinbefore by way of example.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Biogen, Inc.
Maraganore, John M
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(ii) TITLE OF INVENTION: THROMBIN RECEPTOR ANTAGONISTS

(iii) NUMBER OF SEQUENCES: 38

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/847,561
(B) FILING DATE: 02-MAR-1992

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Val Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn Ile
1 5 10 15

Glu Lys Ile Ser
20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Val Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn Ile
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Cys Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Leu Val Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn Ile
1 5 10 15

Glu Lys Ile

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /note= "residue 6 is ornithine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Leu Val Arg Ile Gly Xaa His Ser Arg Thr Arg Tyr Glu Arg Asn Ile
1 5 10 15

Glu Lys Ile

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Val Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Asp Arg Asn Ile
1 5 10 15

Glu Lys Ile

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site

(B) LOCATION: 3
(D) OTHER INFORMATION: /note= "Xaa is beta-homoarginine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Val Xaa Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn Ile
1 5 10 15

Glu Lys Ile

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 19
(D) OTHER INFORMATION: /note= "Xaa is cyclohexylalanine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Val Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn Ile
1 5 10 15

Glu Lys Xaa Gly
20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Xaa is an N-terminal mercaptopropionic acid"

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 1..19
(D) OTHER INFORMATION: /note= "the N-terminal mercaptopropionic acid is optionally disulfide bonded to the C-terminal cysteine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Xaa Val Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn Ile
1 5 10 15

Glu Lys Cys

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Xaa at position 1 is mercaptopropionic acid"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 2
(D) OTHER INFORMATION: /note= "Xaa at position 2 is cyclohexylalanine"

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /note= "The N-terminal mercaptopropionic acid residue is optionally disulfide bonded to the C-terminal cysteine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Xaa Xaa Val Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn
1 5 10 15

Ile Glu Lys Cys
20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 10
(D) OTHER INFORMATION: /note= "Xaa is cyclohexylalanine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Val Arg Ile Gly Lys His Ser Arg Xaa Arg Tyr Glu Arg Asn Ile
1 5 10 15

Glu Lys Ile

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 12
(D) OTHER INFORMATION: /note= "Tyrosine is diiodinated at the 3- and 5- positions"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Leu Val Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn Ile
1 5 10 15

Glu Lys Ile

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Xaa is guanadinobenzoic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Xaa Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn Ile Glu Lys
1 5 10 15

Ile

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Arg Ile Gly Lys His Ser Arg Thr Arg Tyr Glu Arg Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Leu Val Arg Ile Gly Lys His Ser Arg
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Leu Val Arg Ser Gly Lys His Ser Arg
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Leu Val Arg Met Gly Lys His Ser Arg
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Leu Val Arg Ile Gly Lys His Ser Lys Thr Arg Tyr Glu Lys Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu Val Arg Ile Gly Lys His Ser His Thr Arg Tyr Glu His Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Val Arg Ile Gly Lys His Ser Ala Thr Arg Tyr Glu Ala Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Leu Val Arg Ile Gly Lys His Ser Arg Thr Cys Tyr Glu Arg Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGTATCTGCT TCAACCACCA GTCCTCCCAG CCGCAGACCA CCAAAACCTG CTCCCCGGGT 60

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GAATCCTCCT GCTACAACAA ACAGTGGCGT ATCGGTAAAC ACTCCCGTAC CCGTTACGAA 60

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CGTAACATCG AAAAAACCAT CATCGAACGT GGTTGCGGTT GCCCGACCGT TAAACCGGGT 60

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATCAAACTGT CCTGCTGCGA ATCCGAAGTT TGCAACAACG C 41

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CAGGTTTTGG TGGTCTGCGG CTGGGAGGAC TGGTGGTTGA AGCAGATACG 50

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTACGGGAGT GTTTACCGAT GGACCACTGT TTGTTGTAGC AGGAGGATTC ACCCGGGGAG 60

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ACGGTCGGGC AACCGCAACC ACGTTCGATG ATGGTTTTTT CGATGTTACG TTCGTAACGG 60

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGCCGCGTTG TTGCAAACTT CGGATTCGCA GCAGGACAGT TTGATACCCG GTTTA 55

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4403 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 2665..2733

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AAATTGTAAA CGTTAATATT TTGTTAATAT TTTGTTAAAA TTCGCGTTAA ATTTTTGTTA 60

AATCAGCTCA TTTTTTAACC AATAGGCCGA AATCGGCAAA ATCCCTTATA AATCAAAAGA 120

ATAGACCGAG ATAGGGTTGA GTGTTGTTCC AGTTTGGAAC AAGAGTCCAC TATTAAAGAA 180

CGTGGACTCC AACGTCAAAG GGCGAAAAAC CGTCTATCAG GGCGATGGCC CACTACGTGA 240

ACCATCACCC TAATCAAGTT TTTTGGGGTC GAGGTGCCGT AAAGCACTAA ATCGGAACCC 300

TAAAGGGATG CCCCGATTTA GAGCTTGACG GGGAAAGCCG GCGAACGTGG CGAGAAAGGA 360

AGGGAAGAAA GCGAAAGGAG CGGGCGCTAG GGCGCTGGCA AGTGTAGCGG TCACGCTGCG 420

CGTAACCACC ACACCCGCCG CGCTTAATGC GCCGCTACAG GGCGCGTCAG GTGGCACTTT 480

TCGGGGAAAT GTGCGCGGAA CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA 540

TCCGCTCATG AGACAATAAC CCTGATAAAT GCTTCAATAA TATTGAAAAA GGAAGAGTAT 600

GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT GCCTTCCTGT 660

TTTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT GAAGATCAGT TGGGTGCACG 720

AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT TTCGCCCCGA 780

AGAACGTTTT CCAATGATGA GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG 840

TATTGACGCC GGGCAAGAGC AACTCGGTCG CCGCATACAC TATTCTCAGA ATGACTTGGT 900

TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA GAGAATTATG 960

CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC TTACTTCTGA CAACGATCGG 1020

AGGACCGAAG GAGCTAACCG CTTTTTTGCA CAACATGGGG GATCATGTAA CTCGCCTTGA 1080

TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC GAGCGTGACA CCACGATGCC 1140

TGTAGCAATG GCAACAACGT TGCGCAAACT ATTAACTGGC GAACTACTTA CTCTAGCTTC 1200

CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC TTCTGCGCTC 1260

GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA GCCGGTGAGC GTGGGTCTCG 1320

CGGTATCATT GCAGCACTGG GGCCAGATGG TAAGCCCTCC CGTATCGTAG TTATCTACAC 1380

GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC 1440

ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT AGATTGATTT 1500

AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA ATCTCATGAC 1560

CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA GACCCCGTAG AAAAGATCAA 1620

AGGATCTTCT TGAGATCCTT TTTTTCTGCG CGTAATCTGC TGCTTGCAAA CAAAAAAACC 1680

ACCGCTACCA GCGGTGGTTT GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT 1740

AACTGGCTTC AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC CGTAGTTAGG 1800

CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA TCCTGTTACC 1860

AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG TTGGACTCAA GACGATAGTT 1920

ACCGGATAAG GCGCAGCGGT CGGGCTGAAC GGGGGGTTCG TGCACACAGC CCAGCTTGGA 1980

GCGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT 2040

TCCCGAAGGG AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG 2100

CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG GGTTTCGCCA 2160

CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG GGGCGGAGCC TATGGAAAAA 2220

CGCCAGCAAC GCGGCCTTTT TACGGTTCCT GGCCTTTTGC TGGCCTTTTG CTCACATGTT 2280

CTTTCCTGCG TTATCCCCTG ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA 2340

TACCGCTCGC CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG AAGCGGAAGA 2400

GCGCCCAATA CGCAAACCGC CTCTCCCCGC GCGTTGGCCG ATTCATTAAT GCAGCTGGCA 2460

CGACAGGTTT CCCGACTGGA AAGCGGGCAG TGAGCGCAAC GCAATTAATG TGAGTTAGCT 2520

CACTCATTAG GCACCCCAGG CTTTACACTT TATGCTTCCG GCTCGTATGT TGTGTGGAAT 2580

TGTGAGCGGA TAACAATTTC ACACAGGAAA CAGCTATGAC CATGATTACG AATTTAATAC 2640

GACTCACTAT AGGGAAAGCT TTTTTTGGAG ATTTTCAACG TGAAAAAATT ATTATTCGCA 2700

ATTCCTTTAG TTGTTCCTTT CTATTCTCAC AGCCGTATCT GCTTCAACCA CCAGTCCTCC 2760

CAGCCGCAGA CCACCAAAAC CTGCTCCCCG GGTGAATCCT CCTGCTACAA CAAACAGTGG 2820

CGTATCGGTA AACACTCCCG TACCCGTTAC GAACGTAACA TCGAAAAAAC CATCATCGAA 2880

CGTGGTTGCG GTTGCCCGAC CGTTAAACCG GGTATCAAAC TGTCCTGCTG CGAATCCGAA 2940

GTTTGCAACA ACGCGGCCGC AGAACAAAAA CTCATCTCAG AAGAGGATCT GAATGGGATC 3000

GAAGGACGTG GGGCCGCAGA AACTGTTGAA AGTTGTCTGG CAAAACCTCA TACAGAAAAT 3060

TCATTTACTA ACGTCTGGAA AGACGACAAA ACTTTAGATC GTTACGCTAA CTATGAGGGC 3120

TGTCTGTGGA ATGCTACAGG CGTTGTGGTT TGTACTGGTG ACGAAACTCA GTGTTACGGT 3180

ACATGGGTTC CTATTGGGCT TGCTATCCCT GAAAATGAGG GTGGTGGCTC TGAGGGTGGC 3240

GGTTCTGAGG GTGGCGGTTC TGAGGGTGGC GGTACTAAAC CTCCTGAGTA CGGTGATACA 3300

CCTATTCCGG GCTATACTTA TATCAACCCT CTCGACGGCA CTTATCCGCC TGGTACTGAG 3360

CAAAACCCCG CTAATCCTAA TCCTTCTCTT GAGGAGTCTC AGCCTCTTAA TACTTTCATG 3420

TTTCAGAATA ATAGGTTCCG AAATAGGCAG GGTGCATTAA CTGTTTATAC GGGCACTGTT 3480

ACTCAAGGCA CTGACCCCGT TAAAACTTAT TACCAGTACA CTCCTGTATC ATCAAAAGCC 3540

ATGTATGACG CTTACTGGAA CGGTAAATTC AGAGACTGCG CTTTCCATTC TGGCTTTAAT 3600

GAGGATCCAT TCGTTTGTGA ATATCAAGGC CAATCGTCTG ACCTGCCTCA ACCTCCTGTC 3660

AATGCTGGCG GCGGCTCTGG TGGTGGTTCT GGTGGCGGCT CTGAGGGTGG CGGCTCTGAG 3720

GGTGGCGGTT CTGAGGGTGG CGGCTCTGAG GGTGGCGGTT CCGGTGGCGG CTCCGGTTCC 3780

GGTGATTTTG ATTATGAAAA AATGGCAAAC GCTAATAAGG GGGCTATGAC CGAAAATGCC 3840

GATGAAAACG CGCTACAGTC TGACGCTAAA GGCAAACTTG ATTCTGTCGC TACTGATTAC 3900

GGTGCTGCTA TCGATGGTTT CATTGGTGAC GTTTCCGGCC TTGCTAATGG TAATGGTGCT 3960

ACTGGTGATT TTGCTGGCTC TAATTCCCAA ATGGCTCAAG TCGGTGACGG TGATAATTCA 4020

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CCTTATGTCT TTGGCGCTGG TAAACCATAT GAATTTTCTA TTGATTGTGA CAAAATAAAC 4140

TTATTCCGTG GTGTCTTTGC GTTTCTTTTA TATGTTGCCA CCTTTATGTA TGTATTTTCG 4200

ACGTTTGCTA ACATACTGCG TAATAAGGAG TCTTAATAAG AATTCACTGG CCGTCGTTTT 4260

ACAACGTCGT GACTGGGAAA ACCCTGGCGT TACCCAACTT AATCGCCTTG CAGCACATCC 4320

CCCTTTCGCC AGCTGGCGTA ATAGCGAAGA GGCCCGCACC GATCGCCCTT CCCAACAGTT 4380

GCGCAGCCTG AATGGCGAAT GGG 4403

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGAGTGTTTA CCGATACGCC ACTGTTTGTT 30

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AAACACTCCG CTACCCGTTA CGAAGCTAAC 30

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

AAACACTCCC ATACCCGTTA CGAACATACC 30

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AAACACTCCA AAACCCGTTA CGAAAAAAAC 30

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CTCTAAAGCT TTTTTTGGAG ATTTTCAACG TGAAAAAATT 40

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CTTTCAACAG TTTCTGCGGC CCCACGTCCT TCGATCCCAT TCAGATCCTC TTCTGAGAT 59

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Asn Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu Glu Lys Asn Glu Ser
1 5 10 15

Gly Gly Gly Cys
20

CLAIMS

We claim:

1. A thrombin receptor antagonist comprising the formula:

$Y-A_1-W-X_1-X_2-X_3-X_4-A_2-Z$; wherein

Y is hydrogen, a peptide of from 1 to 10 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 30 backbone atoms;

each of A_1 and A_2, either the same or different, are selected from the group consisting of a positively charged amino acid and an acyl or alkyl chain comprising from 1 to 10 backbone atoms and a positively charged side group;

W and each X is any amino acid, either the same or different; and

Z is OH, a peptide of from 1 to 11 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 33 backbone atoms;

with the proviso that at least one of W, X_1 and X_2 is not a basic amino acid; and wherein

said antagonist is optionally cyclized by a covalent bond between Y and Z.

2. The thrombin receptor antagonist according to claim 2, wherein Y has the formula: $Y_1-B_n-C_m$, wherein

Y_1 is hydrogen, a peptide of from 1 to 6 amino acids, either the same or different, or an acyl or alkyl chain comprising from 1 to 18 backbone atoms;

B is a hydrophobic amino acid;

C is an acidic amino acid, either the same or different;

n is 0 or 1; and

m is an integer from 1 to 3.

3. The thrombin inhibitor according to claim 2, wherein

B, if present, is Asp; and

C_m is selected from Val, Leu-Val, Val-Val, Leu-Leu-Val, Leu-Val-Val, Ile-Leu-Val and Ile-Val-Val.

4. The thrombin receptor antagonist according to claim 1, wherein A_1 is Arg.

5. The thrombin receptor antagonist according to claim 1, wherein A_2 is Arg.

6. The thrombin receptor antagonist according to claim 1, wherein W is Ile, D-Cys or Ala.

7. The thrombin receptor antagonist according to claim 6, wherein W is D-Cys.

8. The thrombin receptor antagonist according to claim 1, wherein X_2 is an anionic amino acid.

9. The thrombin receptor antagonist according to claim 1, wherein X_3 is His.

10. The thrombin receptor antagonist according to claim 2, wherein X_4 is Ser.

11. The thrombin receptor antagonist according to claim 3, wherein

C_m is Leu-Val

A_1 is Arg;

W is D-Cys;

X_2 is Lys or Orn;

X_3 is His;

X_4 is Ser; and

A_2 is Arg.

12. A thrombin receptor antagonist of the formula:

Leu-Val-Arg-(D-Cys)-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Cys-Glu-Lys-Ile.

13. A thrombin receptor antagonist of the formula:

Leu-Val-Arg-Ile-Cys-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-(D-Cys).

14. The thrombin receptor antagonist according to claim 2, consisting of the formula (SEQ ID NO:4) :

Leu-Val-Arg-(D-Cys)-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-(D-Cys)-Glu-Lys-Ile.

15. A thrombin receptor antagonist of the formula:

SEQ ID NO:11: MPR-Cha-Val-Arg-Ile-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn-Ile-Glu-Lys-Cys.

16. A thrombin receptor antagonist of the formula:

Leu-Val-Arg-D-Cys-Gly-Lys-His-Ser-Arg-Thr-Arg-Tyr-Glu-Arg-Asn.

17. A thrombin receptor antagonist of the formula:

Leu-Val-Arg-D-Cys-Gly-Lys-His-Ser-Arg.

18. A composition for inhibiting thrombin receptor-mediated processes comprising a pharmaceutically effective amount of a thrombin

receptor antagonist according to claim 1 and a pharmaceutically acceptable carrier.

19. A method for inhibiting thrombin-induced platelet activation in a patient comprising the step of treating said patient with a composition according to claim 18.

20. A method for inhibiting thrombin-induced smooth muscle cell proliferation in a patient comprising the step of administering to said patient a composition according to claim 18.

21. The method according to claim 20, wherein said composition is administered to said patient through a device inserted into said patient, wherein a portion of said device is characterized by aperture means through which said composition is capable of being passed.

22. The method according to claim 21, wherein said device is a weeping balloon catheter.

23. A method for treating or preventing neurodegenerative disease in a patient comprising the step of administering to said patient a composition according to claim 18.

24. A method for treating or preventing bone resorption in a patient comprising the step of administering to said patient a composition according to claim 18.

25. The method according to claim 24, wherein said patient is suffering from osteoporosis.

26. A method for treating or preventing thrombin-induced inflammation in a patient, said method comprising the step of administering to said patient a composition according to claim 18.

27. The method according to claim 26, wherein said inflammation is caused by a disease selected from the group consisting of adult respiratory distress syndrome, septic shock, septicemia and reperfusion damage.

28. A method for inhibiting platelet-dependent thrombosis in a patient comprising the step of administering to said patient a composition according to claim 18.

29. A fusion protein comprising the formula:

PP_L-T-PP_R; wherein

T is a thrombin receptor antagonist of the formula: Y-A_1-W-X_1-X_2-X_3-X_4-A_2-Z; wherein

Y is hydrogen or a peptide of from 1 to 10 amino acids;

each A is a positively charged amino acid, either the same or different;

W and each X is any amino acid, either the same or different; and

Z is OH or a peptide of from 1 to 11 amino acids;

with the proviso that at least one of W, X_1 and X_2 is not a basic amino acid; and

PP_L and PP_R are both portions of a polypeptide of the formula: PP_L-BL-PP_R; wherein

BL forms a ß-loop structure in said polypeptide; and

each of PP_L and PP_R are characterized by an antiparallel ß-strand structure in said polypeptide.

FIGURE 1

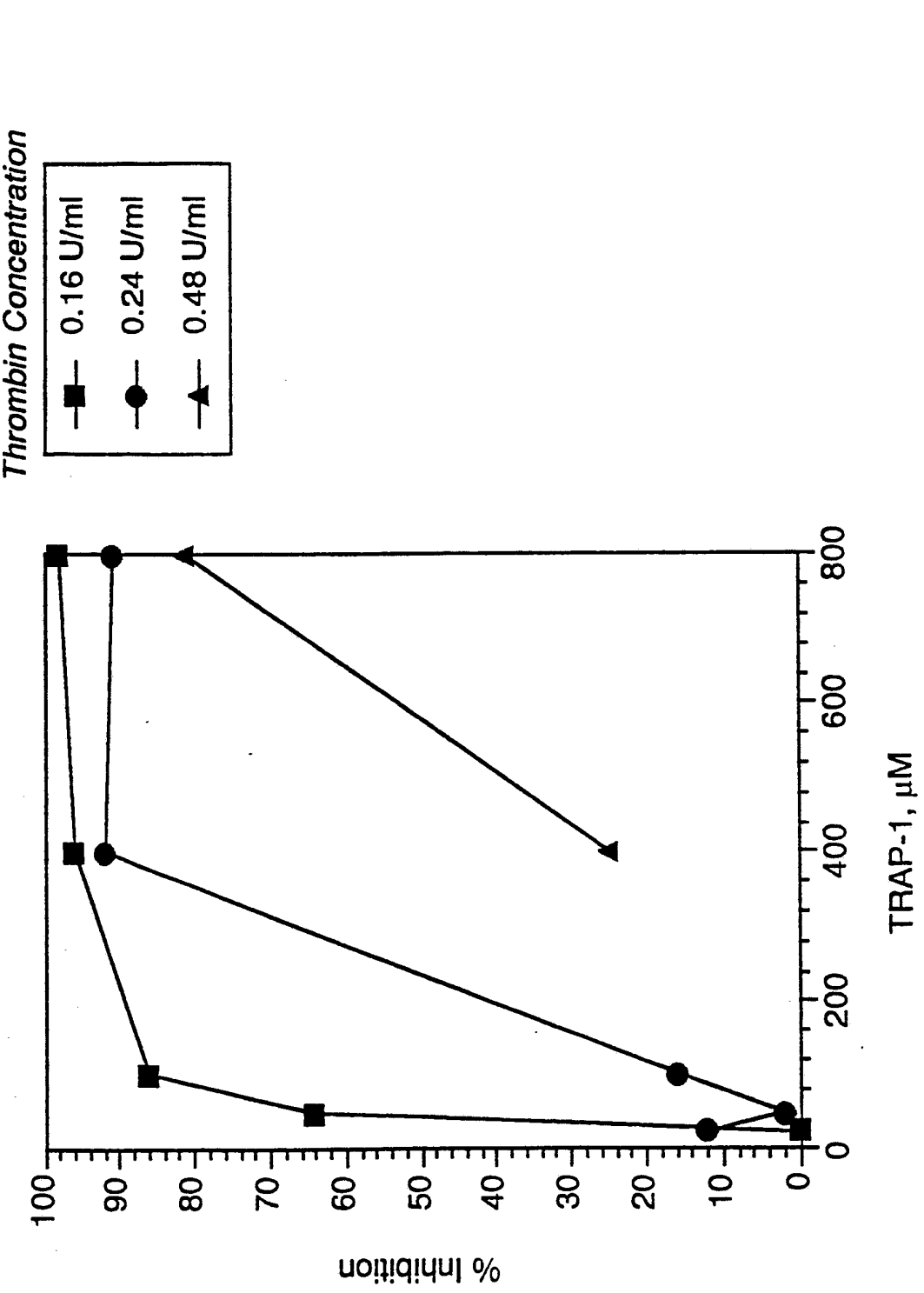
Thrombin Concentration
0.16 U/ml
0.24 U/ml
0.48 U/ml
% Inhibition
100
90
80
70
60
50
40
30
20
10
0
0
200
400
600
800
TRAP-1, µM

FIGURE 2

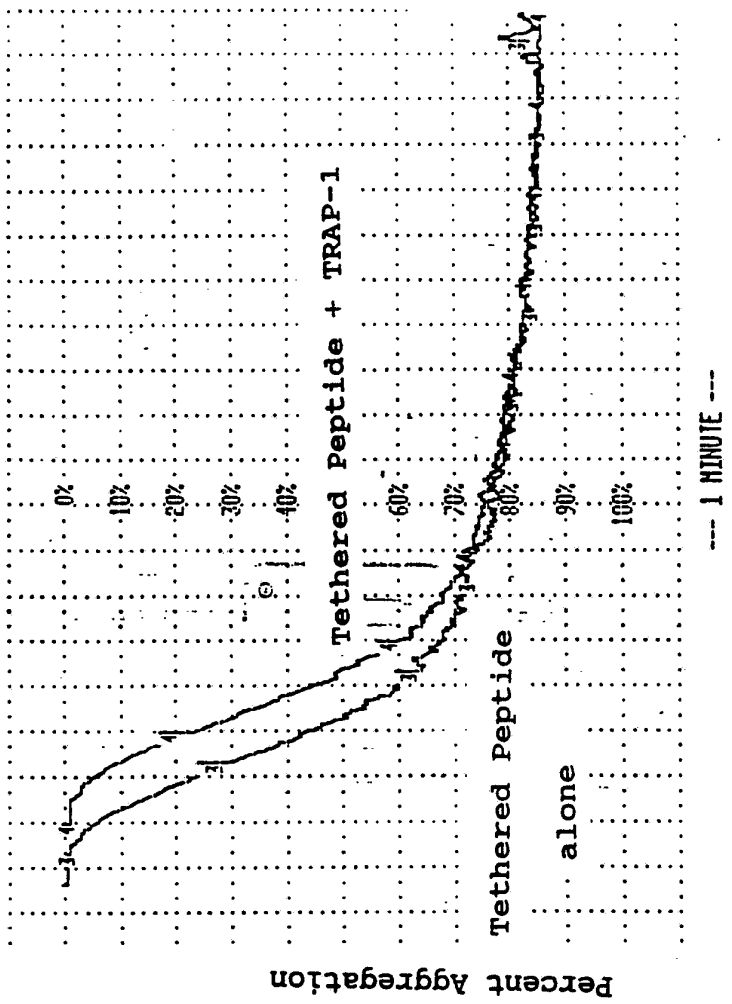
Tethered Peptide + TRAP-1
Tethered Peptide alone
0%
10%
20%
30%
40%
60%
70%
80%
90%
100%
Percent Aggregation
--- 1 MINUTE ---

FIGURE 3

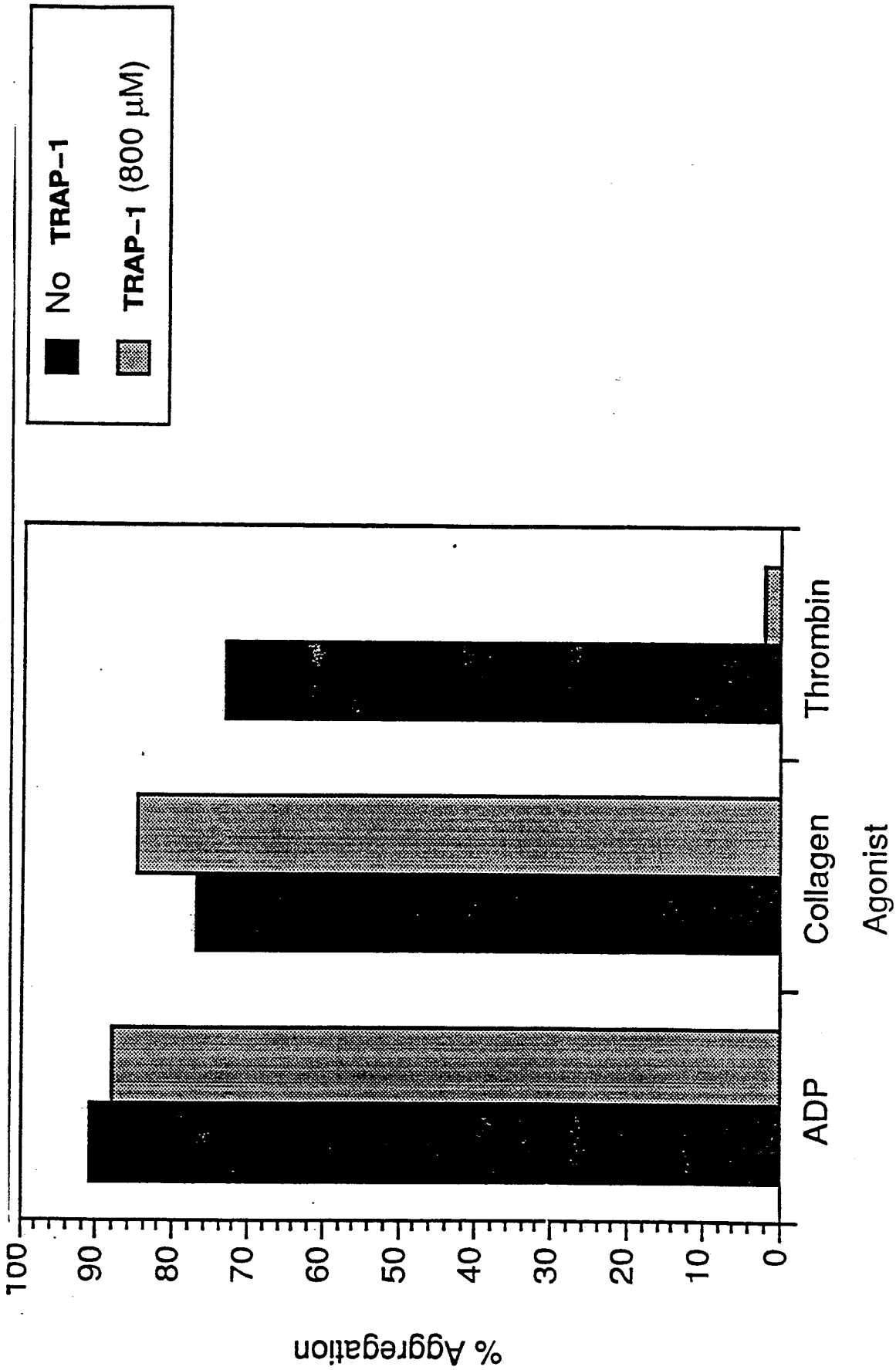
No TRAP-1
TRAP-1 (800 µM)
% Aggregation
100
90
80
70
60
50
40
30
20
10
0
ADP
Collagen
Thrombin
Agonist

FIGURE 4

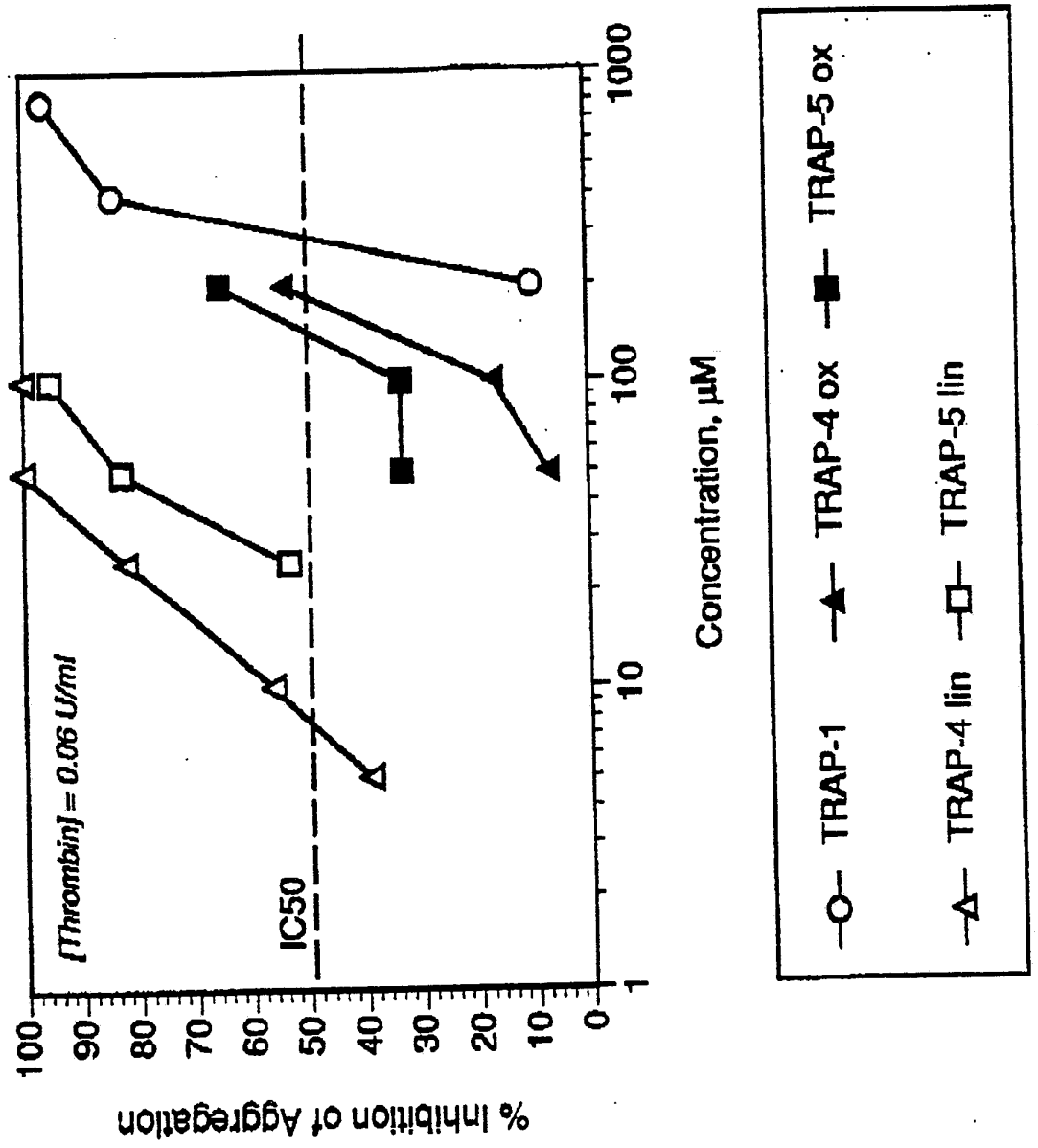
% Inhibition of Aggregation
100
90
80
70
60
50
40
30
20
10
0
1
10
100
1000
Concentration, μM
[Thrombin] = 0.06 U/ml
IC50
TRAP-1
TRAP-4 ox
TRAP-5 ox
TRAP-4 lin
TRAP-5 lin

FIGURE 5

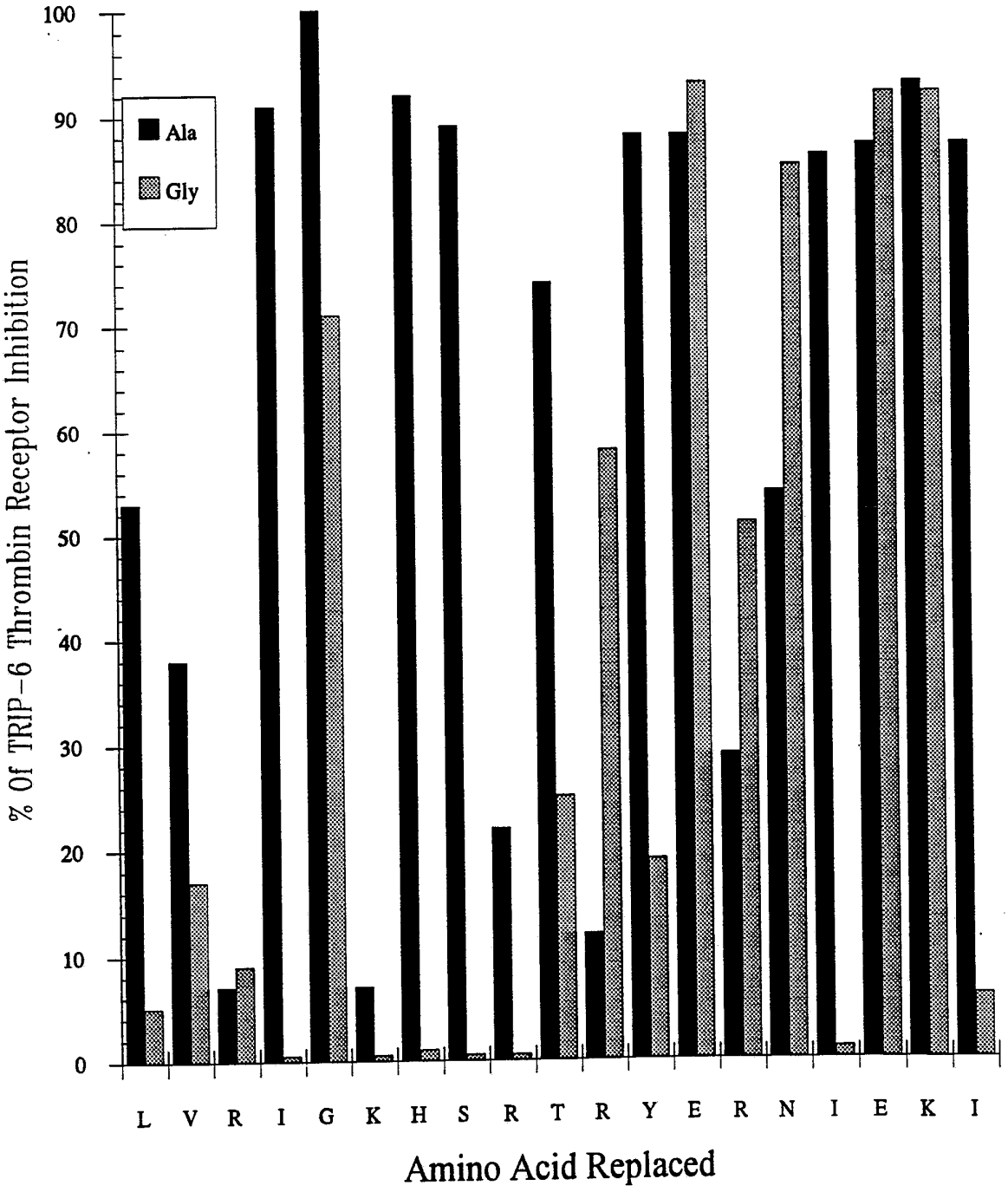
100
90
80
70
60
50
40
30
20
10
0
Ala
Gly
% Of TRIP-6 Thrombin Receptor Inhibition
L V R I G K H S R T R Y E R N I E K I
Amino Acid Replaced

FIGURE 6

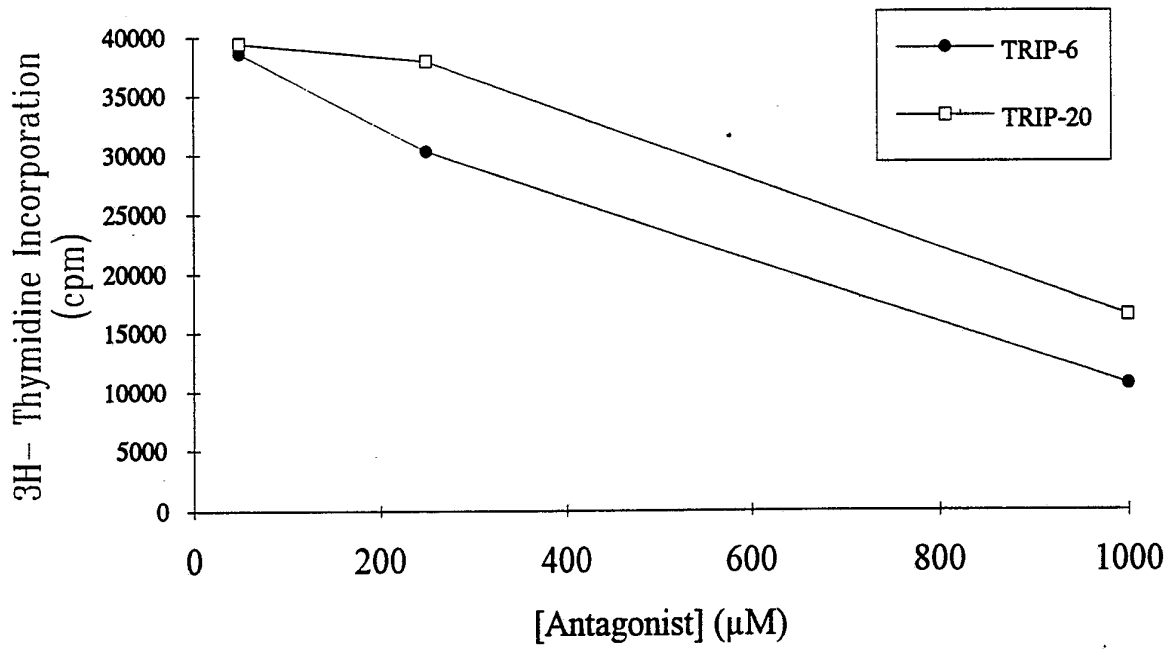
3H- Thymidine Incorporation (cpm)
40000
35000
30000
25000
20000
15000
10000
5000
0
0
200
400
600
800
1000
[Antagonist] (μM)
TRIP-6
TRIP-20

FIGURE 7

```
AAATTGTAAACGTTAATATTTTGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTA   60
AATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGA  120
ATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAA  180
CGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGA  240
ACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCC  300
TAAAGGGATGCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGA  360
AGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCG  420
CGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCAGGTGGCACTTT  480
TCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA  540
TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTAT  600
GAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGT  660
TTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACG  720
AGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGA  780
AGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCG  840
TATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGT  900
TGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATG  960
CAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGG 1020
AGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGA 1080
TCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCC 1140
TGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTC 1200
CCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTC 1260
GGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCG 1320
CGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACAC 1380
GACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTC 1440
ACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTT 1500
```

FIGURE 7 (cont')

AAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGAC 1560

CAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAA 1620

AGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACC 1680

ACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGT 1740

AACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGG 1800

CCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACC 1860

AGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTT 1920

ACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGA 1980

GCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCT 2040

TCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCG 2100

CACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCA 2160

CCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAA 2220

CGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTT 2280

CTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGA 2340

TACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGA 2400

GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCA 2460

CGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCT 2520

CACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAAT 2580

TGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTTAATAC 2640

GACTCACTATAGGGAAAGCTTTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCA 2700

ATTCCTTTAGTTGTTCCTTTCTATTCTCACAGCCGTATCTGCTTCAACCACCAGTCCTCC 2760

CAGCCGCAGACCACCAAAACCTGCTCCCCGGGTGAATCCTCCTGCTACAACAAACAGTGG 2820

CGTATCGGTAAACACTCCCGTACCCGTTACGAACGTAACATCGAAAAAACCATCATCGAA 2880

CGTGGTTGCGGTTGCCCGACCGTTAAACCGGGTATCAAACTGTCCTGCTGCGAATCCGAA 2940

GTTTGCAACAACGCGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGGATC 3000

GAAGGACGTGGGGCCGCAGAAACTGTTGAAAGTTGTCTGGCAAAACCTCATACAGAAAAT 3060

FIGURE 7 (cont')

TCATTTACTAACGTCTGGAAAGACGACAAAACTTTAGATCGTTACGCTAACTATGAGGGC 3120

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ACATGGGTTCCTATTGGGCTTGCTATCCCTGAAAATGAGGGTGGTGGCTCTGAGGGTGGC 3240

GGTTCTGAGGGTGGCGGTTCTGAGGGTGGCGGTACTAAACCTCCTGAGTACGGTGATACA 3300

CCTATTCCGGGCTATACTTATATCAACCCTCTCGACGGCACTTATCCGCCTGGTACTGAG 3360

CAAAACCCCGCTAATCCTAATCCTTCTCTTGAGGAGTCTCAGCCTCTTAATACTTTCATG 3420

TTTCAGAATAATAGGTTCCGAAATAGGCAGGGTGCATTAACTGTTTATACGGGCACTGTT 3480

ACTCAAGGCACTGACCCCGTTAAAACTTATTACCAGTACACTCCTGTATCATCAAAAGCC 3540

ATGTATGACGCTTACTGGAACGGTAAATTCAGAGACTGCGCTTTCCATTCTGGCTTTAAT 3600

GAGGATCCATTCGTTTGTGAATATCAAGGCCAATCGTCTGACCTGCCTCAACCTCCTGTC 3660

AATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTCTGAGGGTGGCGGCTCTGAG 3720

GGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGTGGCGGTTCCGGTGGCGGCTCCGGTTCC 3780

GGTGATTTTGATTATGAAAAAATGGCAAACGCTAATAAGGGGGCTATGACCGAAAATGCC 3840

GATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTAC 3900

GGTGCTGCTATCGATGGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCT 3960

ACTGGTGATTTTGCTGGCTCTAATTCCCAAATGGCTCAAGTCGGTGACGGTGATAATTCA 4020

CCTTTAATGAATAATTTCCGTCAATATTTACCTTCTTTGCCTCAGTCGGTTGAATGTCGC 4080

CCTTATGTCTTTGGCGCTGGTAAACCATATGAATTTTCTATTGATTGTGACAAAATAAAC 4140

TTATTCCGTGGTGTCTTTGCGTTTCTTTTATATGTTGCCACCTTTATGTATGTATTTTCG 4200

ACGTTTGCTAACATACTGCGTAATAAGGAGTCTTAATAAGAATTCACTGGCCGTCGTTTT 4260

ACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCC 4320

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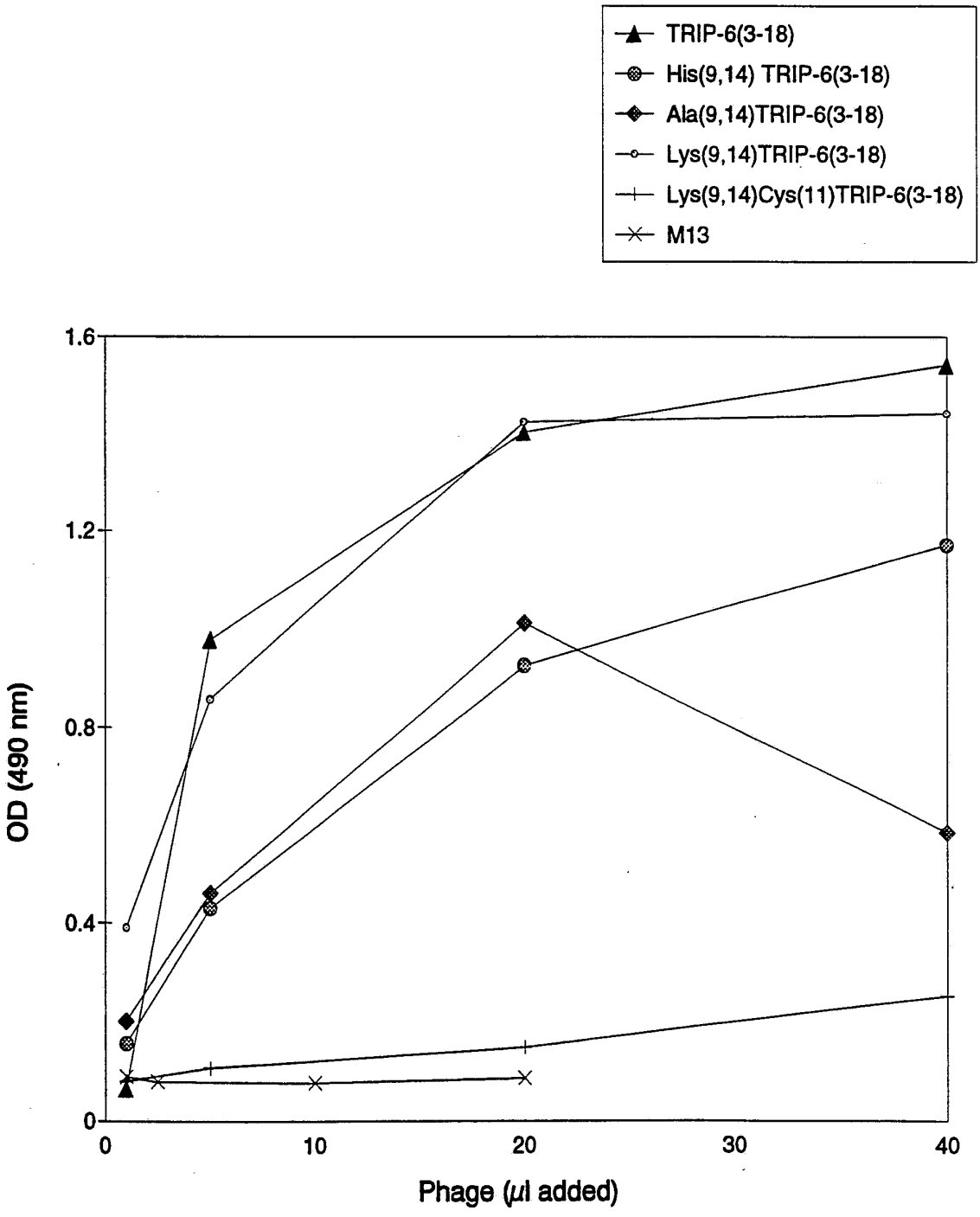
GCGCAGCCTGAATGGCGAATGGG 4403

FIGURE 8


TRIP-6(3-18)
His(9,14) TRIP-6(3-18)
Ala(9,14)TRIP-6(3-18)
Lys(9,14)TRIP-6(3-18)
Lys(9,14)Cys(11)TRIP-6(3-18)
M13
OD (490 nm)
1.6
1.2
0.8
0.4
0
0
10
20
30
40
Phage (µl added)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/01901

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N9/74; C12N15/62; C07K7/00; A61K37/02
A61K37/54

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO,A,9 214 750 (THE REGENTS OF THE UNIVERSITY OF CALIFORNAI, US) 3 September 1992 see page 18, line 21 - page 20, line 25 the claims	1-6,8,9, 11,18-28
A	EP,A,0 303 983 (BEHRINGWERKE AG, DE) 22 February 1989	
A	WO,A,8 803 151 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS) 5 May 1988	
A	EP,A,0 220 157 (WASHINGTON UNIVERSITY, US) 29 April 1987	

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 JULY 1993	21.07.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	NAUCHE S.A.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 263, 1988, BALTIMORE, MD US pages 11729 - 11735 NOE, G. ET AL.; 'The use of sequence specific antibodies to identify a secondary binding site in thrombin' see the whole document ---	1-5, 8-11, 18-28
A	BIOCHEMISTRY. vol. 26, 1987, EASTON, PA US pages 6165 - 6177 Friezner Degen, S.J. et al.; 'Nucleotide sequence of the gene for human prothrombin.' -----	

INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/US93/01901

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

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

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

Remark: Although claims 19-28 are directed to a method of treatment of the human/animal body (Rule 39.1(iv)) the search has been carried out and based on the alleged effects of the compound/composition.

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

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

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

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

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

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

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

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

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

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9301901
SA 71628

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 07/07/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9214750	03-09-92	AU-A-	1456892	15-09-92
EP-A-0303983	22-02-89	DE-A-	3727610	02-03-89
		AU-B-	612683	18-07-91
		AU-A-	2105888	23-02-89
		JP-A-	1070498	15-03-89
		US-A-	5071954	10-12-91
WO-A-8803151	05-05-88	AU-A-	8239987	25-05-88
		EP-A-	0328552	23-08-89
		JP-T-	2501028	12-04-90
EP-A-0220157	29-04-87	US-A-	4704451	03-11-87
		AU-B-	595249	29-03-90
		AU-A-	6387886	16-04-87
		CA-A-	1299814	28-04-92
		DE-A-	3682368	12-12-91
		JP-A-	62174098	30-07-87
		US-A-	4704380	03-11-87