

**(19) AUSTRALIAN PATENT OFFICE**

(54) Title

Use of transthyretin peptide/protein fusions to increase the serum half-life of  
pharmacologically active peptides/proteins

(51)<sup>6</sup> International Patent Classification(s)

**C12N** 15/09 (2006.01) 47/34  
**A61K** 38/00 (2006.01) 20060101ALI2006031  
**A61K** 38/17 (2006.01) OBMJP **A61K**  
**A61K** 38/44 (2006.01) 47/36  
**A61K** 47/32 (2006.01) 20060101ALI2006031  
**A61K** 47/34 (2006.01) OBMJP **A61K**  
**A61K** 47/36 (2006.01) 47/42  
**A61K** 47/42 (2006.01) 20060101ALI2006031  
**A61K** 47/48 (2006.01) OBMJP **A61K**  
**A61P** 3/10 (2006.01) 47/48  
**A61P** 7/04 (2006.01) 20060101ALI2006031  
**C07K** 14/435 OBMJP **A61P**  
(2006.01) 3/10 20060101ALI2006031  
**C07K** 14/475 OBMJP **A61P**  
(2006.01) 7/04  
**C07K** 14/605 20060101ALI2006031  
(2006.01) OBMJP **C07K**  
**C07K** 17/08 (2006.01) 14/435  
**C07K** 19/00 (2006.01) 20060101ALI2006031  
**C12N** 15/09 OBMJP **C07K**  
20060101AFI2006031 14/475  
OBMJP **A61K** 20060101ALI2006031  
38/00 OBMJP **C07K**  
20060101ALI2006031 14/605  
OBMJP **A61K** 20060101ALI2006031  
38/17 OBMJP **C07K**  
20060101ALI2005100 17/08  
8BMEP **A61K** 20060101ALI2006031  
38/44 OBMJP **C07K**  
20060101ALI2005100 19/00  
8BMEP **A61K** 20060101ALI2006031  
47/32 OBMJP  
20060101ALI2006031 PCT/US03/10443  
OBMJP **A61K**

(21) Application No: 2003224844

(22) Application Date: 2003 .04 .03

(87) WIPO No: W003/086444

(30) Priority Data

(31) Number	(32) Date	(33) Country
10/117,109	2002 .04 .04	US

(43) Publication Date : 2003 .10 .27

(43) Publication Journal Date : 2003 .12 .04

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(56) Related Art

Furuya, H. et al. Biochem. Biophys. Res. Comm. 1989, vol. 163, pp. 851-859  
 Matsumoto, T. et al. Biosci. Biotech. Biochem. 1993, vol. 57, pp. 414-418

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

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 October 2003 (23.10.2003)

PCT

(10) International Publication Number  
WO 03/086444 A1

- (51) International Patent Classification<sup>7</sup>: **A61K 38/00**
- (21) International Application Number: PCT/US03/10443
- (22) International Filing Date: 3 April 2003 (03.04.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
10/117,109 4 April 2002 (04.04.2002) US
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- (81) Designated States (*national*): AL, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**  
*with international search report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 03/086444 A1

(54) Title: USE OF TRANSFERRIN PEPTIDE/PROTEIN FUSIONS TO INCREASE THE SERUM HALF-LIFE OF PHARMACOLOGICALLY ACTIVE PEPTIDES/PROTEINS

(57) Abstract: The present invention provides a means for increasing the serum half-life of a selected biologically active agent by utilizing transferrin (TTR) as a fusion partner with a biologically active agent. Specifically, the present invention provides substantially homogenous preparations of TTR (or a TTR variant)-biologically active agent fusions and PEG-TTR (PEG-TTR variant)-biologically active agent fusions. As compared to the biologically active agent alone, the TTR-biologically active agent fusion and/or PEG-TTR-biologically active agent fusion has substantially increased serum half-life.

USE OF TRANSTHYRETIN PEPTIDE/PROTEIN FUSIONS TO INCREASE THE  
SERUM HALF-LIFE OF PHARMACOLOGICALLY ACTIVE  
PEPTIDES/PROTEINS

This application is a Continuation in Part of U. S. Application No. 10/117,109,  
5 filed 04 April 2002, which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Any discussion of the prior art throughout the specification should in no way be  
considered as an admission that such prior art is widely known or forms part of common  
general knowledge in the field.

10 Proteins, peptides and other drug molecules for therapeutic use are currently  
available in suitable forms in adequate quantities largely as a result of the advances in  
recombinant DNA technologies. The availability of such peptides and proteins has  
engendered advances in protein formulation and chemical modification. Chemical  
modification of biologically active peptides, proteins, oligonucleotides and other drugs  
15 for purposes of extending the serum half-life of such bioactive agents has been  
extensively studied. The ability to extend the serum half-life of such agents allows for  
the therapeutic potential of the agent to be realized without the need for high dosages  
and frequent administration.

Chemical modification used to extend the half-lives of proteins *in vivo* includes  
20 the chemical conjugation of a water soluble polymer, such as polyethylene glycol (PEG),  
to the protein of interest. A variety of approaches have been used to attach the  
polyethylene glycol molecules to the protein (PEGylation). For example, Royer (U.S.  
Patent No. 4,002,531) states that reductive alkylation was used for attachment of  
polyethylene glycol molecules to an enzyme. Davis *et al.* (U. S. Patent No. 4,179,337)  
25 disclose PEG: protein conjugates involving, for example, enzymes and insulin. Shaw  
(U.S. Patent No. 4,904,584) disclose the modification of the number of lysine —

- 2 -

residues in proteins for the attachment of polyethylene glycol molecules via reactive amine groups. Hakimi et al. (U.S. Patent No. 5,834,594) disclose substantially non-immunogenic water soluble PEG:protein conjugates, involving for example, the proteins IL-2, interferon alpha, and IL-1ra. The methods of Hakimi et al. involve the utilization of unique linkers to connect the various free amino groups in the protein to PEG. Kinstler et al. (U.S. Patent Nos. 5,824,784 and 5,985,265) teach methods allowing for selectively N-terminally chemically modified proteins and analogs thereof, including G-CSF and consensus interferon.

Other approaches designed to extend the serum half-life of bioactive agents include: conjugation of the peptides to a large, stable protein which is too large to be filtered through the kidneys (e.g., serum albumin); G. D. Mao et al., *Biomat., Art. Cells, Art. Org.* 17:229-244 (1989); use of low- and high-density lipoproteins as transport vehicles and to increase serum half-life; P. Chris de Smidt et al., *Nuc. Acids. Res.*, 19(17):4695-4700 (1991); the use of the Fc region of immunoglobulins to produce Fc-protein fusions; PCT WO 98/28427 (Mann et al, and references cited therein); and the use of the Fc domain to increase in vivo half-life of one or more biologically active peptides; PCT WO 00/24782 (Feige et al, and references cited therein).

Transthyretin (TTR) (formerly called prealbumin) is a 56kDa tetrameric serum protein that plays important physiological roles as a transporter of thyroxine and retinol-binding protein; Hamilton and Benson, *Cell. Mol. Life Sci.*, 58:1491-1521 (2001), and references cited therein. Blaney et al., in U.S. Patent No. 5,714,142, describe the exploitation of TTR by endowing the drug to be administered with functionality that allows it to bind specifically to

- 3 -

the protein. Specifically, Blaney et al. demonstrate that covalent attachment of a peptide, protein, nucleotide, oligonucleotide, oligosaccharide or other drug to a transthyretin-selective ligand will

5 reversibly bind the drug to TTR and thereby increase the serum half-life of the agent based on the affinity of the ligand for TTR. It is stated that the intrinsic activity of the drug is not adversely affected and the resulting drug-TTR ligand conjugate will still be small

10 enough to be orally absorbed.

#### SUMMARY OF THE INVENTION

It has been found, surprisingly and

15 importantly, that TTR (or a TTR variant), and in particular, a TTR or TTR variant which has been chemically modified via conjugation to a water soluble polymer, e.g., can be used as a fusion partner with a biologically active agent to increase the serum half-

20 life of the biologically active agent. Accordingly, the present invention provides a means for increasing the serum half-life of a selected biologically active agent.

The present invention thus relates to

25 substantially homogenous preparations of TTR (or a TTR variant)-biologically active agent fusions and PEG-TTR (PEG-TTR variant)-biologically active agent fusions. As compared to the biologically active agent alone, the TTR-biologically active agent fusion and/or PEG-TTR-

30 biologically active agent fusion has substantially increased serum half-life.

The present invention further relates to TTR-biologically active agent fusions and PEG-TTR-biologically active agent fusions, in a

35 pharmaceutically acceptable carrier, to provide a pharmacologically active compound.

The present invention further relates to the preparation of TTR variants. Specifically, TTR proteins are modified such that cysteine residue(s) are engineered into the TTR protein sequence. The TTR variants are recoverable in high yield and are then chemically modified via conjugation of a water soluble polymer at the cysteine residue  
5 to provide a chemically modified TTR variant which can then be fused to a selected biologically active agent.

The present invention further relates to processes for preparing pharmacologically active compounds. For example, the principal embodiment of the method for making the substantially homogenous preparation of a PEG-TTR-peptide  
10 fusion comprises: (a) engineering a cysteine residue into a specific amino acid position within the amino acid sequence of said TTR to provide a variant of said TTR; (b) conjugating a polyethylene glycol to said TTR variant at said cysteine residue to provide a PEG-TTR; (c) fusing said PEG-TTR to a peptide of interest to provide a PEG-TTR-peptide fusion; and (d) isolating said PEG-TTR-peptide fusion.

15 The present invention also relates to methods of treatment of individuals using the pharmacologically active compounds as above.

According to a first aspect, the present invention provides a method for manufacturing a fusion product comprising fusing a biologically active agent to transthyretin (TTR) or a TTR variant, wherein the biologically active agent comprises a  
20 protein or peptide selected from the group consisting of cytokines, hematopoietic factors, interleukins, growth factors, trophic factors, and hormones, and wherein the fusion product exhibits increased serum half life *in vivo* compared to the biologically active agent.

According to a second aspect, the present invention provides a fusion product  
25 comprising a biologically active agent fused to a transthyretin (TTR) or a TTR variant, wherein the biologically active agent comprises a protein or peptide selected from the group consisting of cytokines, hematopoietic factors, interleukins, growth factors, trophic factors, and hormones.

According to a third aspect, the present invention provides a nucleic acid that  
30 comprises a nucleotide sequence that encodes the fusion product of the second aspect.

According to a fourth aspect, the present invention provides a vector that comprises the nucleic acid of the third aspect.

According to a fifth aspect, the present invention provides a host cell transformed or transfected with the vector of the fourth aspect.

5 According to a sixth aspect, the present invention provides a method for manufacturing a fusion product of the second aspect that is a fusion protein, the method comprising expressing a nucleic acid that comprises a nucleotide sequence that encodes the fusion product as a fusion protein.

10 According to a seventh aspect, the present invention provides a substantially homogenous preparation of a fusion product according to the second aspect.

According to an eighth aspect, the present invention provides a process for preparing a substantially homogenous preparation of a TTR-biologically active agent fusion product comprising: (a) fusing TTR to a biologically active agent according to the method of the first aspect, to provide a TTR-biologically active agent fusion product;  
15 and (b) isolating said TTR-biologically active agent fusion product.

According to a ninth aspect, the present invention provides a process for preparing a substantially homogenous preparation of a TTR variant-biologically active agent fusion product comprising engineering a cysteine residue into a specific amino acid position within the amino acid sequence of TTR to provide a variant of said TTR;  
20 and, subsequently, fusing TTR variant to a biologically active agent according to the method of the first aspect, to provide a TTR variant-biologically active agent fusion product; and isolating said TTR variant biologically active agent fusion product.

According to a tenth aspect, the present invention provides a process for preparing a substantially homogenous preparation of a PEG-TTR-biologically active agent fusion  
25 product comprising: (a) conjugating a polyethylene glycol to TTR to provide a PEG-TTR; and, subsequently, the process of the invention using said PEG-TTR.

According to an eleventh aspect, the present invention provides a process for preparing a substantially homogenous preparation of a PEG-TTR variant-biologically active agent fusion product comprising: (a) engineering a cysteine residue into a specific  
30 amino acid position within the amino acid sequence of TTR to provide a variant TTR;

(b) conjugating a polyethylene glycol to said TTR variant to provide a PEG-TTR variant and, subsequently, fusing PEG-TTR variant to a biologically active agent according to the method of the first aspect, to provide a PEG-TTR variant-biologically active agent fusion product; and isolating said PEG-TTR variant-biologically active agent fusion  
5 product.

According to a twelfth aspect, the present invention provides a process for preparing a substantially homogeneous preparation of a PEG-TTR-biologically active agent fusion product comprising: (a) fusing TTR to a biologically active agent according to claim 3 to provide a TTR-biologically active agent fusion product; (b) conjugating a  
10 polyethylene glycol to said TTR-biologically active agent to provide a PEG-TTR-biologically active agent fusion; and (c) isolating the PEG-TTR-biologically active agent fusion product.

According to a thirteenth aspect, the present invention provides a process for preparing a substantially homogenous preparation of a PEG-TTR variant-biologically  
15 active agent fusion product comprising: (a) engineering a cysteine residue into a specific amino acid position within the amino acid sequence of TTR to provide a TTR variant; (b) fusing said TTR variant to a biologically active agent according to the first aspect to provide a TTR variant-biologically active agent fusion product; (c) conjugating a polyethylene glycol to said TTR variant to provide a PEG-TTR variant-biologically  
20 active agent fusion product; and (d) isolating the PEG-TTR variant-biologically active agent fusion product.

According to a fourteenth aspect, the present invention provides a process for preparing a PEG-TTR-biologically active agent fusion product or a PEG-TTR variant-biologically active agent fusion product comprising: starting with a fusion product  
25 according to the second aspect; and conjugating a polyethylene glycol thereto.

According to a fifteenth aspect, the present invention provides a method of treating thrombocytopenia comprising administering a therapeutically effective dose of a preparation of the fusion product of the first aspect.

According to a sixteenth aspect, the present invention provides a method of  
30 treating non-insulin dependent diabetes comprising administering a therapeutically effective dose of a preparation of the fusion product of the first aspect.



- 4c -

According to a seventeenth aspect, the present invention provides a fusion product produced by the method of the first aspect or the process of the invention.

According to an eighteenth aspect, the present invention provides use of a fusion product of the first aspect for the preparation of a medicament for treating  
5 thrombocytopenia.

According to a nineteenth aspect, the present invention provides use of a fusion product of the first aspect for the preparation of a medicament for treating non-insulin dependent diabetes.

Unless the context clearly requires otherwise, throughout the description and the  
10 claims, the words "comprise", "comprising", and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an SDS gel that depicts the purification of an *E. coli* expressed,  
15 recombinant human transthyretin (TTR) variant (C10A/G83C) with a Bradykinin peptide fused to the C-terminus of TTR. Lane 1 contains Novex Mark 12 molecular weight standards, and lanes 2-7 contain the following respectively: cell lysate, post-heating supernatant, pool from Q-sepharose chromatography step, pool from ———

- 5 -

phenyl sepharose chromatography step, pool from hydroxyapatite chromatography step, and pool from source Q chromatography step.

5           Figure 2 demonstrates by size exclusion chromatography that fusion of peptides to the amino-terminus or carboxy-terminus of a TTR variant, TTR(C10A/G83C), does not alter its oligomeric structure. Solid line is TTR(C10A/G83C), dashed line  
10 is parathyroid hormone (PTH) fused to the amino-terminus of TTR(C10A/G83C), and the dotted line is Bradykinin fused to the carboxy-terminus of TTR(C10A/G83C).

15           Figure 3 demonstrates by size exclusion chromatography that fusion of proteins to the amino-terminus or carboxy-terminus of a TTR variant, TTR(C10A), does not alter its oligomeric structure. Solid line is TTR(C10A), dashed line is IL-1-ra fused  
20 to the carboxy-terminus of TTR(C10A), and the dotted line is IL-1-ra fused to the amino-terminus of TTR(C10A).

            Figure 4 shows the binding observed using  
25 BIAcore of various TPO-mimetic peptide (TMP) constructs to human MPL receptor: ■ Fc-TMP, ● TMP(m)-TTR, ▲ TMP(m)-TTR-PEG5K, ▼ TMP(m)-TTR-PEG20K.

            Figure 5 shows that injection of TMP(m)-TTR-  
30 PEG5K induces platelet formation in mice. The following symbols correspond to the corresponding constructs: ■ Carrier, ● Fc-TMP, ▲ TTR-TMP, ▼ TMP(m)-TTR, and ◆ TMP(m)-TTR-PEG5K.

- 6 -

Figure 6 demonstrates by size exclusion chromatography that native TTR and TTR(C10A) maintain a similar oligomeric configuration (an apparent tetramer). Solid line is native TTR and the dashed line is TTR(C10A).

Figure 7 demonstrates by size exclusion chromatography that conjugation of PEG to TTR increases its molecular size in a predictable uniform manner. Solid lines indicate no PEG conjugated, dashed lines indicate 5K PEG fused, and dotted lines indicate 20K PEG fused. The following constructs were used: A) TMP-TTR(C10A/A37C), B) TMP-TTR(C10A/D38C), C) TMP-TTR(C10A/A81C), and D) TMP-TTR(C10A/G83C).

Figure 8 is an SDS gel that depicts the extent of pegylation of various TMP-TTR constructs involving TTR variants having a non-native cysteine engineered in at one of four different locations. Lane 1 contains Novex Mark 12 molecular weight standards; lane 2 is unpegylated TMP-TTR(C10A/A37C); lanes 3 - 6 are 5K pegylated versions of TMP-TTR(C10A/A37C), TMP-TTR(C10A/D38C), TMP-TTR(C10A/A81C), and TMP-TTR(C10A/G83C) respectively; lanes 7 - 10 are 20K pegylated versions of TMP-TTR(C10A/A37C), TMP-TTR(C10A/D38C), TMP-TTR(C10A/A81C), and TMP-TTR(C10A/G83C), respectively.

Figures 9A-C compare the competitive binding of Fc-TMP and TMP-TTR to human MPL by BIAcore analysis. A) ■ Fc-TMP, ● TMP-TTR(C10A/A37C), ▲ TMP-TTR(C10A/D38C), ▼ TMP-TTR(C10A/A81C), ◆ TMP-TTR(C10A/G83C). B) ■ Fc-TMP, 5K pegylated versions of TMP-TTR(C10A/A37C) (●), TMP-TTR(C10A/D38C) (▲), TMP-TTR(C10A/A81C) (▼), TMP-TTR(C10A/G83C) (◆). C) ■ Fc-

- 7 -

TMP, 20K pegylated versions of TMP-TTR(C10A/A37C) (●), TMP-TTR(C10A/D38C) (▲), TMP-TTR(C10A/A81C) (▼), TMP-TTR(C10A/G83C) (◆).

5           Figures 10A-C show that injection of TMP-TTR with PEG conjugated to engineered cysteines induces platelet formation in mice. A) ■ TTR(C10A), ● Fc-TMP, ▲ TMP-TTR(C10A/A37C), ▼ TMP-TTR(C10A/D38C) (carboxamidomethylated), ◆ TMP-TTR(C10A/A81C), ◄ TMP-TTR(C10A/G83C). B) ■ TTR(C10A), ● Fc-TMP, 5K  
10           pegylated versions of TMP-TTR(C10A/A37C) (▲), TMP-TTR(C10A/D38C) (▼), TMP-TTR(C10A/A81C) (◆), TMP-TTR(C10A/G83C) (◄). C) ■ TTR(C10A), ● Fc-TMP, 20K  
15           pegylated versions of TMP-TTR(C10A/A37C) (▲), TMP-TTR(C10A/D38C) (▼), TMP-TTR(C10A/A81C) (◆), TMP-TTR(C10A/G83C) (◄).

Figure 11 shows that injection of PTH-TTR with PEG conjugated to engineered cysteines induces  
20           ionized calcium release in mice. The following symbols correspond to the corresponding constructs: ■ TTR(C10A), ● PTH-Fc, ▲ PTH-TTR, ▼ PTH-TTR(C10A/K15A/A37C) (carboxamidomethylated), ◆ 5K  
25           pegylated version of PTH-TTR(C10A/K15A/A37C), ◄ 20K  
            pegylated version of PTH-TTR(C10A/K15A/A37C), ► PTH-TTR(C10A/K15A/G83C) (carboxamidomethylated), ● 5K  
            pegylated version of PTH-TTR(C10A/K15A/G83C), and ★ 20K  
            pegylated version of PTH-TTR(C10A/K15A/G83C).

30           Figure 12 shows that injection of Glucagon-like Peptide 1 (GLP1)-TTR with PEG conjugated to engineered cysteines lowers blood glucose levels in mice. The following symbols correspond to the  
            corresponding constructs: ■ TTR(C10A), ● GLP1-Fc, ▲

- 8 -

GLP1-TTR(C10A/K15A/G83C) (PEG 5K), and ▼ GLP1-TTR(C10A/K15A/G83C) (PEG 20K).

Figure 13 shows that injection of TMP-TTR conjugates with fused CH2 domains increase serum platelet levels in mice. The following symbols correspond to the corresponding constructs: ■ TTR(C10A), ● Fc-TMP, ▲ TMP-TTR(C10A)-CH2, ▼ TTR(C10A)-CH2-TMP, and ◆ TMP-CH2-TTR(C10A).

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Figure 14 shows that injection of and carboxy-terminal fusions of TMP with pegylated TTR increases blood platelet counts in mice. The following symbols correspond to the corresponding constructs: ■ TTR(C10A), ● Fc-TMP, ▲ TTR(C10A/K15A/A37C)-TMP (PEG 20K), ▼ TTR(C10A/K15A/A81C)-TMP (PEG 20K), ◆ TTR(C10A/K15A/G83C)-TMP (PEG 20K), ◀ TMP-TTR(C10A/K15A/A37C) (PEG 20K), ▶ TMP-TTR(C10A/K15A/A81C) (PEG 20K), ● TMP-TTR(C10A/K15A/G83C) (PEG 20K).

20

Figures 15 A-C show that injection of pegylated TMP-TTR fusions containing a K15A alteration increases blood platelet counts in mice. The following symbols correspond to the corresponding constructs: A) ■ TTR(C10A), ● Fc-TMP, ▲ TMP-TTR(C10A/K15A/A37C) (carboxyamidomethylated), and ▼ TMP-TTR(C10A/K15A/A81C) (carboxyamidomethylated); B) ■ TTR(C10A), ● Fc-TMP, ▲ TMP-TTR(C10A/K15A/A37C) (PEG 5K), ▼ TMP-TTR(C10A/K15A/A81C) (PEG 5K), and ◆ TMP-TTR(C10A/K15A/G83C) (PEG 5K); C) ■ TTR(C10A), ● Fc-TMP, ▲ TMP-TTR(C10A/K15A/A37C) (PEG 20K), ▼ TMP-TTR(C10A/K15A/A81C) (PEG 20K), and ◆ TMP-TTR(C10A/K15A/G83C) (PEG 20K).

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- 9 -

DETAILED DESCRIPTION OF THE INVENTION

For purposes of describing the present invention, the following terms are defined as set forth below.

The term "biologically active agent" refers to any chemical material or compound useful for prophylactic, therapeutic or diagnostic application.

The term "pharmacologically active compound" refers to a compound suitable for administration to a mammalian, preferably a human individual, which induces a desired local or systemic effect.

The terms "peptide", "polypeptide" and "protein" describe a type of biologically active agents, and the terms are used interchangeably herein to refer to a naturally occurring, recombinantly produced or chemically synthesized polymer of amino acids. The terms are intended to include peptide molecules containing as few as 2 amino acids, chemically modified polypeptides, consensus molecules, analogs, derivatives or combinations thereof.

Any number of peptides may be used in conjunction with the present invention. Of particular interest are peptides that mimic the activity of erythropoietin (EPO), thrombopoietin (TPO), Glucagon-like Peptide 1 (GLP-1), parathyroid hormone (PTH), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 receptor antagonist (IL-1ra), leptin, cytotoxic T-lymphocyte antigen 4 (CTLA4), TNF-related apoptosis-inducing ligand (TRAIL), tumor growth factor-alpha and beta (TGF- $\alpha$  and TGF- $\beta$ , respectively), and growth hormones. The terms "-mimetic peptide" and "-agonist peptide" refer to a peptide having biological

- 10 -

activity comparable to a protein (e.g., GLP-1, PTH, EPO, TPO, G-CSF, etc.) that interacts with a protein of interest. These terms further include peptides that indirectly mimic the activity of a protein of interest, such as by potentiating the effects of the natural ligand of the protein of interest. Thus, the term "EPO-mimetic peptide" comprises any peptides that can be identified or derived as having EPO-mimetic subject matter; see, for example, Wrighton et al., *Science*, 273:458-63 (1996); and Naranda et al., *Proc. Natl. Acad. Sci. USA* 96:7569-74 (1999). Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

The term "TPO-mimetic peptide" (TMP) comprises peptides that can be identified or derived as having TPO-mimetic subject matter; see, for example, Cwirla et al., *Science*, 276:1696-9 (1997); U.S. Patent Nos. 5,869,451 and 5,932,946; and PCT WO 00/24782 (Liu et al, and references cited therein), hereby incorporated by reference in its entirety. Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

The term "G-CSF-mimetic peptide" comprises any peptides that can be identified as having G-CSF-mimetic subject matter; see, for example, Paukovits et al., *Hoppe-Seylers Z. Physiol. Chem.* 365:303-11 (1984). Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

- 11 -

The term "CTLA4-mimetic peptide" comprises any peptides that can be identified or derived as described in Fukumoto et al., *Nature Biotech.* 16:267-70 (1998). Those of ordinary skill in the art appreciate  
5 that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

Peptide antagonists are also of interest,  
10 particularly those antagonistic to the activity of TNF, leptin, any of the interleukins, and proteins involved in complement activation (e.g., C3b). The term "-antagonist peptide" or "inhibitor peptide" refers to a peptide that blocks or in some way interferes with the  
15 biological activity of the associated protein of interest, or has biological activity comparable to a known antagonist or inhibitor of the associated protein of interest. Thus, the term "TNF-antagonist peptide" comprises peptides that can be identified or derived as  
20 having TNF-antagonistic subject matter; see, for example, Takasaki et al., *Nature Biotech.*, 15:1266-70 (1997). Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by  
25 following the disclosed procedures with different peptide libraries.

The terms "IL-1 antagonist" and "IL-1ra-mimetic peptide" comprises peptides that inhibit or down-regulate activation of the IL-1 receptor by IL-1.  
30 IL-1 receptor activation results from formation of a complex among IL-1, IL-1 receptor, and IL-1 receptor accessory protein. IL-1 antagonist or IL-1ra-mimetic peptides bind to IL-1, IL-1 receptor, or IL-1 receptor accessory protein and obstruct complex formation among  
35 any two or three components of the complex. Exemplary IL-1 antagonist or IL-1ra-mimetic peptides can be



- 12 -

identified or derived as described in U.S. Pat. Nos.  
5,608,035, 5,786,331, 5,880,096. Those of ordinary  
skill in the art appreciate that each of these  
references enables one to select different peptides  
5 than actually disclosed therein by following the  
disclosed procedures with different peptide libraries.

The term "VEGF-antagonist peptide" comprises  
peptides that can be identified or derived as having  
VEGF-antagonistic subject matter; see, for example,  
10 Fairbrother, *Biochem.*, 37:17754-64 (1998). Those of  
ordinary skill in the art appreciate that each of these  
references enables one to select different peptides  
than actually disclosed therein by following the  
disclosed procedures with different peptide libraries.

15 The term "MMP inhibitor peptide" comprises  
peptides that can be identified or derived as having  
MMP inhibitory subject matter; see, for example,  
Koivunen, *Nature Biotech.*, 17:768-74 (1999). Those of  
ordinary skill in the art appreciate that each of these  
20 references enables one to select different peptides  
than actually disclosed therein by following the  
disclosed procedures with different peptide libraries.

Targeting peptides are also of interest,  
including tumor-homing peptides, membrane-transporting  
25 peptides, and the like.

Exemplary peptides may be randomly generated  
by various techniques known in the art. For example,  
solid phase synthesis techniques are well known in the  
art, and include those described in Merrifield, *Chem.*  
30 *Polypeptides*, pp. 335-61 (Katsoyannis and Panayotis  
eds.) (1973); Merrifield, *J. Am. Chem. Soc.*, 85:2149  
(1963); Davis et al., *Biochem. Intl.*, 10:394-414  
(1985); Stewart and Young, *Solid Phase Peptide*  
*Synthesis* (1969); U.S. Pat. No. 3,941,763; Finn et al.,  
35 *The Proteins*, 3rd ed., 2:105-253 (1976); and Erickson  
et al., *The Proteins*, 3rd ed., 2:257-527 (1976). Solid

- 13 -

phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides.

Phage display is another useful method in  
5 generating peptides for use in the present invention. It has been stated that affinity selection from libraries of random peptides can be used to identify peptide ligands for any site of any gene product; Dedman et al., *J. Biol. Chem.*, 268:23025-30 (1993).  
10 Phage display is particularly well suited for identifying peptides that bind to such proteins of interest as cell surface receptors or any proteins having linear epitopes; Wilson et al., *Can. J. Microbiol.*, 44:313-29 (1998); Kay et al., *Drug Disc. Today*, 3:370-8 (1998). Such proteins are extensively  
15 reviewed in Herz et al., *J. Receptor & Signal Transduction Res.*, 17(5):671-776 (1997), which is hereby incorporated by reference.

The peptides may also be made in transformed  
20 host cells using recombinant DNA techniques. To do so, a recombinant DNA molecule coding for the peptide is prepared. Methods of preparing such DNA and/or RNA molecules are well known in the art. For instance, sequences coding for the peptides could be excised from  
25 DNA using suitable restriction enzymes. The relevant sequences can be created using the polymerase chain reaction (PCR) with the inclusion of useful restriction sites for subsequent cloning. Alternatively, the DNA/RNA molecule could be synthesized using chemical  
30 synthesis techniques, such as the phosphoramidite method. Also, a combination of these techniques could be used.

Additional biologically active agents  
contemplated for use include recombinant or naturally  
35 occurring proteins, whether human or animal, hormones, cytokines, hematopoietic factors, growth factors,

antiobesity factors, trophic factors, anti-inflammatory factors, and enzymes. Such proteins would include but are not limited to interferons (see, U. S. Patent Nos. 5,372,808, 5,541,293, 4,897,471, and 4,695,623, hereby incorporated by reference including drawings), interleukins (see, U. S. Patent No. 5,075,222, hereby incorporated  
5 by reference including drawings), erythropoietins (see, U. S. Patent Nos. 4,703,008, 5,441,868, 5,618,698, 5,547,933, and 5,621,080, hereby incorporated by reference including drawings), granulocyte-colony stimulating factors (see, U. S. Patent Nos. 4,810,643, 4,999,291, 5,581,476, 5,582,823, and PCT Publication No. 94/17185, hereby incorporated by reference including drawings), stem cell factor (PCT Publication Nos.  
10 91/05795, 92/17505 and 95/17206, hereby incorporated by reference including drawings), novel erythropoietin stimulating protein (NESP) (PCT Publication No. 94/09257, hereby incorporated by reference including drawings), osteoprotegerin (PCT Publication No. 97/23614, hereby incorporated by reference including drawings), interleukin-1 receptor antagonist (IL-1ra) (PCT Publication Nos. 91/08285 and  
15 92/16221) and leptin (OB protein) (PCT publication Nos. 96/40912, 96/05309, 97/00128, 97/01010 and 97/06816, hereby incorporated by reference including figures).

In addition, biologically active agents can also include but are not limited to insulin, gastrin, prolactin, adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), human  
20 chorionic gonadotropin (HCG), motilin, interferons (alpha, beta, gamma), interleukins (IL-1 to IL-12), tumor necrosis factor (TNF), tumor necrosis factor-binding protein (TNF-bp), brain derived neurotrophic factor (BDNF),

- 15 -

glial derived neurotrophic factor (GDNF), neurotrophic factor 3 (NT3), fibroblast growth factors (FGF), neurotrophic growth factor (NGF), bone growth factors such as osteoprotegerin (OPG), insulin-like growth factors (IGFs), macrophage colony stimulating factor (M-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), megakaryocyte derived growth factor (MGDF), keratinocyte growth factor (KGF), thrombopoietin, platelet-derived growth factor (PDGF), colony stimulating growth factors (CSFs), bone morphogenetic protein (BMP), superoxide dismutase (SOD), tissue plasminogen activator (TPA), urokinase, streptokinase and kallikrein.

Transthyretin (TTR) contemplated for use in the present invention will have the DNA and amino acid sequences of TTR as reported in Mita et al., *Biochem. Biophys. Res. Commun.*, 124(2):558-564 (1984). These sequences have been deposited in Genbank as accession number K02091. The 127 amino acid TTR sequence used herein does not include the signal sequence (amino acids 1-20) of the K02091 sequence and is depicted below as SEQ ID NO:1.

SEQ ID NO:1

GPTGTGESKCPMLVKVLDVAVRGSPAINVAVHVFRKAADDTWEPFASGKTSSESGEL  
HGLTTEEEFVEGIYKVEIDTKSYWKALGISPFHEAEVVFTANDSGPRRYTIAAL  
LSPYSYSTTAVVTNPKE

The term "TTR variant" refers to a molecule or sequence that is a modified form of a native TTR. For example, a native TTR comprises sites that may be removed because they provide structural features or biological activity that are not required for the fusion molecules of the present invention. Thus, the term "TTR variant" comprises a molecule or sequence that lacks one or more native TTR sites or residues or

- 16 -

that has had one or more native TTR sites or residues replaced with a different amino acid or that has had one or more residues added to the sequence. For purposes of an example, a TTR variant wherein the Alanine residue at amino acid sequence position 37 has been replaced with a Cysteine residue, will be designated TTR variant (A37C); and a TTR variant wherein both the Alanine residue at amino acid sequence position 37 and the Glycine residue at amino acid sequence position 83 have both been replaced with a Cysteine residue will be designated TTR variant (A37C/G83C).

In one embodiment, a TTR or TTR variant fused to a biologically active agent may be fused to a third protein or protein fragment that further stabilizes the TTR-biologically active agent fusion protein, and thereby increases the half-life of the resulting fusion in serum. Examples of such additional proteins or fragments thereof that can be used in such methods and compositions include the Fc domain or CH2 domain of an immunoglobulin, or any other protein domain that one of skill in the art would recognize as having properties that would increase protein stability (see, e.g., Example 29 below). Such protein groups can be fused to the carboxy or amino terminus of the TTR-biologically active agent fusion protein, or can be placed between the TTR and the biologically active agent. It is contemplated that linkers or spacers can be placed, as needed, between each of the domains of the fusion protein to facilitate their desired activity.

In another embodiment, the TTR or TTR variant of the invention can be chemically crosslinked to the biologically active agent. Cross-linking of proteins can be performed by using, for example, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) according to established, published procedures. Additional cross-

- 17 -

linking agents are readily available and can be identified by one of skill in the art. For details on the above procedure, see, e.g., Karpovsky et al, J. Exp. Med. 160, 1686-1701 (1984); Perez et al, Nature, 5 316, 354-356 (1985) or Titus et al, Journal of Immunology, 139, 3153-3158 (1987).

In another embodiment, a molecule can be covalently linked to the fusion protein such that stability and/or half-life in serum are increased. For 10 example, a preferred TTR or TTR variant may be chemically modified using water soluble polymers such as polyethylene glycol (PEG). The PEG group may be of any convenient molecular weight and may be straight chain or branched. The average molecular weight of the 15 PEG will preferably range from about 2 kDa to about 100 kDa, more preferably from about 5 kDa to about 50 kDa, most preferably about 20 kDa.

The PEG groups will generally be attached to the compounds of the invention via acylation, reductive 20 alkylation, Michael addition, thiol alkylation or other chemoselective conjugation/ligation methods through a reactive group on the peg moiety (e.g., an aldehyde, amino, ester, thiol, -haloacetyl, maleimido or hydrazino group) to a reactive group on the target 25 compound (e.g., an aldehyde, amino, ester, thiol, -haloacetyl, maleimido or hydrazino group).

Other water soluble polymers used include copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, polyvinyl alcohol, polyvinyl 30 pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran.

A DNA molecule encoding the peptide of 35 interest, protein of interest, TTR or TTR variant can be prepared using well known recombinant DNA technology

- 18 -

methods such as those set forth in Sambrook et al.  
(*Molecular Cloning: A Laboratory Manual*, Cold Spring  
Harbor Laboratory Press, Cold Spring Harbor, NY [1989])  
and/or Ausubel et al., eds, *Current Protocols in*  
5 *Molecular Biology*, Green Publishers Inc. and Wiley and  
Sons, NY (1994). A gene or cDNA encoding the protein  
of interest or fragment thereof may be obtained for  
example by screening a genomic or cDNA library with a  
suitable probe. Suitable probes include, for example,  
10 oligonucleotides, cDNA fragments, or genomic DNA  
fragments, that are expected to have some homology to  
the gene encoding the protein of interest, such that  
the probe will hybridize with the gene encoding the  
protein of interest under selected hybridization  
15 conditions. An alternate means of screening a DNA  
library is by polymerase chain reaction "PCR"  
amplification of the gene encoding the protein of  
interest. PCR is typically accomplished using  
oligonucleotide "primers" which have a sequence that is  
20 believed to have sufficient homology to the gene to be  
amplified such that at least a sufficient portion of  
the primer will hybridize with the gene.

Alternatively, a gene encoding the peptide of  
interest or protein of interest may be prepared by  
25 chemical synthesis using methods well known to the  
skilled artisan such as those described by Engels et  
al., *Angew. Chem. Intl. Ed.*, 28:716-734 (1989). These  
methods include, *inter alia*, the phosphotriester,  
phosphoramidite, and H-phosphonate methods for nucleic  
30 acid synthesis. A preferred method for such chemical  
synthesis is polymer-supported synthesis using standard  
phosphoramidite chemistry. Typically, the DNA encoding  
the protein of interest will be several hundred  
nucleotides in length. Nucleic acids larger than about  
35 100 nucleotides can be synthesized as several fragments  
using these methods. The fragments can then be ligated

- 19 -

together to form a gene coding for the full length protein of interest. Usually, the DNA fragment encoding the amino terminus of the polypeptide will have an ATG, which encodes a methionine residue. This  
5 methionine may or may not be present on the mature form of the protein of interest. The methionine can be removed inside the cell or during the process of secretion. Preferred TTR polypeptides may include TTR with the nucleic acid sequence altered to optimize  
10 expression in *E. coli* and to introduce convenient restriction sites. A general discussion of codon optimization for expression in *E. coli* is described in Kane, *Curr. Opin. Biotechnol.*, 6:494-500 (1995).

Once the genes encoding the protein of  
15 interest and the TTR polypeptide have been obtained, they may be modified using standard methods to create restriction endonuclease sites at the 5' and/or 3' ends. Creation of the restriction sites permits the genes to be properly inserted into amplification and/or  
20 expression vectors. Addition of restriction sites is typically accomplished using PCR, where one primer of each PCR reaction typically contains, *inter alia*, the nucleotide sequence of the desired restriction site.

The gene or cDNA encoding the peptide of  
25 interest, or protein of interest can be inserted into an appropriate expression vector for expression in a host cell. The vector is selected to be functional in the particular host cell employed (i.e., the vector is compatible with the host cell machinery such that  
30 amplification and/or expression of the gene encoding the protein of interest can occur).

Typically, the vectors used in any of the host cells will contain a promoter (also referred to as a "5' flanking sequence") and other regulatory elements  
35 as well such as an enhancer(s), an origin of replication element, a transcriptional termination



- 20 -

element, a ribosome binding site element, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element. Each of these elements is discussed below.

- 5 Optionally, the vector may contain a "tag" DNA sequence, *i.e.*, an oligonucleotide sequence located at either the 5' or 3' end of the fusion DNA construct. The tag DNA encodes a molecule such as hexaHis, *c-myc*, FLAG (Invitrogen, San Diego, CA) or another small  
10 immunogenic sequence. When placed in the proper reading frame, this tag will be expressed along with the fusion protein, and can serve as an affinity tag for purification of the fusion protein from the host cell. Optionally, the tag can subsequently be removed  
15 from the purified fusion protein by various means such as using a selected peptidase for example.

The promoter may be homologous (*i.e.*, from the same species and/or strain as the host cell), heterologous (*i.e.*, from a species other than the host  
20 cell species or strain), hybrid (*i.e.*, a combination of promoters from more than one source), synthetic, or it may be the native protein of interest promoter. Further, the promoter may be a constitutive or an inducible promoter. As such, the source of the  
25 promoter may be any unicellular prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the promoter is functional in, and can be activated by, the host cell machinery.

- 30 The promoters useful in the vectors of this invention may be obtained by any of several methods well known in the art. Typically, promoters useful herein will have been previously identified by mapping and/or by restriction endonuclease digestion and can  
35 thus be isolated from the proper tissue source using the appropriate restriction endonucleases. In some

- 21 -

cases, the full nucleotide sequence of the promoter may be known. Here, the promoter may be synthesized using the methods described above for nucleic acid synthesis or cloning.

5           Where all or only a portion of the promoter sequence is known, the complete promoter may be obtained using PCR and/or by screening a genomic library with suitable oligonucleotide and/or 5' flanking sequence fragments from the same or another  
10 species.

          Suitable promoters for practicing this invention are inducible promoters such as the *lux* promoter, the *lac* promoter, the arabinose promoter, the *trp* promoter, the *tac* promoter, the *tna* promoter,  
15 synthetic lambda promoters (from bacteriophage lambda), and the T5 or T7 promoters. Preferred promoters include the *lux*, and *lac* promoters.

          The origin of replication element is typically a part of prokaryotic expression vectors  
20 whether purchased commercially or constructed by the user. In some cases, amplification of the vector to a certain copy number can be important for optimal expression of the protein or polypeptide of interest. In other cases, a constant copy number is preferred.  
25 In any case, a vector with an origin of replication that fulfills the requirements can be readily selected by the skilled artisan. If the vector of choice does not contain an origin of replication site, one may be chemically synthesized based on a known sequence, and  
30 ligated into the vector.

          The transcription termination element is typically located 3' of the end of the fusion protein DNA construct, and serves to terminate transcription of the RNA message coding for the fusion polypeptide.  
35 Usually, the transcription termination element in prokaryotic cells is a G-C rich fragment followed by a

- 22 -

poly T sequence. While the element is easily cloned from a library or even purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis such as those

5 described above.

Expression vectors typically contain a gene coding for a selectable marker. This gene encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical  
10 selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline, chloramphenicol, or kanamycin for prokaryotic host cells, (b) complement auxotrophic deficiencies of the cell; or (c) supply critical  
15 nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, the chloramphenicol resistance gene, and the tetracycline resistance gene.

The ribosome binding element, commonly called  
20 the Shine-Dalgarno sequence in prokaryotes, is necessary for the initiation of translation of mRNA. The element is typically located 3' to the promoter and 5' to the coding sequence of the fusion protein DNA construct. The Shine-Dalgarno sequence is varied but  
25 is typically a polypurine (i.e., having a high A-G content). Many Shine-Dalgarno sequences have been identified, each of which can be readily synthesized using methods set forth above and used in a prokaryotic vector.

30 Where one or more of the elements set forth above are not already present in the vector to be used, they may be individually obtained and ligated into the vector. Methods used for obtaining each of the elements are well known to the skilled artisan and are  
35 comparable to the methods set forth above (i.e., synthesis of the DNA, library screening, and the like).

- 23 -

Each element may be individually ligated into the vector by cutting the vector with the appropriate restriction endonuclease(s) such that the ends of the element to be ligated in and the ends of the vector are compatible for ligation. In some cases, it may be necessary to "blunt" the ends to be ligated together in order to obtain a satisfactory ligation. Blunting can be accomplished by first filling in "sticky ends" using an enzyme such as Klenow DNA polymerase or T4 DNA polymerase in the presence of all four nucleotides. This procedure is well known in the art and is described for example in Sambrook *et al.*, *supra*.

Alternatively, two or more of the elements to be inserted into the vector may first be ligated together (if they are to be positioned adjacent to each other) and then ligated into the vector.

Another method for constructing the vector is to conduct all ligations of the various elements simultaneously in one reaction mixture. Here, many nonsense or nonfunctional vectors may be generated due to improper ligation or insertion of the elements, however the functional vector may be identified by expression of the selectable marker. Proper sequence of the ligation product can be confirmed by digestion with restriction endonucleases or by DNA sequencing.

After the vector has been constructed and a fusion protein DNA construct has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for fusion protein expression.

Host cells suitable for the present invention are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, JM109, DH5 $\alpha$ , DH10, and MC1061) are well-known host cells for use in preparing recombinant polypeptides. The choice of bacterial strain is typically made so that the strain and the

- 24 -

expression vector to be used are compatible. Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in practicing this invention in conjunction with appropriate expression vectors.

Insertion (also referred to as "transformation" or "transfection") of the vector into the selected host cell may be accomplished using such methods as calcium phosphate precipitation or electroporation. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook et al., *supra*.

The host cells containing the vector (*i.e.*, transformed or transfected host cells) may be cultured using one or more standard media well known to the skilled artisan. The selected medium will typically contain all nutrients necessary for the growth and survival of the host cells. Suitable media for culturing *E. coli* cells, are, for example, Luria broth ("LB"), YT broth, SOB, SOC, and/or Terrific Broth ("TB").

There are several ways to prepare the DNA construct encoding the fusion protein which comprises the TTR gene, the gene encoding the peptide or protein of interest, and, optionally, a DNA molecule encoding a linker peptide which is located between the two genes.

In one procedure, the TTR gene and gene encoding the protein of interest (the "fusion partner genes") can be ligated together in either orientation (*e.g.*, TTR gene at the 5' or 3' end of the construct). Where a linker DNA molecule is to be included, it can first be ligated to one of the fusion partner genes, and that construct can then be ligated to the other fusion partner gene. Ligations are typically

- 25 -

accomplished using DNA ligase enzyme in accordance with the manufacturer's instructions.

A separate procedure provides for first ligating one fusion partner gene into the selected vector, after which the other fusion partner gene can be ligated into the vector in a position that is either 3' or 5' to the first fusion partner gene. Where a linker DNA molecule is to be included, the linker DNA molecule may be ligated to either fusion partner gene either before or after that gene has been ligated into the vector.

The TTR-TMPs of the present invention can be used to treat conditions generally known as those that involve an existing megakaryocyte/platelet deficiency or an expected megakaryocyte/platelet deficiency (*e.g.*, because of planned surgery or platelet donation). Such conditions will usually be the result of a deficiency (temporary or permanent) of active Mpl ligand *in vivo*. The generic term for platelet deficiency is thrombocytopenia, and hence the methods and compositions of the present invention are generally available for treating thrombocytopenia in patients in need thereof. Thrombocytopenia (platelet deficiencies) may be present for various reasons, including chemotherapy and other therapy with a variety of drugs, radiation therapy, surgery, accidental blood loss, and other specific disease conditions.

Exemplary specific disease conditions that involve thrombocytopenia and may be treated in accordance with this invention are: aplastic anemia, idiopathic thrombocytopenia, metastatic tumors which result in thrombocytopenia, systemic lupus erythematosus, splenomegaly, Fanconi's syndrome, vitamin B12 deficiency, folic acid deficiency, May-Hegglin anomaly, Wiskott-Aldrich syndrome, and paroxysmal nocturnal hemoglobinuria. Also, certain

- 26 -

treatments for AIDS result in thrombocytopenia (e.g., AZT). Certain wound healing disorders might also benefit from an increase in platelet numbers.

With regard to anticipated platelet  
5 deficiencies, e.g., due to future surgery, a compound of the present invention could be administered several days to several hours prior to the need for platelets. With regard to acute situations, e.g., accidental and massive blood loss, a compound of this invention could  
10 be administered along with blood or purified platelets.

The TMP compounds of this invention may also be useful in stimulating certain cell types other than megakaryocytes if such cells are found to express Mpl receptor. Conditions associated with such cells that  
15 express the Mpl receptor, which are responsive to stimulation by the Mpl ligand, are also within the scope of this invention.

The TMP compounds of this invention may be used in any situation in which production of platelets  
20 or platelet precursor cells is desired, or in which stimulation of the c-Mpl receptor is desired. Thus, for example, the compounds of this invention may be used to treat any condition in a mammal wherein there is a need of platelets, megakaryocytes, and the like.  
25 Such conditions are described in detail in the following exemplary sources: WO95/26746; WO95/21919; WO95/18858; WO95/21920 and are incorporated herein.

The TMP compounds of this invention may also be useful in maintaining the viability or storage life  
30 of platelets and/or megakaryocytes and related cells. Accordingly, it could be useful to include an effective amount of one or more such compounds in a composition containing such cells.

The therapeutic methods, compositions and  
35 compounds of the present invention may also be employed, alone or in combination with other cytokines,

- 27 -

soluble Mpl receptor, hematopoietic factors, interleukins, growth factors or antibodies in the treatment of disease states characterized by other symptoms as well as platelet deficiencies. It is anticipated that the inventive compound will prove useful in treating some forms of thrombocytopenia in combination with general stimulators of hematopoiesis, such as IL-3 or GM-CSF. Other megakaryocytic stimulatory factors, i.e., meg-CSF, stem cell factor (SCF), leukemia inhibitory factor (LIF), oncostatin M (OSM), or other molecules with megakaryocyte stimulating activity may also be employed with Mpl ligand. Additional exemplary cytokines or hematopoietic factors for such co-administration include IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), SCF, GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), consensus interferon, IFN-beta, or IFN-gamma. It may further be useful to administer, either simultaneously or sequentially, an effective amount of a soluble mammalian Mpl receptor, which appears to have an effect of causing megakaryocytes to fragment into platelets once the megakaryocytes have reached mature form. Thus, administration of an inventive compound (to enhance the number of mature megakaryocytes) followed by administration of the soluble Mpl receptor (to inactivate the ligand and allow the mature megakaryocytes to produce platelets) is expected to be a particularly effective means of stimulating platelet production. The appropriate dosage would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

35        In non-insulin dependent diabetes mellitus (NIDDM), also known as type 2 diabetic patients, the



- 28 -

administration of glucagon-like peptide-1 (GLP-1) has antidiabetic properties. However, GLP-1 is rapidly degraded by dipeptidyl peptidase IV (DPP-IV) after its release *in vivo*. Thus, it is an advantage of the present invention that a GLP-1 peptide or variant thereof can be fused to a TTR polypeptide of the invention to stabilize GLP-1 and increase its half life *in vivo*. Accordingly, in another embodiment of the invention, a TTR-GLP1 fusion protein as described herein can be used to treat conditions generally known to involve non-insulin dependent diabetes mellitus (NIDDM), which is also known as type II diabetes.

One of skill in the art will recognize that the sequence of a GLP-1 peptide can be varied such that it retains its insulinotropic effects. Particular examples of such variations known in the art include, for example, GLP-1(7-34), (7-35), (7-36) or (7-37), Gln<sup>3</sup>-GLP-1(7-37), D-Gln<sup>3</sup>-GLP-1(7-37), Thr<sup>16</sup>-Lys<sup>18</sup>-GLP-1(7-37), and Lys<sup>18</sup>-GLP-1(7-37). Additional examples of GLP-1 variants are described in U.S. Patent Nos. 5,118,666, 5,545,618, 5,977,071, and WO 02/46227 and in Adelhorst et al., J. Biol. Chem. 269:6275 (1994), which are incorporated by reference. Accordingly, any GLP-1 peptide can be used to generate fusion proteins of the invention, as long as the GLP-1 fusion protein is capable of binding and inducing a signal through its cognate receptor. Receptor binding and activation can be measured by standard assays (U.S. Patent No. 5,120,712).

The dose of fusion protein effective to normalize a patient's blood glucose will depend on a number of factors among which are included the subject's weight, age, severity of their inability to regulate blood glucose, the route of administration, the bioavailability, the pharmacokinetic profile of the

- 29 -

fusion protein and the formulation as is discussed more fully below.

The therapeutic methods, compositions and compounds of the present invention may also be  
5 employed, alone or in combination with other diabetes treatments, including but not limited to insulin, DPPIV-inhibitors and the like. The dosage of the GLP-1 fusion protein would be adjusted to compensate for such additional components in the therapeutic composition.  
10 Progress of the treated patient can be monitored by conventional methods, such as, for example, the monitoring of blood glucose levels.

The present invention also provides pharmaceutical compositions of the inventive compounds.  
15 Such pharmaceutical compositions may be for administration for injection, or for oral, nasal, transdermal or other forms of administration, including, e.g., by intravenous, intradermal, intramuscular, intramammary, intraperitoneal,  
20 intrathecal, intraocular, retrobulbar, intrapulmonary (e.g., aerosolized drugs) or subcutaneous injection (including depot administration for long term release); by sublingual, anal, vaginal, or by surgical  
25 implantation, e.g., embedded under the splenic capsule, brain, or in the cornea. The treatment may consist of a single dose or a plurality of doses over a period of time. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of a compound of the invention together with  
30 pharmaceutically acceptable diluents, preservatives, stabilizers, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate, citrate, etc.), pH and ionic strength;  
35 additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80, etc.), anti-oxidants

- 30 -

(e.g., ascorbic acid, sodium metabisulfite),  
preservatives (e.g., Thimersol, benzyl alcohol) and  
bulking substances (e.g., lactose, mannitol);  
incorporation of the material into particulate  
5 preparations of polymeric compounds such as polylactic  
acid, polyglycolic acid, etc. or into liposomes.  
Hyaluronic acid may also be used, and this may have the  
effect of promoting sustained duration in the  
circulation. The pharmaceutical compositions  
10 optionally may include still other pharmaceutically  
acceptable liquid, semisolid, or solid diluents that  
serve as pharmaceutical vehicles, excipients, or media,  
including but are not limited to, polyoxyethylene  
sorbitan monolaurate, magnesium stearate, methyl- and  
15 propylhydroxybenzoate, starches, sucrose, dextrose, gum  
acacia, calcium phosphate, mineral oil, cocoa butter,  
and oil of theobroma. Such compositions may influence  
the physical state, stability, rate of *in vivo* release,  
and rate of *in vivo* clearance of the present proteins  
20 and derivatives. See, e.g., Remington's Pharmaceutical  
Sciences, 18th Ed. (1990, Mack Publishing Co., Easton,  
PA 18042) pages 1435-1712 which are herein incorporated  
by reference. The compositions may be prepared in  
liquid form, or may be in dried powder, such as  
25 lyophilized form. Implantable sustained release  
formulations are also contemplated, as are transdermal  
formulations.

Controlled release formulation may be  
desirable. The drug could be incorporated into an  
30 inert matrix which permits release by either diffusion  
or leaching mechanisms e.g., gums. Slowly degenerating  
matrices may also be incorporated into the formulation,  
e.g., alginates, polysaccharides. Another form of a  
controlled release of this therapeutic is by a method  
35 based on the Oros therapeutic system (Alza Corp.),  
i.e., the drug is enclosed in a semipermeable membrane

- 31 -

which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

- Also contemplated herein is pulmonary
- 5 delivery of the present protein (or derivatives thereof). The protein (or derivative) is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream. (Other reports of this include Adjei et al.,
  - 10 Pharmaceutical Research 7:565-569 (1990); Adjei et al., International Journal of Pharmaceutics 63:135-144 (1990) (leuprolide acetate); Braquet et al., Journal of Cardiovascular Pharmacology 13 (suppl.5): s.143-146 (1989) (endothelin-1); Hubbard et al., Annals of
  - 15 Internal Medicine 3:206-212 (1989) (1-antitrypsin); Smith et al., J. Clin. Invest. 84:1145-1146 (1989) (1-proteinase); Oswein et al., "Aerosolization of Proteins", Proceedings of Symposium on Respiratory Drug Delivery II, Keystone, Colorado, March, 1990
  - 20 (recombinant human growth hormone); Debs et al., The Journal of Immunology 140:3482-3488 (1988) (interferon- and tumor necrosis factor ) and Platz et al., U.S. Patent No. 5,284,656 (granulocyte colony stimulating factor).

- 25 Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of
- 30 which are familiar to those skilled in the art.

- Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II
- 35 nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler,

- 32 -

manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

All such devices require the use of  
5 formulations suitable for the dispensing of the inventive compound. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to diluents, adjuvants and/or carriers useful  
10 in therapy.

The inventive compound should most advantageously be prepared in particulate form with an average particle size of less than 10 $\mu$ m (or microns), most preferably 0.5 to 5 $\mu$ m, for most effective delivery  
15 to the distal lung.

Carriers include carbohydrates such as trehalose, mannitol, xylitol, sucrose, lactose, and sorbitol. Other ingredients for use in formulations may include DPPC, DOPE, DSPC and DOPC. Natural or  
20 synthetic surfactants may be used. Polyethylene glycol may be used (even apart from its use in derivatizing the protein or analog). Dextrans, such as cyclodextran, may be used. Bile salts and other related enhancers may be used. Cellulose and cellulose  
25 derivatives may be used. Amino acids may be used, such as use in a buffer formulation.

The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician, considering  
30 various factors which modify the action of drugs, *e.g.* the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the dose should be in the range of 0.1  $\mu$ g to 100 mg of  
35 the inventive compound per kilogram of body weight per day, preferably 0.1 to 1000  $\mu$ g/kg; and more preferably

- 33 -

0.1 to 150  $\mu\text{g/kg}$ , given in daily doses or in equivalent doses at longer or shorter intervals, *e.g.*, every other day, twice weekly, weekly, or twice or three times daily.

5           The inventive compounds may be administered by an initial bolus followed by a continuous infusion to maintain therapeutic circulating levels of drug product. As another example, the inventive compounds may be administered as a one-time dose. Those of  
10 ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient. The frequency of dosing will depend on the pharmacokinetic parameters of  
15 the agents and the route of administration. The optimal pharmaceutical formulation will be determined by one skilled in the art depending upon the route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990,  
20 Mack Publishing Co., Easton, PA 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface area or organ size.

25           Appropriate dosages may be ascertained through use of established assays for determining serum levels in conjunction with appropriate dose-response data. The final dosage regimen will be determined by the attending physician, considering various factors  
30 which modify the action of drugs, *e.g.* the drug's specific activity, the severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other  
35 clinical factors. As studies are conducted, further information will emerge regarding the appropriate

- 34 -

dosage levels and duration of treatment for various diseases and conditions.

The following Examples are intended for illustration purposes only, and should not be construed to limit the invention in any way.

Example 1

10 This example describes the preparation of DNA for native recombinant human transthyretin (TTR) and the following TTR variants: TTR(C10A), TTR(C10A/A37C), TTR(C10A/D38C), TTR(C10A/A81C), TTR(C10A/G83C), and TTR(C10A/K15A/G83C).

15 The expression plasmid pAMG21 is available from the ATCC under accession number 98113, which was deposited on July 24, 1996 (see PCT WO 97/23614, published 3 July 1997 for a description of pAMG21). DNA sequence coding for TTR, TTR variants or TTR-peptide fusions was placed under control of the LuxPR promoter in pAMG21.

The bacterial host GM221 is an *E.coli* K-12 strain that has been modified to contain both the temperature sensitive lambda repressor cI857s7 in the early *ebg* region and the lacI<sup>0</sup> repressor in the late *ebg* region (68 minutes). The presence of these two repressor genes allows the use of this host with a variety of expression systems, however both of these repressors are irrelevant to the expression from luxP<sub>a</sub>.  
25 The untransformed host has no antibiotic resistances. The ribosomal binding site of the cI857s7 gene has been modified to include an enhanced RBS. It has been inserted into the *ebg* operon between nucleotide position 1170 and 1411 as numbered in Genbank accession number M64441Gb\_Ba with deletion of the intervening *ebg* sequence. The construct was delivered to the  
35

- 35 -

chromosome using a recombinant phage called MMeBg-cI857s7 enhanced RBS #4 into F'tet/393. After recombination and resolution only the chromosomal insert described above remains in the cell. It was  
5 renamed F'tet/GM101. F'tet/GM101 was then modified by the delivery of a lacI<sup>c</sup> construct into the *ebg* operon between nucleotide position 2493 and 2937 as numbered in the Genbank accession number M64441Gb\_Ba with the deletion of the intervening *ebg* sequence. The  
10 construct was delivered to the chromosome using a recombinant phage called AGebg-lacI<sup>o</sup> #5 into F'tet/GM101. After recombination and resolution only the chromosomal insert described above remains in the cell. It was renamed F'tet/GM221. The F'tet episome  
15 was cured from the strain using acridine orange at a concentration of 25 µg/ml in LB. The cured strain was identified as tetracycline sensitive and was stored as GM221.

Oligonucleotides (1.0 nm each) were  
20 synthesized by phosphoramidite method. Nucleotides were, in some cases, altered for optimized expression in *E. coli*. These codon changes did not result in changes in the amino acid sequence. Each of the oligonucleotides utilized in this example are listed in  
25 Table 1.

PCR was performed with the Expand Long Polymerase according to the manufacturer's protocol (Boehringer Mannheim). PCR products were verified by agarose gel electrophoresis, purified and digested with  
30 NdeI and XhoI (New England Biolabs). Expression vector pAMG21 was digested in the same manner and then treated with calf intestinal phosphatase (Boehringer Mannheim). The vector and insert were purified from an agarose gel, then mixed and ligated by T4 DNA ligase (New  
35 England Biolabs). Ligation was done at 4°C for 2 hrs.



- 36 -

Each ligation mixture was transformed by electroporation into the host strain GM221 described above with a Biorad GenePulser (Biorad Laboratories) using 2.5V, 25uFD, and 200 ohms in a cuvette with a gap  
5 length of about 2 mm. After electroporation, the cells were allowed to recover in 1 ml of Luria broth (LB) for about one hour at 37°C with gentle shaking. The entire transformation mix was plated on LB agar containing 50ug/ml kanamycin. Colonies were screened for presence  
10 of the desired molecular weight by PCR using oligonucleotides directed against flanking vector sequence. The PCR products were evaluated by agarose gel electrophoresis. Positive clones were further screened for the ability to produce the recombinant  
15 protein product and finally verified by nucleotide sequencing.

The DNA and amino acid sequences of TTR are known (Mita, S et al., Biochem. Biophys. Res. Commun. 124 (2), 558-564 [1984]). These sequences have been  
20 deposited in Genbank as accession number K02091. The cDNA of native TTR excluding the signal peptide was cloned from a cDNA library derived from human liver (Clontech). Specifically, an oligonucleotide encoding eight codons of the TTR 5' (Oligo 2693-79) end and an  
25 oligonucleotide encoding seven codons of TTR 3' end including a terminating codon (Oligo 2693-80) were synthesized and used to amplify the full-length mature TTR with Expand Long polymerase using human liver cDNA library as template. The resulting PCR fragment was  
30 digested with NdeI and XhoI, gel purified and ligated with NdeI/XhoI restricted expression vector pAMG21. After 2 hours at 4°C, the ligation mixture was electroporated into GM221 cells. Single colonies were picked and plasmid DNA was prepared and sequenced. One  
35 resulting plasmid (strain #5316) was shown to have the

- 37 -

correct DNA sequence of native TTR (plus a methionine at the N-terminus) and was used for expression. This DNA sequence is identified in SEQ ID NO:2.

Mutant TTR(C10A) was made by using

- 5 oligonucleotide 2693-80 above and oligonucleotide 2820-88 (encompasses the first 11 codons of native TTR in which the codon Cys at the tenth position was changed to Ala). The PCR procedure and the process for selecting the expression strain were similar to that  
10 described above. The resulting strain (strain #5619) had the DNA sequence identified in SEQ ID NO:3.

Plasmid 5619 was further modified by replacing the amino acids at the following positions: A37, D38, A81 and G83, with the amino acid Cysteine.

- 15 As described below, each pair of the complementary oligonucleotides harboring the desired mutations was used in conjunction with TTR 5' and 3' primers described above in a standard two-step PCR procedure designed for site-specific mutagenesis. Each of the  
20 forward primers were used with a TTR 3' primer and each of the reverse primers were used with a TTR 5' primer in a 20-cycle PCR in which plasmid derived from strain 5619 was used as the template. The resulting PCR amplified 5' and 3' fragments were mixed and used as  
25 the template for the second step PCR to generate the full-length mutants. Subsequent cloning and sequencing procedures were similar to those already described. The following oligonucleotides were utilized: TTR(A37C) forward (Oligo 2823-91); TTR(A37C) reverse (Oligo 2823-92); TTR(D38C) forward (Oligo 2823-93); TTR(D38C)  
30 reverse (Oligo 2823-94); TTR(A81C) forward (Oligo 2823-95); TTR(A81C) reverse (Oligo 2823-96); TTR(G83C) forward (Oligo 2823-97); TTR(G83C) reverse (Oligo 2823-98). The resulting *E. coli* strains containing the  
35 plasmids are described as follows:  
TTR(C10A/A37C) (strain 5641) had the DNA sequence

- 38 -

identified in SEQ ID NO:4. TTR(C10A/D38C)(strain 5642)  
 had the DNA sequence identified in SEQ ID NO:5.  
 TTR(C10A/A81C)(strain 5643) had the DNA sequence  
 identified in SEQ ID NO:6. TTR(C10A/G83C)(strain 5651)  
 5 had the DNA sequence identified in SEQ ID NO:7.  
 The Lys in the 15th position in strain 5651  
 was further mutagenized to Ala using oligonucleotides  
 2953-67 and 2953-68 by a procedure similar to that  
 described for strains 5641, 5642, 5643 and 5651.  
 10 The resulting strain, TTR(C10A/K15A/G83C)(strain 5895)  
 had the DNA sequence identified in SEQ ID NO:8.

Table 1

15	Oligo	Sequence	SEQ ID Number
	2693-79	GAGGAATAACATATGGGTCCAACCTGGTACCGGTGAA	18
	2693-80	CCGCGGATCCTCGAGATTATCCTTGGGATTGCTGA	19
	2820-88	GAGGAATAACATATGGGTCCAACCTGGTACCGGTGAA	
20		TCCAAGGCTCCT	20
	2823-91	AGAAAGGCTTGTGATGACACCTGG	21
	2823-92	CCAGGTGTCATCACAAGCCTTTCT	22
	2823-93	AGAAAGGCTGCTTGTGACACCTGG	23
	2823-94	CCAGGTGTCACAAGCAGCCTTTCT	24
25	2823-95	TACTGGAAGTGTCTTGGCATCTCC	25
	2823-96	GGAGATGCCAAGACACTTCCAGTA	26
	2823-97	AAGGCACCTTTCATCTCCCCATTC	27
	2823-98	GAATGGGGAGATGCAAAGTGCCTT	28
	2953-67	CTGATGGTCCAGTTCTAGAT	29
30	2953-68	ATCTAGAACTGCGACCATCAG	30

Example 2

This example describes the preparation of  
 35 various TMP-TTR fusions. Several fusion proteins  
 containing TTR and a TMP were prepared. Each of the  
 oligonucleotides utilized in this example are listed in  
 Table 2.

A fragment containing the TMP was first  
 40 amplified from a strain harboring a plasmid encoding a  
 full-length TMP-Fc fusion (see PCT Publication No.  
 00/24770) using oligonucleotides 2743-96 which encodes

- 39 -

the first 7 codons of the TMP plus a 12 nucleotide 5' extension including a NdeI site and 2743-97 which encodes the first 7 codons of native TTR and the last 7 codons of the TMP of interest. The resulting PCR  
 5 fragment was mixed with plasmid derived from strain 5619 and the mixture was used as a template for oligonucleotide primers 2743-96 and 2693-80 to amplify full-length TMP-TTR. Similar procedures described above were used for cloning and expression. The  
 10 resulting strain, TMP-TTR (strain 5513) had the DNA sequence identified in SEQ ID NO:9.

The TMP was then introduced to the N-terminus of strains 5641, 5642, 5643 and 5651, respectively. Plasmid 5513 was digested with XbaI, the resulting  
 15 XbaI/XbaI insert containing the TMP and the first 18 codons of TTR(C10A) was gel purified and ligated with XbaI restricted, phosphatase treated and gel purified vector derived from 5641, 5642, 5643 and 5651. DNA sequencing was performed to select the correct  
 20 orientation for each fusion. The resulting *E. coli* strains containing the plasmids are described as follows: TMP-TTR(C10A/A37C) (strain 5704) had the DNA sequence identified in SEQ ID NO:10. TMP-TTR(C10A/D38C) (strain 5705) had the DNA sequence  
 25 identified in SEQ ID NO:11. TMP-TTR(C10A/A81C) (strain 5706) had the DNA sequence identified in SEQ ID NO:12. TMP-TTR(C10A/G83C) (strain 5707) had the DNA sequence identified in SEQ ID NO:13.

30 Table 2

Oligo	Sequence	SEQ ID Number
2743-96	GAGGAATAACATATGATCGAAGGTCCGACTCTGCGT	31
2743-97	TTCACCGGTACCAGTTGGACCTGCGCGTGTGCAAG	
35	CCATT	32

Example 3

- 40 -

This example describes the preparation of PTH (1-34)-TTR(C10A/K15A/G83C) fusion. Each of the oligonucleotides utilized in this example are listed in Table 3.

Two new oligonucleotides, oligonucleotide 2694-01, which encodes the first 7 codons of human PTH, and oligonucleotide 2694-03, which encodes the first 7 codons of TTR and amino acids 28-34 of PTH, were synthesized to make the fusion. Oligonucleotides 2694-01 and 2694-03 were used in a 20-cycle PCR procedure as described above to amplify PTH (1-34) with the TTR linker. The template for this reaction was a strain which harbors a plasmid encoding a PTH1-34-Fc fusion (see PCT Publication No. 01/81415). The resulting PCR mixture was combined with strain 5895 and used as the template to amplify the full length PTH (1-34)-TTR(C10A/K15A/G83C) using primers 2694-01 and 2693-80. After sequence confirmation, the resulting expression strain containing the new plasmid was designated PTH-TTR(C10A/K15A/G83C) (strain 5920) and had the DNA sequence identified in SEQ ID NO:14.

Table 3

Oligo	Sequence	SEQ ID Number
2694-01	GAGGAATAACATATGCTCTGTTCTGAAATCCAG	33
2694-03	TTCACCGGTACCAGTTGGACCAAAGTTATGAACGTC	34

Example 4

This example describes the preparation of an IL-1ra-TTR(C10A) fusion and a TTR(C10A)-GSGS-IL-1ra fusion. Each of the oligonucleotides utilized in this example are listed in Table 4.

To prepare the IL-1ra-TTR(C10A) fusion, two oligonucleotides, oligonucleotide 2823-13, which encodes the first 7 codons of the human protein IL-1ra, and oligonucleotide 2823-14, which encodes the last 7

- 41 -

amino acids of IL-1ra and the first 7 amino acids of TTR, were synthesized. The plasmid derived from a strain which expresses IL-1ra (see PCT Publication No. 91/08285) was amplified using oligonucleotides 2823-13 and 2823-14. The resulting PCR product was mixed with plasmid purified from strain 5619 and used as a template to amplify full-length IL-1-ra-TTR(C10A) using oligonucleotide primers 2823-13 and 2693-80. The PCR product was cloned, sequenced and expressed as described above. The resultant strain containing the new plasmid was designated IL-1ra-TTR(C10A) (strain 5644) and had the DNA sequence identified in SEQ ID NO:15.

To make TTR(C10A)-IL-1ra, the following two oligonucleotides, oligonucleotide 2787-32, which encodes the last 7 amino acids of TTR, the first 7 amino acids of IL-1-ra between which a GSGS linker was introduced, and oligonucleotide 2787-33, which encodes the last 7 codons of IL-1-ra, were synthesized. These two oligonucleotide primers were used to amplify plasmid 2693, and the resulting PCR product was mixed with plasmid 5619, and together these were used as a template to amplify full-length TTR(C10A)-IL-1ra using primers 2787-33 and 2693-79. The PCR product was cloned, sequenced and expressed as described above. The resultant strain containing the new plasmid was designated TTR(C10A)-IL-1ra (strain 5645) and had the DNA sequence identified in SEQ ID NO:16.

Table 4

Oligo	Sequence	SEQ ID Number
2823-13	GAGGAATAACATATGCGACCGTCCGGACGTAA	35
2823-14	TTCTACTTCCAGGAAGACGAAGGTCCAAC TGGTACC	36
2787-32	GTCGTCAACCAATCCCAAGGAAGGTAGTGGTAGCCGA CCGTCCGGCCGTAAGAGC	37
2787-33	CCGCGGATCCTCGAGATTATTCGTCTTCCTGGAAGT AGAA	38

- 42 -

Example 5

This example describes the preparation of  
 5 TTR(C10A/G83C)-Bradykinin. Each of the  
 oligonucleotides utilized in this example are listed in  
 Table 5.

Plasmid purified from strain 5651 was used  
 for PCR with oligonucleotide primer 2693-79 and  
 10 oligonucleotide primer 2943-47, which is a TTR 3'  
 primer containing a PstI restriction site. This PCR  
 product was gel purified and restriction digested with  
 NdeI and PstI. The resulting DNA fragment was used in  
 a ligation mixture containing AMG21, digested with NdeI  
 15 and XhoI, and the annealed oligonucleotide linkers  
 2943-48, which encodes the GSGSG linker, and  
 oligonucleotide 2943-49, which encodes the Bradykinin  
 antagonist peptide KRPPGFSP with PstI 5' and XhoI 3'  
 overlapping ends. GM121 was transformed with this  
 20 ligation product and DNA was purified from the  
 kanamycin resistant colonies. The DNA sequence was  
 then confirmed in the resistant colonies. The  
 confirmed strain was grown at 30°C and induced for  
 expression in a 10-liter fermentation described below.  
 25 The new strain was designated TTR(C10A/G83C)-Bradykinin  
 (strain 5914) and had the DNA sequence identified in  
 SEQ ID NO:17.

Table 5

30	Oligo	Sequence	SEQ ID
			Number
	2693-79	GAGGAATAACATATGGGTCCAACCTGGTACCGGTGAA	39
	2943-47	AATATACTGCAGTGGTGGAAATAGGAG	40
	2943-48	GTCGTCACCAATCCCAAGGAAGGATCAGGATCCGA	
35		AAACGTCCGCCGGGTTCTCCCCGCTGTAATC	41
	2943-49	TCGAGATTACAGCGGGCAGAAACCCGCCGACGTTTT	
		CCGGATCCTGATCCTTCCTGGGATGGTGACGACTGCA	42

EXAMPLE 6

- 43 -

This example describes the recombinant expression of TTR and the TTR fusion constructs in *E. coli*. Each of the newly constructed TTR or TTR fusions were first examined for soluble expression at temperatures ranging from 16°C to 37°C. For this purpose, cultures (25 ml) of GM221 expressing each of the TTR or TTR fusions were grown in LB medium supplemented with 50 µg/ml kanamycin at 37°C until the optical density (OD) at 600nm reached 0.5 to 1.0. The cultures were then placed in shakers with temperature settings at 16°C, 20°C, 25°C, 30°C, 34°C and 37°C, respectively. The induction of gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 20 ng/ml. After 6 hours, the bacterial cultures were examined by microscopy for the presence of inclusion bodies. Often soluble or partial soluble expression could be achieved by growing the cultures at temperatures lower than 30°C for TTR and its fusions, and this temperature was used for large-scale expression. In cases where soluble expression could not be achieved, temperatures at which the level of expression was at the highest were used for large-scale shakers or fermentors.

Large-scale expression was normally done in 4 liter flasks. Four to eight 4 liter shakers containing 1 liter of LB was inoculated with overnight cultures of TTR or its fusion strains. Expression was done essentially as described above. Cells were collected by centrifugation.

The fermentation stage, employing aseptic technique, begins with the inoculation from a seed culture of strains produced in a shake flask containing



- 44 -

500 mL of sterilized Luria broth. When this culture obtained the appropriate cell density (0.8 - 2 at 600nm), the contents were used to inoculate a 20 liter fermentor containing 10 liter of complex based growth medium. The fermentor is maintained at 30°C and pH 7 with dissolved oxygen levels kept at 30% saturation. When the cell density reached an optical density of 10-12 OD units at 600 nm, at which point the culture was induced by the addition of N-(3-oxo-hexanoyl) homoserine lactone. At 6 hours post-induction the cells were harvested from the fermentor by centrifugation.

#### EXAMPLE 7

15 This example describes the purification of TTR(C10A/G83C)-Bradykinin. About 193 g of *E. coli* paste from clone 5914 stored at -80°C was defrosted in 1447 ml of 50 mM tris HCl, 5 mM EDTA, pH 8.0. 50 tablets of Sigma protease inhibitor cocktail 1-873-580 (Saint Louis, MO) was dissolved in the cell suspension and the suspension was passed through a model 110-Y microfluidizer (Microfluidics, Newton, MA) twice at 12,000 PSI. The lysate (Figure 1, Lane 2) was 25 centrifuged at 11,325 x g for 50 min 4°C. The supernatant was removed as the soluble fraction. The soluble fraction was heated in a 65°C water bath for 30 minutes in polypropylene bottles, at which time the temperature of the contents was 63°C. The soluble 30 fraction was centrifuged at 11,325 x g for 50 minutes 4°C. The supernatant was removed as Heat Soluble (Figure 1, Lane 3). The heat soluble fraction was filtered through a 0.45 µm cellulose acetate filter with two prefilters and then loaded on to a 240 ml

- 45 -

Q-sepharose fast flow (5 cm ID) column (Amersham Pharmacia Biotech, Piscataway, NJ) at 20 ml/min equilibrated in Q-Buffer A (20 mM tris HCl, 2.5 mM EDTA, pH 8.0) at room temperature (about 23°C). Column

5 was washed with about 2300 ml Q-Buffer A at 20 ml/min. Q-column was eluted with a 15 column volume linear gradient to 60% Q-Buffer B (20 mM tris HCl, 1 M NaCl, 2.5 mM EDTA, pH 8.0) followed by a 2 column volume step to 100% Q-Buffer B. Fractions containing the TTR

10 fusion as determined by SDS-PAGE were pooled into a single Q-pool (1150 ml) (Figure 1, Lane 4) and 1.77 g of DTT was added. The Q-pool was gently stirred for 30 min at room temperature (about 23°C). To the Q-pool,

15 410 ml of 3.8 M ammonium sulfate pH 7.0 was slowly added and the pH was lowered from about 7.5 to 7.0 by slow addition of 1 M HCl. About one-half of the Q-pool was then loaded on to an 84 ml phenyl sepharose high performance column (2.6 cm ID) (Amersham Pharmacia Biotech) in P-Buffer A (50 mM  $\text{NaH}_2\text{PO}_4$ , 1 M ammonium

20 sulfate, pH 7.0) at 10 ml/min. The column was washed with about 170 ml P-Buffer A followed by three step elutions using 50%, 80%, and 100% P-Buffer B (50 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.0). The remaining half of the Q-pool was then processed using the same protocol as the first

25 half. Fractions containing the TTR fusion as determined by SDS-PAGE were pooled into a single P-pool (260 ml) (Figure 1, Lane 5) and the P-pool was dialyzed against 4 L of HA-Buffer A (10 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.0) for 2 hours at room temperature (about 23 °C) using 20.4 mm

30 diameter 8 kDa cutoff dialysis tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA). The dialysis buffer was changed with a fresh 4 L of HA-Buffer A and dialysis was continued for approximately an additional 15 hours. The P-pool was removed from dialysis and 600

35  $\mu\text{l}$  of 1 M DTT was added followed by incubation at room

- 46 -

temperature (about 23 °C) for about 1 hour. P-pool was loaded on to a 105 ml (2.6 cm) type 1 ceramic hydroxyapatite column (Bio-Rad Inc., Hercules, CA) at 10 ml/min in HA-Buffer A. Column was washed with  
5 approximately 210 ml HA-Buffer A at 10 ml/min followed by 4 steps of 12.5%, 25%, 50%, and 100% HA-Buffer B (400 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.0). The flowthrough was pooled as HA-pool (340 ml) (Figure 1, Lane 6) and 524 mg of DTT was added followed by incubation at room temperature  
10 (about 23°C) for 1 hour.

About one-half of the HA-pool was loaded on to a 47 ml source 15Q (2.6 cm ID) column (Amersham Pharmacia Biotech) at 10 ml/min followed by a wash with about 250 ml Q-Buffer A. Column was eluted with a 20  
15 column volume linear gradient from 10% to 50% Q-Buffer B followed a step of 2 column volumes of 100% Q-Buffer B. The remaining half of the HA-Pool was then processed using the same protocol as the first half. Fractions containing the TTR fusion as determined by  
20 SDS-PAGE were pooled into a single Q2-pool (260 ml) and concentrated to about 75 ml using a stirred cell with a 10 kDa membrane. Q2-pool (Figure 1, Lane 7) was then filtered through a 0.22  $\mu\text{m}$  cellulose acetate filter and the protein concentration was determined to be 16.9  
25 mg/ml using a calculated extinction coefficient of 18,450  $\text{M}^{-1} \text{cm}^{-1}$ . The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Falmouth, MA). The nucleic acid content was determined to be negligible,  
30 since the ratio of the absorbance at 260 nm over 280 nm was determined to be 0.52.

EXAMPLE 8

- 47 -

This example demonstrates that fusing a peptide to either the C-terminus or N-terminus of TTR(C10A/G83C) does not have a significant impact on its oligomeric structure. TTR(C10A/G83C), PTH-  
5 TTR(C10A/K15A/G83C), and TTR(C10A/G83C)-Bradykinin in 20 mM tris pH 8.0 and about 250 mM NaCl were reduced with 9 mM DTT for about 1 hour at room temperature (about 23 °C). About 50 µg of the reduced TTR was injected on to a Biosep-Sec-S 3000 column (7.8 mm ID X  
10 300 mm) (Phenomenex, Torrance, CA) in SEC-Buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, pH 6.7) at 1 ml/min. Bio-Rad molecular weight standards (151-1901) were used to calibrate the column and calculate the approximate molecular size of the injected samples. As can be seen  
15 in Figure 2, TTR(C10A/G83C) eluted at approximately 8.8 min corresponding to a molecular size of 49 kDa, which is comparable to the calculated molecular weight of the tetramer at 55 kDa. PTH-TTR(C10A/K15A/G83C) eluted at about 8.6 min corresponding to a molecular size of 67  
20 kDa, which is close to the calculated 71 kDa for the tetramer. TTR(C10A/G83C)-Bradykinin eluted at about 8.7 min corresponding to a molecular size of 57 kDa, which is also close to the calculated 60 kDa for the tetramer.

25

EXAMPLE 9

This example demonstrates that fusing a protein containing disulfide bonds to either the C-terminus or N-terminus of TTR(C10A) does not have a  
30 significant impact on its oligomeric structure. About 50 µg each of TTR(C10A), IL-1-ra-TTR(C10A), and TTR(C10A)-IL-1-ra was injected on to a Biosep-Sec-S 3000 column (7.8 mm ID X 300 mm) (Phenomenex) in SEC-  
35 Buffer at 1 ml/min. Bio-Rad molecular weight standards

- 48 -

(151-1901) were used to calibrate the column and calculate the approximate molecular weight of the injected samples. As can be seen in Figure 3, TTR(C10A) elutes at approximately 8.8 min, which  
5 corresponds to a molecular size of 49 kDa which is comparable to the calculated molecular weight of the tetramer at 55 kDa. The IL-1-ra-TTR(C10A) fusion eluted at about 7.9 min corresponding to a molecular size of 188 kDa, which is noticeably larger than that  
10 expected for the tetramer at 124 kDa. Similarly, TTR(C10A)-IL-1-ra eluted at about 7.9 min, again corresponding to a molecular size of 188 kDa compared to the 124 kDa expected for the tetramer. These size discrepancies are likely due to differences in the  
15 shape of the molecule, since size exclusion chromatography is shape dependant and the standards are calibrated for globular proteins.

EXAMPLE 10

20 This example compares the binding of a TMP sequence fused to the carboxy-terminus of human immunoglobulin Fc (Fc-TMP) and TMP(m)-TTR to soluble human myeloproliferative leukemia (MPL) receptor. In  
25 addition, this example shows the effect of pegylation of the native TTR cysteine on the binding of the TMP fusion to the MPL receptor. The preparation of the pegylated TTR fusions is described in detail in Example 13.

30 For this example, human MPL receptor was covalently bound to a BIAcore CM5 chip at  $R_L = 1300 R_L$  using the EDC/NHS chemistry as per the manufacturer's instructions (BIAcore, Uppsala, Sweden). All samples were passed over the chip at 50  $\mu$ l/min in Dulbecco's  
35 PBS (Gibco BRL, Gaithersburg, MD) with 0.1 mg/ml bovine serum albumin and 0.005% P20 (polyoxyethylenesorbitan).

The equilibrium endpoint was taken 3 min post injection. As can be seen in Figure 4, Fc-TMP shows superior binding characteristics compared to TMP(m)-TTR. Further, this figure demonstrates that pegylation of the native TTR cysteine (Cys 10) interferes with the binding of TMP to the MPL receptor. The binding of TMP(m)-TTR-PEG5K showed a significantly repressed binding response compared to its non-pegylated counterpart, and TMP(m)-TTR-PEG20K showed an even more severe inhibition. This indicates that the presence of PEG on cysteine 10 likely causes steric interference for binding of the fused TMP to the MPL receptor, and larger PEGs produce more interference.

### EXAMPLE 11

The collected blood was analyzed for a complete and differential count using an ADVIA 120 automated blood analyzer with murine software (Bayer Diagnostics, New York, NY). As seen in Figure 5, Fc-TMP showed the greatest response with platelet count peaking at  $4.3 \times 10^{12}$  platelets  $L^{-1}$  on day 5, which is over 3.4 times baseline at  $1.2 \times 10^{12}$  platelets  $L^{-1}$ . TMP(m)-TTR-PEG 5K was a moderate responder peaking at

- 50 -

2.3 X 10<sup>12</sup> platelets L<sup>-1</sup> which is just under twice the baseline level. The non-pegylated form of TMP(m)-TTR shows very little response at 1.5 X 10<sup>12</sup> platelets L<sup>-1</sup> which is only 20% over the baseline level. The non-pegylated form of TMP(m)-TTR shows better binding *in vitro* than its pegylated counterparts (Figure 4), but it has poor performance *in vivo* compared to TMP(m)-TTR-PEG 5K. This indicates that PEG is required to improve the biological half-life of the TTR construct, and this more than compensates for the reduced affinity for the receptor.

#### EXAMPLE 12

This example demonstrates that mutation of cysteine 10 on TTR to alanine TTR(C10A) does not have a significant impact on its oligomeric structure. About 50 µg each of TTR and TTR(C10A) was injected on to a Biosep-Sec-S 3000 column (7.8 mm ID X 300 mm) (Phenomenex) in SEC-Buffer at 1 ml/min. Bio-Rad molecular weight standards (151-1901) were used to calibrate the column and calculate the approximate molecular size of the injected samples. As can be seen in Figure 6, TTR(C10A) elutes at approximately 8.8 min, which corresponds to a molecular size of 57 kDa which is similar to the calculated molecular weight of the tetramer at 55 kDa. This data combined with the observation that both forms of TTR are resistant to precipitation at 65°C (data not shown) indicates that mutation of cysteine 10 to alanine does not have a significant impact on the structure or stability of TTR.

#### EXAMPLE 13

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- 51 -

This example demonstrates that mutation of alanine 37 to cysteine TMP-TTR(C10A/A37C), aspartate 38 to cysteine TMP-TTR(C10A/D38C), alanine 81 to cysteine TMP-TTR(C10A/A81C), or glycine 83 to cysteine TMP-TTR(C10A/G83C) in a cysteine 10 to alanine background does not have a significant impact on the oligomeric structure of TTR. In addition, this example demonstrates that pegylation of these mutant forms of TTR with a 5K or 20K PEG produces two distinct species of TTR with significantly greater molecular size than the unpegylated form. The pegylation of TTR was carried out by first reducing about 8 ml of the TTR (7.28 mg/ml) with 10 mM DTT for 30 minutes at 30 °C in the presence of 50 mM tris HCl, pH 8.5. The reduced TTR was then desalted using a 26 ml SEPHADEX™ G25 medium column (2.6 cm ID) (Amersham Pharmacia Biotech) at 2.5 ml/min in 20 mM tris HCl, pH 8.5. The concentration was then determined by measuring the absorbance of the reduced TTR at 280 nm and using the calculated extinction coefficient (29,450 M<sup>-1</sup> for TMP-TTR(C10A/A37C) (5.14 mg/ml). One-half (4.6 ml) of the reduced sample was then immediately mixed with 810 µl of 5 mM methoxy-PEG-maleimide 5K (Shearwater Corporation, Huntsville, AL) and the remaining half was mixed with 1620 µl 2.5 mM methoxy-PEG-maleimide 20K (Shearwater Corporation). The reaction was allowed to proceed at 30 °C for 30 min and was quenched by the addition of 46 µl 1 M DTT. Each pegylated sample was then loaded on to a 5 ml HiTrap Q-sepharose column at 2.5 ml/min and washed with several column volumes of Q-Buffer A (20 mM tris HCl, pH 8.0) at 5 ml/min. The columns were eluted with a linear gradient to 40% Q-Buffer B (20 mM tris HCl, 1 M NaCl, pH 8.0) followed by a 2 column volume step to 100% Q-Buffer B. Peak



- 52 -

fractions were pooled and the concentration determined by measuring the absorbance of the pool at 280 nm. About 50 µg of each sample was injected on to a Biosep-Sec-S 3000 column (7.8 mm ID X 300 mm) (Phenomenex) in SEC-Buffer at 1 ml/min. Bio-Rad molecular weight standards (151-1901) were used to calibrate the column and calculate the approximate molecular size of the injected samples. As can be seen in Figure 7, the apparent molecular size of the 4 non-pegylated TMP-TTR constructs is between 40 and 45 kDa which is noticeably lower than the expected 70 kDa tetramer. This retarded elution time is likely due to a slight interaction of the TMP-TTR construct with the size exclusion resin, which has been observed with several other TMP constructs (data not shown). After conjugation with the 5K PEG, the apparent molecular size increases to between 421 and 428 kDa (1.53 - 1.64 minutes more advanced elution than the unpegylated counterparts), which is much greater than the expected 90 kDa. The observation of an exaggerated molecular weight of pegylated molecules on size exclusion chromatography is frequently observed phenomenon (data not shown). The 20K PEG constructs elute earlier than the largest calibration standard (670 kDa) showing a 1.28 - 1.40 minutes more advanced elution than their 5K pegylated counterparts. This data taken together demonstrates that all 4 engineered mutant forms of TMP-TTR can be pegylated drastically increasing their apparent molecular size.

About 2 µg of the pegylated TMP-TTR constructs were analyzed by SDS-PAGE (Figure 8). This figure demonstrates by gel shift that most of the TMP-TTR monomers were modified by only one methoxy-PEG-maleimide, and the reaction was nearly complete leaving very little unmodified monomer.

- 53 -

EXAMPLE 14

This example demonstrates that Fc-TMP, TMP-TTR(C10A/A37C), TMP-TTR(C10A/D38C), TMP-TTR(C10A/A81C), and TMP-TTR(C10A/G83C) have similar affinities for binding human MPL receptor *in vitro*. For this example, Fc-TMP was bound to a BIAcore protein G chip at high density as per the manufacturer's instructions (BIAcore, Uppsala, Sweden). Test proteins were preincubated with 5 nM MPL receptor in Binding Buffer (Dulbecco's PBS (Gibco BRL, Gaithersburg, MD) with 0.1 mg/ml bovine serum albumin and 0.005% P20 (polyoxyethylenesorbitan) for >2 hours at room temperature (about 23 °C). For non-pegylated proteins, 0.1 mg/ml heparin was added to prevent non-specific binding. All samples were then passed over the chip at 50 µl/min in Binding Buffer. The equilibrium endpoint was taken 3 min post injection. As can be seen in Figure 9, all TTR constructs showed similar affinity for the MPL receptor with affinities ranging from 0.881 to 2.333 nm, while the Fc-TMP construct had affinities ranging from 3.276 to 5.369 nm.

25 EXAMPLE 15

This example shows the effect of injecting pegylated TMP-TTR constructs into mice on blood platelet count. For this example 170 BDF1 mice were split into 17 groups and injected (day 0) subcutaneously with 50 µg test protein per kg animal (TMP fusion construct, Fc-TMP, or a TTR(C10A) control). Each group was divided in half and bled (140 µl) on alternate time points (day 0, 3, 5, 7, 10, 12, and 14).

- 54 -

Mice were anesthetized with isoflurane prior to collection.

The collected blood was analyzed for a complete and differential count using an ADVIA 120 automated blood analyzer with murine software (Bayer Diagnostics, New York, NY). As seen in Figure 10A, Fc-TMP showed the greatest response with platelet count rising to over  $4.2 \times 10^{12}$  platelets  $L^{-1}$  on day 5 which is 3 times baseline at  $1.4 \times 10^{12}$  platelets  $L^{-1}$ . All 4 of the non-pegylated TMP-TTR constructs performed better than the control, but not as well as Fc-TMP with platelet counts between  $1.8$  and  $2.9 \times 10^{12}$  platelets  $L^{-1}$  on day 5, which is between a 29% and 107% improvement over baseline. As can be seen in Figure 10B, addition of a 5K PEG group to the engineered cysteine of all 4 TMP-TTR constructs substantially improves efficacy with platelet counts between  $3.7$  and  $4.4 \times 10^{12}$  platelets  $L^{-1}$  (2.8 to 3.4 times baseline).

Also as can be seen in Figure 10C, conjugation of a 20K PEG to TMP-TTR results in an additional, but less dramatic improvement in efficacy with platelet counts between  $4.2$  and  $4.6 \times 10^{12}$  platelets  $L^{-1}$  (3.2 to 3.5 times baseline). Since all of the TMP fusion constructs had similar binding affinities for MPL *in vitro*, this difference is likely due to the effect of PEG conjugation increasing the effective biological half-life of the construct.

#### EXAMPLE 16

This example shows the effect of injecting pegylated PTH-TTR constructs into mice on blood ionized calcium release. For this example 60 male, BDF1, 4 week-old mice were split into 12 groups and injected (day 0) subcutaneously with 8.91 mg test protein per kg animal (PTH fusion construct, PTH-Fc, or a TTR(C10A)

- 55 -

control). Each group was bled (75  $\mu$ l) at time points 0, 24, 48, and 72 hours. Mice were anesthetized with isoflurane prior to collection.

The collected blood was analyzed for ionized calcium using a Ciba\*Corning 634 Ca<sup>++</sup>/pH analyzer. As seen in Figure 11, PTH-Fc, PTH-TTR(C10A/K15A/A37C) (PEG 5K), PTH-TTR(C10A/K15A/A37C) (PEG 20K), PTH-TTR(C10A/K15A/G83C) (PEG 5K), and PTH-TTR(C10A/K15A/G83C) (PEG 20K) showed the greatest response with ionized calcium levels rising between 2.2 and 2.7 mmol per L at 24 hours post-injection, which is 1.7 times baseline at 1.3 mmol per L. At 72 hours post injection, the ionized calcium levels of all groups returned to baseline, except PTH-TTR(C10A/K15A/A37C) (PEG 5K), PTH-TTR(C10A/K15A/G83C) (PEG 5K), and PTH-TTR(C10A/K15A/G83C) (PEG 20K) treated groups that maintained elevated ionized calcium levels between 1.8 and 1.9 mmol per L. The non-pegylated PTH-TTR constructs were equivalent to or slightly better than the TTR(C10A) control at raising serum ionized calcium levels.

#### EXAMPLE 17

This example describes the construction of a PTH-TTR(C10A/K15A/A81C) containing plasmid. The Xba1/Xba1 fragment of 5920 was ligated with the purified vector derived from digesting plasmid 5643 (described in example 1) with Xba1. The *E. coli* strain containing the resulting plasmid is described as 5933 PTH-TTRC10A/K15A/A81C.

#### SEQ ID NO:43:

ATGTCGTGTTCTGAAATCCAGCTGATGCATAACCTGGGTAAACATCTGAACTCTA  
 TGGAACGTGTTGAATGGCTGCGTAAGAACTGCAGGACGTTTCATAACTTTGGTCC  
 AACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAGATGCTGTC  
 CGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGCTGCTGATG

- 56 -

ACACCTGGGAGCCATTTGCCCTCTGGGAAAACCACTGAGTCTGGAGAGCTGCATGG  
 GCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATAGACACC  
 AAATCTTACTGGAAGTGTCTTGGCATCTCCCCATCCATGAGCATGCAGAGGTGG  
 TATTCACAGCCAACGACTCCGGCCCCCGCGCTACACCATGCGGCCCTGCTGAG  
 5 CCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

EXAMPLE 18

This example describes the preparation of a  
 10 GLP-1-TTR(C10A/G83C) fusion and a GLP-1-  
 TTR(C10A/K15A/G83C) fusion. These constructs were  
 cloned using plasmid pAMG21, which is described in  
 example 1. Each of the oligonucleotides utilized in  
 this example are listed in Table 6.

15 The bacterial host GM121 is an *E. coli* K-12 strain  
 that has been modified to contain the *lacI*<sup>0</sup> repressor  
 in the late *ebg* region (68 minutes). The presence of  
 this repressor gene allows the use of this host with a  
 variety of expression systems, however this repressor  
 20 is irrelevant to the expression from *luxPR*. The  
 untransformed host has no antibiotic resistances.  
 Specifically, F'tet/393 was modified by the delivery of  
 a *lacI*<sup>0</sup> construct into the *ebg* operon between  
 nucleotide position 2493 and 2937 as numbered in the  
 25 Genbank accession number M64441Gb\_Ba with the deletion  
 of the intervening *ebg* sequence. The construct was  
 delivered to the chromosome using a recombinant phage  
 called AGebg-*lacI*<sup>0</sup> #5.

After recombination and resolution only the  
 30 chromosomal insert described above remains in the cell.  
 It was renamed F'tet/GM120. F'tet/GM120 was then  
 mutated in the *hsdR* gene to inactivate it. This was  
 renamed F'tet/GM121. The F'tet episome was cured from  
 the strain, verified as tetracycline sensitive and was  
 35 stored as GM121 (ATCC #202174).

PCR was performed with Roche PCR Core Kit  
 (Cat. No. 1 578 553) in 80  $\mu$ l reactions containing 2-4  
 $\mu$ l mini-prep plasmid DNA template, 1  $\mu$ M each

- 57 -

oligonucleotide, 0.2 mM each oligonucleotide, 5% DMSO (Sigma), and 2U Taq DNA polymerase in order to amplify the GLP-1 sequence and a linker. Reaction cycles were 94° C for 5 min followed by 35 cycles of [94° C for 20  
5 sec, 45° C for 30 sec, 72° C for 1 min]. PCR products were purified with QIAquick® PCR Purification Kit according to the manufacturer's protocol (QIAGEN). PCR products and vectors were then digested with NdeI and KpnI (New England Biolabs).

10 Digested DNA was purified from an agarose gel, then mixed and ligated by T4 DNA ligase (New England Biolabs) for 1.5-2 hours at room temperature. Each ligation mixture was transformed by  
15 electroporation into the host strain GM121 described above with a Biorad *E. coli* Pulser at 2.5KV in a cuvette with a gap length of 2 mm. The cells were allowed to recover in 2 ml Terrific Broth (TB) for about 3 hours at 37°C at 250 rpm. 70-100 µl of the recovery culture was plated on LB agar containing 40  
20 ug/ml kanamycin. DNA mini-preps were prepared and correct clones were verified by nucleotide sequencing.

To prepare the GLP-1-TTR(C10A/G83C) fusion, two oligonucleotides, oligonucleotide 1209-85, which binds the luxR promoter region, and 3131-63, which  
25 encodes the last 12 amino acids of the fusion linker and the first 8 amino acids of TTR, were synthesized. A pAMG21 plasmid derived from a strain which expresses a GLP-1 sequence with a N-terminal Met-Lys start followed by a seven Histidine sequence for nickel  
30 column purification, an Aspartic acid-Glutamic acid-Valine-Aspartic acid sequence for cleavage before the first Histidine of GLP-1 by caspase, the GLP-1(A2G) sequence, and a 27 amino acid fusion linker was amplified using oligonucleotides 1209-85 and 3131-63.  
35 The PCR product was cloned and sequenced as described

- 58 -

above. The resultant strain containing the new plasmid was designated GLP-1-TTR(C10A/G83C) (strain 6298) and had the DNA sequence identified in SEQ ID NO:47.

To prepare the GLP-1-TTR(C10A/K15A/G83C)

5 fusion, two oligonucleotides, oligonucleotide 3183-83, which contains an NdeI site and encodes the purification and cleavage sequence described above plus the first six amino acids of GLP-1(A2G), and 3183-84, which encodes the last 6 amino acids of the fusion  
10 linker and the first 8 amino acids of TTR, were synthesized.

A pAMG21 plasmid derived from a strain which expresses a GLP-1 sequence with a N-terminal Met-Lys start followed by a seven Histidine sequence for nickel  
15 column purification, an Aspartic acid-Glutamic acid-Valine-Aspartic acid sequence for cleavage before the first Histidine of GLP-1 by caspase, the GLP-1(A2G) sequence, and a 25 amino acid fusion linker was amplified using oligonucleotides 3183-83 and 3183-84.  
20 The PCR product was cloned and sequenced as described above. The resultant strain containing the new plasmid was designated GLP-1-TTR(C10A/K15A/G83C) (strain 6450) and had the DNA sequence identified in SEQ ID NO:48.

25 Table 6

Oligo	Sequence	SEQ ID Number
1209-85	CGTACAGGTTTACGCAAGAAAATGG	44
3131-63	GGATTACCCGGTACCAGTTGGACCACCACCACCAC	
30 3183-83	CACCACCCGCACTGCCCTGAACCAAGAGC	45
	TGACTAAGCCATATGAAACATCATCACCATCACCAT	
	CATGACGAAGTTGATCACGGTGAAGGTACTTTCAC	46
3183-84	GGATTACCCGGTACCAGTTGGACCACCACCACCAC	
35	CACCGCTAC	47

SEQ ID NO:48

ATGAAACATCATCACCATCACCATCATGACGAAGTTGATCACGGTGAAGGTACTT  
TCACTTCTGACGTTTCTTTATCTGGAAGGTCAGGCTGCTAAAGAATTCATCGC  
TTGGCTGGTTAAAGGTCGTGGTGGTTCTGGTTCTGCTACTGGTGGTTCGGCTCC  
40 ACCGCAAGCTCTGGTTCAGGCAGTGCAGGTTGGTGGTGGTGGTGGTCCAACTG  
GTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTCTAGATGCTGTCCGAGG

5 CAGTCCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGCTGCTGATGACACC  
TGGGAGCCATTTGGCTCTGGGAAACCACTGAGTCTGGAGAGCTGCATGGGCTCA  
CAACTGAGGAGGAAATTTGTGAGAGGATATACAAAGTGGAAATAGACCAAAATC  
TTACTGGAAGGCATCTTGATCTCCCCATTCATGAGCATGCAGAGGTTGTAATTC  
ACAGCTAACGACTCCGCGCCCGCCCTACACCTATGCCGCCCTGCTGAGCCCT  
ACTCCTAATCTCACCACGGCTGTGCTGACCAATCCCAAGGAATAA

SEQ ID NO:49

This example describes the preparation of a  
25 GLP-1(A2G)-K-Fc fusion. This construct was cloned  
using plasmid pAMG33\*, which differs from pAMG21 in  
that the lux protein and promoters are replaced with  
lacI binding sites and an IPTG inducible promoter and  
the ribosomal binding site sequence is shorter (the  
30 sequence between the AatII and ClaI recognition sites  
is replaced with AATTGTGAGCGGATAACAATTGAC  
AAATGCTAAATCTTGATTAATTGTGAGCGGATAACAATTTATCGATTTGATTC  
TAGAAGGAGGAATAA) and some of the sequence after the  
SacII recognition site was deleted (leaving  
35 AATAATAAGTAACGATCCGGTCCAGTAATGACCTCAGAAC  
TCCATCTGGATTGTTCAGAACGCTCGGTTCCGCCGGGCGTTTTTTATTGGTGA  
GAATCGCAGCAACTTGTGCGCCCAATCGAGCCATGTCGTCGTCACGACCCCCCA  
TTCAAGAACAGCAAGCAGCATTGAGAACTTTGGAATCCAGTCCCTCTTCCACCTG  
CTGACCG). Each of the oligonucleotides utilized in  
40 this example are listed in Table 7.

-64-



- 60 -

contains and NdeI site and encodes the purification and cleavage sequence described above plus the first eight amino acids of GLP-1(A2G) , and 2687-50, which encodes the amino acids 18 through 23 of the Fc, were synthesized. A pAMG33\* plasmid derived from a strain which expresses a GLP-1(A2G) sequence with a N-terminal Met start, a 27 amino acid linker, and an Fc sequence was amplified using oligonucleotides 2985-92 and 2687-50. The PCR product was cloned and sequenced as described above except the enzymes used were NdeI and EcoRI. A colony screening step was included which verified the presence of insert by PCR with oligonucleotides directed against upstream vector sequence and the 5 His-Aspartic acid sequence which the insert introduced. The resultant strain containing the new plasmid was designated GLP-1(A2G)-K-Fc (strain 5945) and had the DNA sequence identified in SEQ ID NO:51.

Table 7

20	Oligo	Sequence	SEQ ID
			Number
	2985-92	AGACCTGTACATATGAAACATCATCACCATCACCAT CATGACGAAGTTGATCACGGTGAAGGTACTTTCAC TTCTG	50
25	2687-50	GGGGGAAGAGGAAACTGAC	51

## SEQ ID NO: 52

ATGAAACATCATCACCATCACCATCATGACGAAGTTGATCACGGTGAAGGTACTT  
TCACTTCTGACGTTTCTTCTTATCTGGAAGGTCAGGCTGCTAAAGAATTCATCGC  
30 TTGGCTGGTTAAAGGTCGTGGTGGTTCTGGTTCTGCTACTGGTGGTTCCGGCTCC  
ACCGCAAGCTCTGGTTCAGGCAGTGCAGCTCATGGTGGTGGTGGTGACAAAA  
CTCACACATGTCCACCGTGCCAGCACCTGAACCTCTGGGGGACCGTCAGTTTTT  
CCTCTTCCCCCAAAACCAAGGACACCTCATGATCTCCCGACCCCTGAGGTC  
ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCCTGAGGTCAAGTTCAACTGGT  
35 ACGTGACGCGCTGGAGGTGCATAATGCCAAGACAAAGCCGCGGAGGAGCAGTA  
CAACAGCACGTACCGTGTGGTCAAGCTCTCACCCTCTGCACACGAGTGGCTG  
AATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCCCATCG  
AGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCT  
GCCCCCATCCCGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTC  
40 AAAGGCTTCTATCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGG  
AGAACAACCTACAAGACCAGCCTCCCGTCTGGACTCCGACGGCTCCTTCTTCT  
CTACAGCAAGCTCACCCTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCA  
TGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGAGAAGAGCCTCTCC  
TGCTCCCGGTAAA

- 61 -

EXAMPLE 20

This example describes the cloning of the CH2  
 5 domain of an immunoglobulin molecule to the TTR(C10A)  
 to generate TMP-CH2-TTRC10A and TTRC10A-CH2-TMP.

The CH2 domain derived from TMP-Fc was linked to  
 the C-terminal end of TTR(C10A), *i.e.*, strain 5619, by  
 a two-step PCR procedure. The CH2 domain (containing  
 10 from 5' to 3': the last 7 codons of TTR, CH2 and a  
 BamH1-XhoI linker) was first amplified by the following  
 oligos:

2973-77:

GTC GTC ACC AAT CCC AAG GAA GGT TCT GGC TCC GGA  
 15 TCA GGG GGA CCG TCA GTT TTC CTC (SEQ ID NO:53), and

2973-78:

CCG CGG ATC CTC GAG ATT AGG ATC CAG AAC CCC CTT  
 TGG CTT TGG AGA TGG T (SEQ ID NO:54).

This fragment was then fused to 5619 in a  
 20 subsequent PCR by oligos 2973-78 and

2973-79:

GAG GAA TAA CAT ATG GGT CCA ACT GGT ACC GGT GAA  
 TCC AAG (SEQ ID NO:55),

followed by NdeI/XhoI digest and cloning into  
 25 similarly restricted pAMG21. The resulting plasmid is  
 described as 6017 (TTRC10A-CH2):

SEQ ID NO:56:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAG  
 30 ATGCTGTCCGAGGCAGTCCCTGCCATCAATGTGGCCGTGCATGTGTTCAAGAAAGGC  
 TGCTGATGACACCTGGGAGCCATTGCTCTGGGAAAACCAAGTGTCTGGAGAG  
 CTGCAATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA  
 TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCATTCATGAGCATGC  
 AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATGCGCGCC  
 35 CTGCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCAAGGAAG  
 GTTCTGGCTCCGGATCAGGGGGACCGTCAGTTTTCCTCTTCCCCCAAAACCCAA  
 GGACACCCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTG  
 AGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACCGCGTGGAGGTGC  
 ATAATGCCAAGACAAAGCCCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGT  
 40 CAGCGTCTCACCCTCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGC

- 62 -

AAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCA  
AAGGGGGTTCTGGATCCTAA

The Xba1/Xba1 fragment of 6017 was replaced with  
5 the corresponding fragment of 5704 as described above  
to construct TMP-TTRC10A-CH2 (Strain 6024):

SEQ ID NO:57:

10 ATGATCGAAGGTCGCACTCTGCGTCAGTGGCTGGCTGCTCGTGGCGGTGGTG  
GCGGAGGGGGTGGCATTTGAGGGCCCAACCCCTTCGCCAATGGCTTGCAGCACGCGC  
AGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAGAT  
15 GCTGTCCGAGGCAGTCCCTGCCATCAATGTGGCCGTGCATGTGTTTCAAGAAAGGCTG  
CTGATGACACCTGGGAGCCATTTCCTCTGGGAAAACCAAGTGAGTCTGGAGAGCT  
GCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAATA  
GACACCAAATCTTACTGGAAGGCACCTGGCATCTCCCCATTCATGAGCATGCAG  
20 AGGTGGTATTTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT  
GCTGAGCCCCCTACTCTATTCCACCACGGCTGTCGTACCAATCCCAAGGAAGGT  
TCTGGCTCCGGATCAGGGGGACCGTCAGTTTTCCTCTTCCCCCAAAACCCAAGG  
ACACCTCATGATCTCCCGGACCCCTGAGGTACATGCGTGGTGGTGGACGTGAG  
25 CCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTCAT  
AATGCCAAGACAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCA  
GCGTCTTCACCGTCTGCAACAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAA  
GGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAA  
GGGGGTCTGGATCCTAA

Construction of TTRC10A-CH2-TMP was done as  
follows: the TMP fragment containing a 5' BamHI linker  
and 3' XhoI linker was amplified by oligos 2694-19 and

30 2974-70:  
GAG GAA TAA GGA TCC ATC GAA GGT CCG ACT CTG CG  
(SEQ ID NO:58).

The amplified fragment was digested with BamHI and  
35 XhoI and was subsequently ligated with similarly  
restricted 6017. The resulting clone is described as  
strain 6104 (TTRC10A-CH2-TMP).

SEQ ID NO:59:

40 ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAG  
ATGCTGTCCGAGGCAGTCCCTGCCATCAATGTGGCCGTGCATGTGTTTCAAGAAAGGC  
TGCTGATGACACCTGGGAGCCATTTCCTCTGGGAAAACCAAGTGAGTCTGGAGAG  
CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGAAA

- 63 -

TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC  
 AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATGCGCGC  
 CTGCTGAGCCCTACTCTTATTCACACGGCTGTGCTCACCATCCCAAGGAAG  
 GTTCTGGCTCCGGATCAGGGGGACCGTCAGTTTCTCTTCCCCCAAAACCCAA  
 5 GGACACCTCATGATCTCCCGACCCCTGAGGTCACATGCGTGGTGGGACGTG  
 AGCCACGAAGACCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGC  
 ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGT  
 CAGCGTCCCTACCGTCTGACACAGGACTGGCTGAATGGCAAGGAGTACAAGTGC  
 AAGGTCTCCAACAAAGCCCTCCAGCCCCATCGAGAAAACCATCTCCAAAGCCA  
 10 AAGGGGTCTTGGATCCATCGAAGTCCGACTCTGCGTCAGTGGCTGGCTGCTCG  
 TGCTGGCGGTGGTGGCGAGGGGTGGCATTTGAGGGCCCAACCCCTTCGCCAATGG  
 CTTCGAGCACGCGCATAA

Another configuration of this fusion was made as  
 15 TMP-CH2-TTR2. The CH2 domain derived from TMP-Fc was  
 first linked to N-terminus of TTRC10A by a two-step  
 PCR. The CH2 domain (containing from 5' to 3': a NdeI-  
 BamHI linker, CH2 and the first 7 codons of TTR C10A)  
 was first amplified by oligos

20

2974-65:

TTC ACC GGT ACC AGT TGG ACC AGA ACC CCC TTT GGC  
 TTT GGA GAT GGT (SEQ ID NO:60), and

2974-66:

25 GAG GAA TAA CAT ATG GGA TCC GGT TCT GGG GGA CCG  
 TCA GTT TTC CTC (SEQ ID NO:61).

This fragment was fused to 5619 in a subsequent  
 PCR by oligos 2974-66 and 2693-80 (example 1), followed  
 by restriction with NdeI/XhoI and cloning into  
 30 similarly restricted pAMG21. The resulting clone is  
 described as 6016 (CH2-TTRC10A):

SEQ ID NO:62:

ATGGGATCCGGTTCTGGGGACCGTCAGTTTCTCTTCCCCCAAAACCCAAAGG  
 35 ACACCTCATGATCTCCCGACCCCTGAGGTCACATGCGTGGTGGGACGTGAG  
 CCACGAAGACCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCAT  
 AATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCA  
 GCGTCCCTACCGTCTGACACAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAA  
 GGTCCTCAACAAAGCCCTCCAGCCCCATCGAGAAAACCATCTCCAAAGCCAAA  
 40 GGGGGTCTGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTGTATGGTCAAAG  
 TTCTAGATGCTGTCAGGAGCAGTCTGCCATCAATGTGGCGTGCATGTGTTTCAG  
 AAAGGCTGCTGATGACACCTGGGAGCCATTGCTCTGGGAAAACCAAGTGAAGTCT  
 GGAGAGCTGCATGGGTCACAACAGGAGGAATTTGTAGAAGGGATATACAAAG

- 64 -

TGGAAATAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCATGA  
GCATGCAGAGGTGGTATTACAGCCAACGACTCCGGCCCCCGCGCTACACCATT  
GCCGCCCTGCTGAGCCCCCTACTCTATTCCACCACGGCTGTCGTCACCAATCCCA  
AGGAATAA

5

The TMP fragment containing a NdeI linker at 5'  
end and a BamHI linker at 3' end was amplified by  
oligos

10

2974-68:

GAG GAA TAA CAT ATG ATC GAA GGT CCG ACT CTG (SEQ ID  
NO:63), and

2974-69:

TAA CAT ATG GGA TCC TGC GCG TGC TGC AAG CCA TTG (SEQ ID

15

NO:64).

This fragment was then digested with NdeI/BamHI  
and ligated with the vector which was similarly  
restricted, gel purified from strain 6016. The

20

resulting clone is described as 6110 (TMP-CH2-TTRC10A):

SEQ ID NO:65:

25

ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTGCTGGCGGTGGTG  
GCGGAGGGGGTGGCATTTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCAGCGGC  
AGGATCCGGTTCTGGGGGACCGTCAGTTTCTCTTCCCCCCCCAAACCAAGGAC  
ACCCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCC  
ACGAAGACCCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAA  
TGCCAAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCAGTACCGTGTGGTCAGC  
GTCCCTCACCGTCCTGCACCGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGG  
TCTCCAACAAAGCCCTCCAGCCCCCATCGAGAAAACCATCTCCAAGCCCAAGG  
GGGTTCTGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTT  
CTAGATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTAGAA  
AGGCTGCTGATGACACCTGGGAGCCATTGCTCTGGGAAAACAGTGAGTCTGG  
AGAGCTGCATGGGCTCACAACAGGAGGAATTTGTAGAGGGATATACAAAGTG  
GAAATAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGC  
ATGCAGAGGTGGTATTACAGCCAACGACTCCGGCCCCCGCGCTACACCATTGC  
CGCCCTGCTGAGCCCCCTACTCTATTCCACCACGGCTGTCGTCACCAATCCCAAG  
GAATAA

35

#### EXAMPLE 21

40

This example describes the construction of  
TTRC10A/K15A-TMP, TTRC10A/K15A/A81C-TMP and  
TTRC10A/K15A/G83C-TMP.

- 65 -

TMP was also cloned at the C- termini of TTR and variants thereof. The full length TMP containing at its N- terminal end a 5-amino acids linker (gsgsg) plus the last 7 amino acids of wt TTR was amplified by the following set of oligonucleotides in a standard PCR procedure.

2694-18:

GTC GTC ACC AAT CCC AAG GAA GGT TCT GGT TCT GGT

10 ATC GAA (SEQ ID NO:66), and

2694 - 19:

CCG CGG ATC CTC GAG ATT ATG CGC GTG CTG CAA GCC

ATT G (SEQ ID NO:67).

15 This PCR fragment was further linked to the 3' end of wt TTR by a second PCR utilizing oligos 2694-19 and 2693-79 as described in example 1. The resulting clone was sequence confirmed and is described as strain 5365 (TTR-TMP):

20 SEQ ID NO:68:

ATGGGTCCAACTGGTACCGGTGAATCCAAGTGTCTCTGATGGTCAAAGTTCTAG  
ATGCTGTCCGAGGCAGTCCCTGCCATCAATGTGGCCGTGCATGTGTTTCAAGAAAGGC  
TGCTGATGACACCTGGGAGCCATTGTCCTCTGGGAAAACCAAGTGTGAGAGAG  
CTGCATGGGCTCACAACCTGAGGAGGAATTGTAGAAGGGATATACAAAGTGGAAA  
25 TAGACACCAAATCTTACTGGAAGGCACCTGGCATCTCCCCATTCCATGAGCATGC  
AGAGGTGGTATTACAGCCAACGACTCCGGCCCCCGCCGTACACCATTTGCCGCC  
CTGCTGAGCCCCCTACTCTATTCCACCGGCTGTGCTCACCATCCCAAGGAAG  
GTTCTGCTTCTGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC  
TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCCTTCGCCAATGGCTT  
30 GCAGCACGCGCATAA

The Xba1/Xba1 fragment of 5365 was then replaced by the corresponding Xba1/Xba1 fragment of strain 5895 to make strain 5921 (TTRC10A/K15A-TMP) as described above:

35 SEQ ID NO:69:

ATGGGTCCAACTGGTACCGGTGAATCCAAGGCTCTCTGATGGTCGCAGTTCTAG  
ATGCTGTCCGAGGCAGTCCCTGCCATCAATGTGGCCGTGCATGTGTTTCAAGAAAGGC  
TGCTGATGACACCTGGGAGCCATTGTCCTCTGGGAAAACCAAGTGTGAGAGAG  
40 CTGCATGGGCTCACAACCTGAGGAGGAATTGTAGAAGGGATATACAAAGTGGAAA  
TAGACACCAAATCTTACTGGAAGGCACCTGGCATCTCCCCATTCCATGAGCATGC

- 66 -

AGAGGTGGTATTACAGCCAACGACTCCGGCCCCCGCGCTACACCATGGCCGCC  
 CTGCTGAGCCCCCTACTCCTATTCACCACGGCTGTCGTACCAATCCCAAGGAAG  
 GTTCTGGTTCTGGTATCGAAGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC  
 TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT  
 5 GCAGCACGCCCATAA

Plasmid 5921 was subsequently modified by  
 replacing the amino acids at the following positions:  
 A37, A81 and G83, with the amino acid Cysteine as  
 10 described in example 1, except that the TTR 3' oligo  
 utilized with the mutation oligos (2693-80) in example  
 1 was replaced with 2694-19, resulting in Strain 5982,  
 containing TTRC10A/K15A/A37C-TMP (SEQ ID NO:70), Strain  
 5983 containing TTRC10A/K15A/A81C-TMP (SEQ ID NO:71),  
 15 and Strain 5984 containing TTRC10A/K15A/G83C-TMP (SEQ  
 ID NO:72).

SEQ ID NO:70:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCCGAGTTCTAG  
 ATGCTGTCCGAGGCAGTCCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC  
 TTGTGATGACACCTGGGAGCCATTGCTCTGGGAAAACCAAGTGAGTCTGGAGAG  
 20 CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA  
 TAGACACCAAATCTTACTGGAAGGCATTGGCATCTCCCCATTCCATGAGCATGC  
 AGAGGTGGTATTACAGCCAACGACTCCGGCCCCCGCGCTACACCATGGCCGCC  
 CTGCTGAGCCCCCTACTCCTATTCACCACGGCTGTCGTACCAATCCCAAGGAAG  
 25 GTTCTGGTTCTGGTATCGAAGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC  
 TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT  
 GCAGCACGCCCATAA

SEQ ID NO:71:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCCGAGTTCTAG  
 ATGCTGTCCGAGGCAGTCCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC  
 TGCTGATGACACCTGGGAGCCATTGCTCTGGGAAAACCAAGTGAGTCTGGAGAG  
 CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA  
 TAGACACCAAATCTTACTGGAAGTGTCTTGGCATCTCCCCATTCCATGAGCATGC  
 35 AGAGGTGGTATTACAGCCAACGACTCCGGCCCCCGCGCTACACCATGGCCGCC  
 CTGCTGAGCCCCCTACTCCTATTCACCACGGCTGTCGTACCAATCCCAAGGAAG  
 GTTCTGGTTCTGGTATCGAAGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC  
 TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT  
 40 GCAGCACGCCCATAA

SEQ ID NO:72:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCCGAGTTCTAG  
 ATGCTGTCCGAGGCAGTCCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC  
 TGCTGATGACACCTGGGAGCCATTGCTCTGGGAAAACCAAGTGAGTCTGGAGAG  
 45 CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA  
 TAGACACCAAATCTTACTGGAAGGCATTGCTCTCCCCATTCCATGAGCATGC

- 67 -

AGAGGTGGTATTTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC  
 CTGCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTACCAATCCCAAGGAAG  
 GTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC  
 TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAGTGGCTT  
 5 GCAGCACGCGCATAA

EXAMPLE 22

This example describes the construction of TMP-  
 10 TTRC10A/K15A/A81C and TMP-TTRC10A/K15A/A37C. The Lys  
 at 15th position of TTR was mutagenized to Ala in  
 strains 5704, 5706 and 5707 by the following methods.  
 Plasmid 5513 was digested with Nde1/Kpn1, the insert  
 harboring TMP fragment and the first 6 amino acids of  
 15 TTR was purified and ligated with Nde1/Kpn1 restricted  
 and gel purified vector derived from strain 5895. The  
 bacterial strain containing the resulting plasmid is  
 described as 5919 (TMP-TTRC10A/K15A/G83C). Plasmid  
 5919 was then digested with Xba1, the resulting  
 20 Xba1/Xba1 fragment containing TMP and the first 18  
 codons of TTR including the C10A and K15A mutations was  
 gel purified and ligated with Xba1 digested,  
 phosphatase treated and gel purified vectors derived  
 from strain 5704 and 5706. The new strains are  
 25 described as 5918 (TMP-TTRC10A/K15A/A81C) and 6023  
 (TMP-TTRC10A/K15A/A37C).

TMP-TTRC10A/K15A/G83C (SEQ ID NO:73):

ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG  
 30 GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC  
 AGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAGAT  
 GCTGTCCGAGGCAGTCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTG  
 CTGATGACACCTGGGAGCCATTTGCCCTCTGGGAAAACAGTGAGTCTGGAGAGCT  
 GCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGATATACAAAGTGGAAATA  
 35 GACACCAAATCTTACTGGAAGGCACTTTGCATCTCCCATTCATGAGCATGCAG  
 AGGTGGTATTTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT  
 GCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTACCAATCCCAAGGAATAA

TMP-TTRC10A/K15A/A81C (SEQ ID NO:74):

40 ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG  
 GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC



- 68 -

AGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCCTAGAT  
 GCTGTCCGAGGCAGTCTTGCATCAATGTGGCCGTGCATGTGTTAGAAAGGCTG  
 CTGATGACACCTGGGAGCCATTTCCTCTGGGAAAACCAAGTGAGTCTGGAGAGCT  
 GCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATA  
 5 GACACCAAATCTTACTGGAAGTGTCTTGGCATCTCCCATTTCCATGAGCATGCAG  
 AGGTGGTATTACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTTCCGCCCT  
 GCTGAGCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

TMP-TTRC10A/K15A/A37C (SEQ ID NO:75):

10 ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGGCGGTGGTG  
 GCGGAGGGGGTGGCATTGAGGGCCCAACCTTCGCCAATGGCTTGCAGCACGCGC  
 AGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCCTAGAT  
 GCTGTCCGAGGCAGTCTTGCATCAATGTGGCCGTGCATGTGTTAGAAAGGCTT  
 GTGATGACACCTGGGAGCCATTTCCTCTGGGAAAACCAAGTGAGTCTGGAGAGCT  
 15 GCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATA  
 GACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCATTTCCATGAGCATGCAG  
 AGGTGGTATTACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTTCCGCCCT  
 GCTGAGCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

#### 20 EXAMPLE 23

This example describes the expression of GLP-1 fusions proteins in *E. coli*. 25-100 ml of a saturated overnight culture was used to inoculate 50 ml  
 25 TB with 20 ug/ml kanamycin in a 250 ml baffled flask and incubated at 37C, 250 rpm overnight. 10-35 ml of these overnight cultures were used to inoculate 1L TB with 20 ug/ml kanamycin in a 2L baffled flask and incubated at 37C, 250 rpm until the optical density at  
 30 600 nm reached approximately 0.7. The cultures were then induced to express recombinant protein by the addition of: 1 ml of ethanol containing 30 ug/ml N-(B - ketocaproyl)-DL-homoserine lactone (Sigma) in the case of pAMG21, or IPTG to 0.1 mM in the case of pAMG33\*.  
 35 The incubation was continued for an additional 2-4 hours and the cells were collected by centrifugation.

#### EXAMPLE 24

40 This example describes the purification of PTH-TTR(C10A/K15A/A81C). About 197 g of *E. coli* paste from

- 69 -

clone 5933 stored at -80°C was defrosted in 1480 ml of 50 mM tris HCl, 5 mM EDTA, pH 8.0. 60 tablets of Sigma protease inhibitor cocktail 1-873-580 (Saint Louis, MO) was dissolved in the cell suspension and the suspension  
5 was passed through a model 110-Y microfluidizer (Microfluidics, Newton, MA) twice at 14,000 PSI. The lysate was centrifuged at 11,325 x g for 50 min 4°C. The supernatant was removed as the soluble fraction. The soluble fraction was heated in a 65°C water bath  
10 for 30 minutes in polypropylene bottles, at which time the temperature of the contents was 63°C. The soluble fraction was centrifuged at 11,325 x g for 50 minutes 4°C. The supernatant was removed as Heat Soluble. The heat soluble fraction was filtered through a 0.45 µm  
15 cellulose acetate filter with two prefilters and then loaded on to a 240 ml Q-sepharose fast flow (5 cm ID) column (Amersham Pharmacia Biotech, Piscataway, NJ) at 25 ml/min equilibrated in Q-Buffer A (20 mM tris HCl, 2.5 mM EDTA, pH 8.0) at room temperature (about 23°C).  
20 Column was washed with about 2200 ml Q-Buffer A at 30 ml/min. Q-column was eluted with a 15 column volume linear gradient to 60% Q-Buffer B (20 mM tris HCl, 1 M NaCl, 2.5 mM EDTA, pH 8.0) followed by a 2 column volume step to 100% Q-Buffer B. Fractions containing  
25 the TTR fusion as determined by SDS-PAGE were pooled into a single Q-pool (1300 ml). To the Q-pool, 464 ml of 3.8 M ammonium sulfate pH 7.2 was slowly added. The solution was centrifuged at 11,325 x g for 50 min 4°C. The supernatant was removed as the ammonium  
30 sulfate soluble fraction and discarded, and the pellet was resuspended in 450 ml 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0 by gentle agitation at room temperature for about 30 min. The solution was centrifuged at 11,325 x g for 50 min 4°C. Supernatant was removed as phosphate buffer

- 70 -

soluble fraction and filtered through a 0.45  $\mu$ m cellulose acetate filter. Added 240  $\mu$ l 1 M dithiothreitol to the phosphate buffer soluble fraction and loaded on to a 105 ml (2.6 cm) type 1 ceramic hydroxyapatite column (Bio-Rad Inc., Hercules, CA) at 10 ml/min in HA-Buffer A. Column was washed with approximately 210 ml HA-Buffer A at 10 ml/min followed by 3 steps of 25%, 50%, and 100% HA-Buffer B (400 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.0). The fractions from the 50% elution were pooled as HA-pool (725 ml) and filtered through a 0.22  $\mu$ m cellulose acetate filter. 1.16 g of dithiothreitol was added to HA-Pool, and the pH was raised to 8.0 using tris base followed by incubation at room temperature for about 30 minutes. Diluted HA-pool with 750 ml water and loaded on to a 50 ml source 15Q (2.6 cm ID) column (Amersham Pharmacia Biotech) at 10 ml/min followed by a wash with about 250 ml Q-Buffer A. Column was eluted with a 20 column volume linear gradient from 10% to 60% Q-Buffer B followed a step of 2 column volumes of 100% Q-Buffer B. Fractions containing the TTR fusion as determined by SDS-PAGE were pooled into a single Q2-pool (170 ml) and filtered through a 0.22  $\mu$ m cellulose acetate filter. The protein concentration was determined to be 3.7 mg/ml using a calculated extinction coefficient of 23,950  $\text{M}^{-1}\text{cm}^{-1}$ . The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Falmouth, MA). The nucleic acid content was determined to be negligible, since the ratio of the absorbance at 260 nm over 280 nm was determined to be 0.61.

EXAMPLE 25

- 71 -

This example describes the purification of TMP-TTR(C10A/D38C). About 170 g of *E. coli* paste from clone 5705 stored at -80°C was defrosted in 1275 ml of 50 mM tris HCl, 5 mM EDTA, pH 8.0. 50 tablets of Sigma protease inhibitor cocktail 1-873-580 (Saint Louis, MO) was dissolved in the cell suspension and the suspension was passed through a model 110-Y microfluidizer (Microfluidics, Newton, MA) twice at 14,000 PSI. The lysate was centrifuged at 11,325 x g for 30 min 4°C.

10 The supernatant was removed as the soluble fraction and discarded. The pellets were resuspended in 1200 ml water using a tissue grinder and 20 more Sigma protease inhibitor tablets were added. The suspension was centrifuged at 11,325 x g for 30 min 4°C. The

15 supernatant was filtered through a Whatman GF/A filter and 2.1 g of dithiothreitol was added followed by incubation at 7 °C for 30 minutes. The reduced sample was loaded on to a 240 ml Q-sepharose fast flow (5 cm ID) column (Amersham Pharmacia Biotech, Piscataway, NJ)

20 at 30 ml/min equilibrated in Q-Buffer A (20 mM tris HCl, 0.02% sodium azide, pH 8.0) at 7 °C. Column was washed with about 1920 ml Q-Buffer A at 30 ml/min. Q-column was eluted with 3 steps of 20%, 35%, and 100% Q-Buffer B (20 mM tris HCl, 1 M NaCl, 0.02% sodium azide,

25 pH 8.0). Added 13 ml 500 mM EDTA pH 8.0 to the flowthrough from the Q-column and centrifuged for 30 min at 11,325 g at 4 °C. Supernatant was discarded, and the pellet was resuspended in 700 ml 4 M urea, 20 mM tris HCl, pH 8.0. The urea solubilized pellet was

30 then filtered through a Whatman GF/A filter and loaded on to a 240 ml Q-sepharose fast flow (5 cm ID) column (Amersham Pharmacia Biotech, Piscataway, NJ) at 30 ml/min equilibrated in Q-Buffer A (20 mM tris HCl, 0.02% sodium azide, pH 8.0) at 7 °C. Column was washed

- 72 -

with about 1920 ml Q-Buffer A at 30 ml/min. Q-column was eluted with 3 steps of 20%, 35%, and 100% Q-Buffer B (20 mM tris HCl, 1 M NaCl, 0.02% sodium azide, pH 8.0) at 15 ml/min. Fractions containing the 35% elution peak were pooled, filtered through a 0.22  $\mu$ m cellulose acetate filter, and 0.5 g of dithiothreitol (10 mM final concentration) was added followed by incubation for 30 min at 7 °C. The 35% Q-pool was then loaded on to a 45 ml (2.6 cm) type 1 ceramic hydroxyapatite column (Bio-Rad Inc., Hercules, CA) at 5 ml/min in 20 mM tris HCl, 350 mM NaCl, pH 8.0 at 7 °C. Column was washed with approximately 70 ml 20 mM tris HCl, 350 mM NaCl, pH 8.0 at 5 ml/min followed by 3 steps of 2.5%, 25%, and 100% HA-Buffer B (400 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0). The fractions from the 2.5% elution were pooled as HA-pool (80 ml) and filtered through a 0.22  $\mu$ m cellulose acetate filter. The protein concentration was determined to be 6.8 mg/ml using a calculated extinction coefficient of 29,450 M<sup>-1</sup> cm<sup>-1</sup>. The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Falmouth, MA). The nucleic acid content was determined to be negligible, since the ratio of the absorbance at 260 nm over 280 nm was determined to be 0.54.

#### EXAMPLE 26

This example describes the refolding and purification of TTR(C10A)-CH2-TMP. About 23 g of *E. coli* paste from clone 6104 stored at -80°C was defrosted in 200 ml of 50 mM tris HCl, 5 mM EDTA, pH 8.0. 10 tablets of Sigma protease inhibitor cocktail 1-873-580 (Saint Louis, MO) was dissolved in the cell suspension and the suspension was passed through a

- 73 -

microfluidizer (Microfluidics, Newton, MA) twice at 12,000 PSI. The lysate was centrifuged at 15,344 x g for 50 min 4 °C. The supernatant was removed as the soluble fraction and discarded. The pellet was

5 resuspended in 200 ml 50 mM tris HCl, 5 mM EDTA, pH 8.0 using a tissue grinder. The suspension was centrifuged at 15,344 x g for 50 min 4 °C. The supernatant was removed as the wash and discarded. The pellet was resuspended in 50 ml 50 mM tris HCl, 5 mM EDTA, pH 8.0

10 using a tissue grinder. The suspension was centrifuged at 14,000 x g for 10 min room temperature. The supernatant was removed as the wash and discarded. The pellets were dissolved in 50 ml 8 M guanidine HCl, 50 mM tris HCl, pH 8.0 using a sonicator for about 1 min.

15 Dissolved protein was reduced for 30 min room temperature by adding 500 µl 1 M DTT. Reduced protein was centrifuged for 30 min at 20 °C at 27,216 g. Supernatant was then added to 4 L 50 mM tris base, 160 mM arginine base, 1 M urea, 1 mM cystamine, 4 mM

20 cysteine, pH 9.5 at 2 ml/min and incubated about 16 hours 4 °C. Refolded protein was then filtered through a Gellman SUPORCAP® 50 and then concentrated to about 500 ml using a Pall Filtron 3 square foot YM10 membrane tangential flow system followed by diafiltration

25 against 2 L 20 mM tris HCl pH 8.0. Concentrated protein was then loaded on to a 45 ml source 15Q (2.6 cm ID) column (Amersham Pharmacia Biotech) at 18 ml/min followed by a wash with about 150 ml Q-Buffer A (20 mM tris HCl pH 8.0). Column was eluted with a 20 column

30 volume linear gradient from 0% to 60% Q-Buffer B followed a step of 2 column volumes of 100% Q-Buffer B. Fractions containing the TTR fusion as determined by SDS-PAGE were pooled into a single Q-pool (29 ml). The Q-Pool was then concentrated to about 6.3 ml using a

35 Millipore CENTRIPREP™ 10 and then passed through a Pall

- 74 -

ACRODISC® MUSTANG™ E membrane filter at 1 ml/min. The protein concentration was determined to be 10.5 mg/ml using a calculated extinction coefficient of 46,410 M<sup>-1</sup> cm<sup>-1</sup>. The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Falmouth, MA). The nucleic acid content was determined to be negligible, since the ratio of the absorbance at 260 nm over 280 nm was determined to be 0.51.

10

EXAMPLE 27

This example describes the purification of GLP1-TTR (C10A/K15A/G83C). About 30 g of *E. coli* paste from clone 6450 stored at -80°C was defrosted in 250 ml of 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0. Cell suspension was passed through a microfluidizer (Microfluidics, Newton, MA) twice at 12,000 PSI. The lysate was centrifuged at 15,344 x g for 50 min 4°C. The supernatant was discarded as the soluble fraction, and the pellet was resuspended in 200 ml deoxycholate using a tissue grinder. The suspension was centrifuged at 15,344 x g for 50 min 4°C. The supernatant was discarded as the wash, and the pellet was resuspended in 200 ml water using a tissue grinder. The suspension was centrifuged at 15,344 x g for 50 min 4°C. The supernatant was discarded as the wash, and the pellet was resuspended in 100 ml water using a tissue grinder. The suspension was centrifuged at 27,216 x g for 30 min room temperature. The supernatant was discarded as the wash, and about 2/3 of the pellets were dissolved in 75 ml 8 M guanidine HCl, 50 mM tris HCl, pH 8.0 by agitation for about 15 min. The suspension was centrifuged at 27,216 x g for 30 min room temperature, and the supernatant was diluted with 18 ml water.

- 75 -

Sample was then loaded on to a 50 ml chelating  
sepharose fast flow column (Amersham Pharmacia Biotech,  
Piscataway, NJ), loaded with  $\text{NiCl}_2$ , at 5 ml/min. After  
washing with about 150 ml Ni-Buffer A (6 M guanidine  
5 HCl, 37.5 ml tris HCl, pH 8.0) at 10 ml/min, eluted  
with two step of 10% and 100% Ni-Buffer B (6 M  
guanidine HCl, 37.5 mM tris HCl, 400 mM imidazole, pH  
8.0). Combined the peak containing the fusion  
construct as Ni-Pool (40 ml) and determined the protein  
10 content to be 6.4 mg/ml by observing the absorbance at  
280 nm in 8 M guanidine HCl using an extinction  
coefficient of  $25,440 \text{ M}^{-1}$ . Added 800  $\mu\text{l}$  500 mM EDTA pH  
8.0 and removed 80 mg of protein for the PEGylation  
reaction. Added 230  $\mu\text{l}$  1 M DTT and incubated for 30  
15 min at 30 °C. Loaded on to a 130 ml SEPHADEX™ G25  
medium column (2.6 cm ID) (Amersham Pharmacia Biotech,  
Piscataway, NJ) at 8 ml/min in 20 mM tris HCl, 6 M  
urea, pH 8.5. Pooled the protein peak as determined by  
absorbance at 280 nm (22 ml) and determined the  
20 concentration to be 3.2 mg/ml by observing the  
absorbance at 280 nm in 20 mM tris HCl, 6 M urea, pH  
8.5 using an extinction coefficient of  $25,440 \text{ M}^{-1}$ .  
Reacted 45% of the buffer exchanged material with 950  
 $\mu\text{l}$  of 5 mM methoxy-PEG-maleimide 5K (Shearwater  
25 Corporation, Huntsville, AL) for 140 min at 30 °C.  
Added 100  $\mu\text{l}$  1 M 2-mercaptoethanol to each reaction to  
quench. Dialyzed reaction against 1 L 25 mM  $\text{NaH}_2\text{PO}_4$ , 3  
M urea, pH 7.25 using a Pierce 10 kDa Slidealyzer for 2  
hour room temperature. Changed the dialysis buffer for  
30 25 mM  $\text{NaH}_2\text{PO}_4$ , 10% sucrose, 2 mM EDTA, pH 7.25 and  
incubated for about 16 hours room temperature. Added  
140  $\mu\text{l}$  5% CHAPS and 7.28  $\mu\text{l}$  2-mercaptoethanol and 0.475  
ml of 3 mg/ml caspase 3 followed by a 2 hour incubation  
at room temperature. Reaction mixture was loaded on to



- 76 -

a 5 ml HiTrap Q-sepharose HP column (Amersham Pharmacia Biotech, Piscataway, NJ) at 1 ml/min in 20 mM tris HCl pH 8.0 followed by about a 15 ml wash in the same buffer. Column was then developed at 5 ml/min using a  
5 linear gradient to 60% 20 mM tris HCl, 1 M NaCl, pH 8.0 followed by a step to 100% of the elution buffer. Fractions containing the TTR fusion as determined by SDS-PAGE were pooled into a single Q-pool (9.5 ml). Concentrated Q-Pool to 3.2 ml using a Millipore  
10 CENTRIPREP™ 30 kDa and filtered through a Pall MUSTANG™ E membrane at about 1 ml/min. Diluted Q-Pool to 6.5 ml with water and added 375 µl acetonitrile. Injected on to a Vydac Protein/Peptide 10 X 250 mm C<sub>4</sub> column (Vydac, Hesperia, CA) in 95% RP-Buffer A (0.1% trifluoroacetic acid) with 5% RP-Buffer B (95%  
15 acetonitrile, 0.1% trifluoroacetic acid) at 5 ml/min. Developed column running a linear gradient to 100% RP-Buffer B. Concentrated protein peak to about 3 ml using a Millipore CENTRIPREP™ 30 kDa and diluted to 15  
20 ml using 20 mM tris HCl pH 8.0. Repeated buffer exchange 3 more times then passed through a Pall MUSTANG™ E membrane at about 1 ml/min. The protein concentration was determined to be 7.7 mg/ml using a calculated extinction coefficient of 25,440 M<sup>-1</sup> cm<sup>-1</sup>.  
25 The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Falmouth, MA). The nucleic acid content was determined to be negligible, since the ratio of the absorbance at 260 nm over 280 nm was  
30 determined to be 0.54.

#### EXAMPLE 28

This example shows the effect of injecting  
35 pegylated GLP1-TTR constructs into mice on blood glucose levels. For this example 40 male, db/db, 9

- 77 -

week-old mice were split into 4 groups and injected (hour 0) intraperitoneal with 7.4 - 16.6 mg test protein per animal (538 pmol monomers for all groups) (5K pegylated GLP1-TTR fusion construct 10 mg, 20K  
5 pegylated GLP1-TTR fusion construct 10 mg, GLP1-Fc 16.6 mg, and a TTR(C10A) control 7.4 mg). Each group was bled at time points 0(baseline measurement), 1, 4, 6, 12, 24, and 48 hours post injection. Food was withheld from the mice for the first 6 hours of the experiment  
10 and replaced after the bleed at the 6 hour time point. Each collected drop of blood per time point was analyzed for glucose content using a One Touch Profile glucose meter and the results are depicted in Figure 12.

15

EXAMPLE 29

This example shows the effect of injecting TMP-TTR constructs with fused antibody CH2 domains into  
20 mice on blood platelet count. For this example 50 female BDF1 mice were split into 5 groups and injected (day 0) subcutaneously with 50 mg test protein per kg animal (TMP fusion construct, Fc-TMP, or a TTR(C10A) control). Each group was divided in half and bled (140  
25 ml) on alternate time points (day 0, 3, 5, 7, and 10). Mice were anesthetized with isoflurane prior to collection.

The collected blood was analyzed for a complete and differential count using an ADVIA 120  
30 automated blood analyzer with murine software (Bayer Diagnostics, New York, NY). As seen in Figure 13, Fc-TMP showed the greatest response with platelet count rising to over  $4.2 \times 10^{12}$  platelets L-1 on day 5 which is 3 times baseline at  $1.4 \times 10^{12}$  platelets L-1. All  
35 three of the CH2 fused TMP-TTR constructs performed better than the control, but not as well as Fc-TMP with

- 78 -

platelet counts between  $2.3 \times 10^{12}$  and  $2.6 \times 10^{12}$   
platelets L-1 on day 5, which is between a 64% and 86%  
improvement over baseline.

5

EXAMPLE 30

This example shows the effect of injecting  
pegylated TTR constructs with TMP fused to the carboxy-  
terminus of pegylated TTR into mice on blood platelet  
10 count. For this example 80 BDF1 mice were split into 8  
groups and injected (day 0) subcutaneously with 50 mg  
test protein per kg animal (TMP fusion constructs, Fc-  
TMP, or a TTR(C10A) control). Each group was divided  
in half and bled (140 ml) on alternate time points (day  
15 0, 3, 5, 7, 10, and 12). Mice were anesthetized with  
isoflurane prior to collection.

The collected blood was analyzed for a  
complete and differential count using an ADVIA 120  
automated blood analyzer with murine software (Bayer  
20 Diagnostics, New York, NY). As seen in Figure 14, Fc-  
TMP and the three amino terminal (TMP-TTR) fusions  
showed the greatest response with platelet count rising  
between  $4.3 \times 10^{12}$  and  $4.6 \times 10^{12}$  platelets L-1 on day 5  
which is over three times baseline at  $1.3 \times 10^{12}$   
25 platelets L-1. All three of the carboxy terminal (TTR-  
TMP) constructs performed better than the control.

EXAMPLE 31

30 This example shows the effect of injecting  
pegylated TTR-TMP constructs containing a K15A  
alteration into mice on blood platelet count. For this  
example 120 BDF1 mice were split into 12 groups and  
injected (day 0) subcutaneously with 50 mg test protein  
35 per kg animal (TMP fusion constructs, Fc-TMP, or a  
TTR(C10A) control) (this study was split into two

- 79 -

batches (PEG 20K in one and the PEG 5K and non-pegylated samples in the other) completed at separate times with repeated controls). Each group was divided in half and bled (140 ml) on alternate time points (day 0, 3, 5, 7, 10, and 12). Mice were anesthetized with isoflurane prior to collection.

The collected blood was analyzed for a complete and differential count using an ADVIA 120 automated blood analyzer with murine software (Bayer Diagnostics, New York, NY). As seen in Figure 15A, the two non-pegylated constructs outperformed the baseline ( $1.3 \times 10^{12}$  platelets L-1) with platelet responses at day 5 rising between  $1.8 \times 10^{12}$  and  $2.0 \times 10^{12}$  platelets L-1. As seen in Figure 15B, Fc-TMP and the three 5K pegylated fusions showed equivalent responses at day 5 with platelet counts rising between  $3.5 \times 10^{12}$  and  $4.4 \times 10^{12}$  platelets L-1 which is at least 2.7 times baseline ( $1.3 \times 10^{12}$  platelets L-1). As seen in Figure 15C, Fc-TMP and the three 20K pegylated fusions showed equivalent responses at day 5 with platelet count rising between  $4.3 \times 10^{12}$  and  $4.6 \times 10^{12}$  platelets L-1 which is over three times baseline at  $1.3 \times 10^{12}$  platelets L-1.

In addition, the 20K pegylated TTR constructs appear to have an improved sustained response with platelets at day 7 ranging from  $3.7 \times 10^{12}$  to  $4.9 \times 10^{12}$  platelets L-1 compared to Fc-TMP at  $3.1 \times 10^{12}$  platelets L-1. This sustained response is maintained at day 10 for the three 20K pegylated TTR constructs with platelets ranging from  $2.3 \times 10^{12}$  to  $3.1 \times 10^{12}$  platelets L-1 compared to Fc-TMP at  $2.0 \times 10^{12}$  platelets L-1.

## THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A method for manufacturing a fusion product comprising fusing a biologically active agent to transthyretin (TTR) or a TTR variant, wherein the biologically active agent comprises a protein or peptide selected from the group consisting of cytokines, hematopoietic factors, interleukins, growth factors, trophic factors, and hormones, and wherein the fusion product exhibits increased serum half life *in vivo* compared to the biologically active agent.
2. A fusion product comprising a biologically active agent fused to a transthyretin (TTR) or a TTR variant, wherein the biologically active agent comprises a protein or peptide selected from the group consisting of cytokines, hematopoietic factors, interleukins, growth factors, trophic factors, and hormones.
3. The method or fusion product according to claim 1 or claim 2, wherein the biologically active agent is fused to TTR.
4. The method or fusion product according to claim 3, wherein said TTR is encoded by the nucleic acid of SEQ ID NO: 2.
5. The method or fusion product according to claim 1 or claim 2, wherein the biologically active agent is fused to a TTR variant.
6. The method or fusion product according to claim 5, wherein the TTR variant is encoded by the nucleic acid of SEQ ID NO: 8.
7. The method or fusion product according to any one of claims 1-6, wherein said TTR or TTR variant is chemically modified with a chemical selected from the group consisting of dextran, poly (n-vinyl pyrrolidone), polyethylene glycols, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohols.

8. The method or fusion product according to any one of claims 1-7, wherein said TTR or TTR variant is chemically modified with polyethylene glycol (PEG).
9. The method or fusion product according to claim 8, wherein the PEG has a  
5 molecular weight of between about 1 kD and 100 kD.
10. The method or fusion product according to claim 9, wherein the PEG has a molecular weight of between about 5 kD and 30 kD.
- 10 11. The fusion product according to any one of claims 2-10 that is a fusion protein comprising a TTR or TTR variant fused to a biologically active agent that comprises a heterologous sequence.
12. The method or fusion product according to any one of claims 1-11, wherein the  
15 biologically active agent is a TPO mimetic peptide (TMP).
13. The method or fusion product according to any one of claims 1-11, wherein the biologically active agent is selected from the group consisting of: a Glucagon-like Peptide-1 (GLP-1), GLP-1(7-34), GLP-1(7-35), GLP-1(7-36), GLP-1(7-37), Gln<sup>9</sup>-GLP-  
20 1(7-37), D-Gln<sup>9</sup>-GLP-1(7-37), Thr<sup>16</sup>-Lys<sup>18</sup>-GLP-1(7-37), and Lys<sup>18</sup>-GLP-1(7-37), wherein the fusion product has insulinotropic activity.
14. The method or fusion product according to any one of claims 1-13, wherein the fusion product further comprises a linker peptide between the TTR and the biologically  
25 active agent.
15. A nucleic acid that comprises a nucleotide sequence that encodes the fusion product of any one of claims 11-14.
- 30 16. The nucleic acid of claim 15 further comprising a promoter operably attached to the nucleotide sequence that encodes the fusion product.
17. A vector that comprises the nucleic acid of claim 15 or 16.

18. The vector of claim 17 that is an expression vector.
19. A host cell transformed or transfected with the vector of claim 18.
- 5 20. A method for manufacturing a fusion product of claim 2 that is a fusion protein, the method comprising expressing a nucleic acid that comprises a nucleotide sequence that encodes the fusion product as a fusion protein.
- 10 21. A substantially homogenous preparation of a fusion product according to any one of claims 2-15.
22. The preparation according to claim 21 in a pharmaceutically acceptable diluent, carrier, or adjuvant.
- 15 23. The preparation according to claim 21 or 22 for use in therapy.
24. The fusion product according to claim 12 for use in treating thrombocytopenia.
- 20 25. The fusion product according to claim 13 for treating non-insulin dependent diabetes.
26. The preparation of claim 21 or 22, wherein the biological agent is TMP, for the manufacture of a medicament for treating thrombocytopenia.
- 25 27. The preparation of claim 21 or 22, wherein the biologically active agent is GLP-1, for the manufacture of a medicament for treating non-insulin dependent diabetes.
28. A process for preparing a substantially homogenous preparation of a TTR-  
30 biologically active agent fusion product comprising: (a) fusing TTR to a biologically active agent according to the method of claim 1, to provide a TTR-biologically active agent fusion product; and (b) isolating said TTR-biologically active agent fusion product.

29. A process for preparing a substantially homogenous preparation of a TTR variant-biologically active agent fusion product comprising engineering a cysteine residue into a specific amino acid position within the amino acid sequence of TTR to provide a variant  
5 of said TTR; and, subsequently, fusing TTR variant to a biologically active agent according to the method of claim 1, to provide a TTR variant-biologically active agent fusion product; and isolating said TTR variant biologically active agent fusion product.

30. A process for preparing a substantially homogenous preparation of a PEG-TTR-  
10 biologically active agent fusion product comprising: (a) conjugating a polyethylene glycol to TTR to provide a PEG-TTR; and, subsequently, the process of claim 28 using said PEG-TTR.

31. A process for preparing a substantially homogenous preparation of a PEG-TTR  
15 variant-biologically active agent fusion product comprising: (a) engineering a cysteine residue into a specific amino acid position within the amino acid sequence of TTR to provide a variant TTR; (b) conjugating a polyethylene glycol to said TTR variant to provide a PEG-TTR variant and, subsequently, fusing PEG-TTR variant to a biologically active agent according to the method of claim 5, to provide a PEG-TTR  
20 variant-biologically active agent fusion product; and isolating said PEG-TTR variant-biologically active agent fusion product.

32. A process for preparing a substantially homogeneous preparation of a PEG-TTR-biologically active agent fusion product comprising: (a) fusing TTR to a biologically  
25 active agent according to claim 3 to provide a TTR-biologically active agent fusion product; (b) conjugating a polyethylene glycol to said TTR-biologically active agent to provide a PEG-TTR-biologically active agent fusion; and (c) isolating the PEG-TTR-biologically active agent fusion product.

30 33. A process for preparing a substantially homogenous preparation of a PEG-TTR variant-biologically active agent fusion product comprising: (a) engineering a cysteine residue into a specific amino acid position within the amino acid sequence of TTR to provide a TTR variant; (b) fusing said TTR variant to a biologically active agent



according to claim 5 to provide a TTR variant-biologically active agent fusion product;  
(c) conjugating a polyethylene glycol to said TTR variant to provide a PEG-TTR  
variant-biologically active agent fusion product; and (d) isolating the PEG-TTR variant-  
biologically active agent fusion product.

5

34. A process for preparing a PEG-TTR-biologically active agent fusion product or a  
PEG-TTR variant-biologically active agent fusion product comprising: starting with a  
fusion product according to claim 2; and conjugating a polyethylene glycol thereto.

10 35. A method of treating thrombocytopenia comprising administering a therapeutically  
effective dose of a preparation of the fusion product of claim 12.

36. A method of treating non-insulin dependent diabetes comprising administering a  
therapeutically effective dose of a preparation of the fusion product of claim 13.

15

37. A fusion product produced by the method of claim 1 or the process of any one of  
claims 28 to 34.

38. Use of a fusion product of claim 12 for the preparation of a medicament for  
20 treating thrombocytopenia.

39. Use of a fusion product of claim 13 for the preparation of a medicament for  
treating non-insulin dependent diabetes.

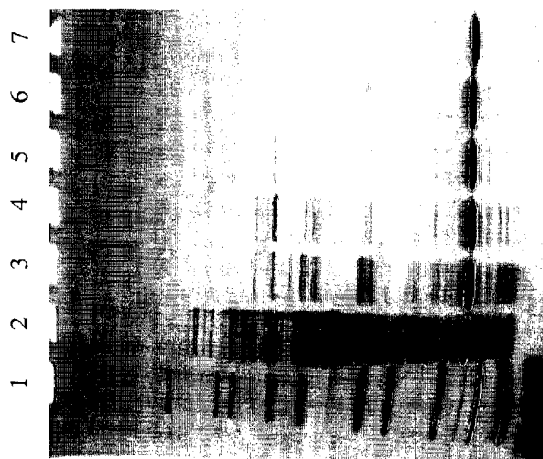
25 40. A method for manufacturing a fusion product; a fusion product; a nucleic acid; a  
vector; a host cell; a substantially homogenous preparation of a fusion product; a process  
for preparing a substantially homogenous preparation of a TTR-biologically active agent  
fusion product; a process for preparing a substantially homogenous preparation of a TTR  
variant-biologically active agent fusion product; a process for preparing a substantially  
30 homogenous preparation of a PEG-TTR-biologically active agent fusion product; a  
process for preparing a substantially homogenous preparation of a PEG-TTR variant-  
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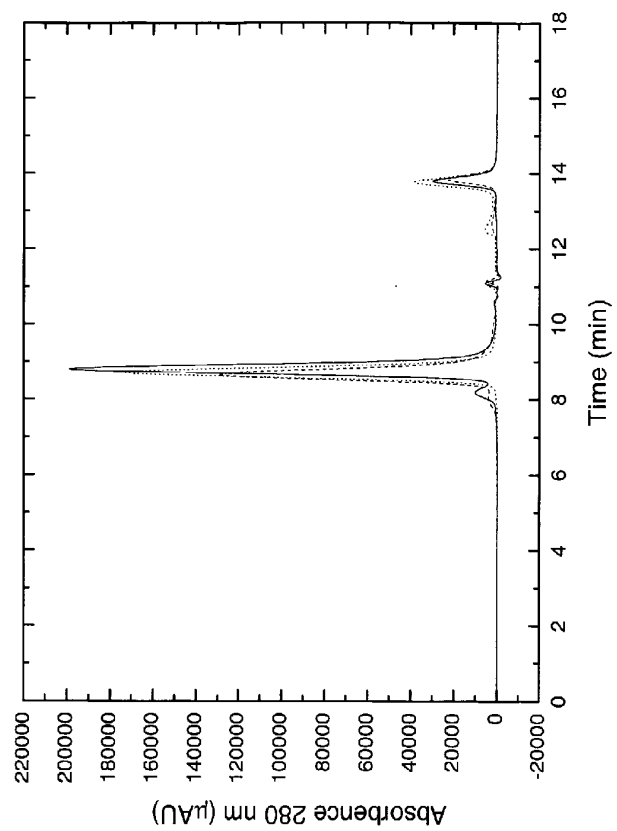
fusion product; a method of treating thrombocytopenia; a method of treating non-insulin dependent diabetes; a fusion product produced by the method of claim 1 or the process of any one of claims 28 to 34; a substantially homogenous preparation of a TTR-biologically active agent fusion product; a substantially homogenous preparation of a

5 TTR variant-biologically active agent fusion product; a substantially homogenous preparation of a PEG-TTR-biologically active agent fusion product; a substantially homogenous preparation of a PEG-TTR variant-biologically active agent fusion product; a PEG-TTR-biologically active agent fusion product or a PEG-TTR variant-biologically active agent fusion product; or use of a fusion product, substantially as herein described

10 with reference to any one or more of the examples but excluding comparative examples.

**Figure 1**



**Figure 2**

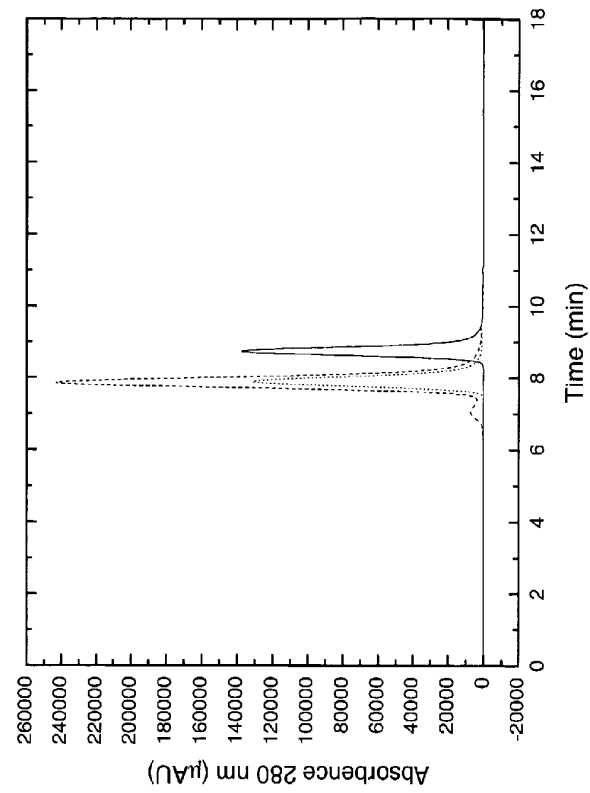
**Figure 3**

Figure 4

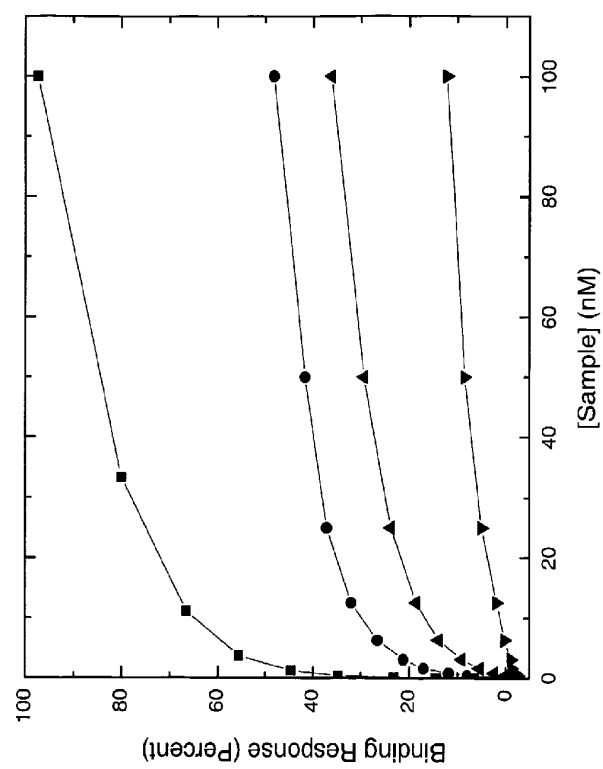
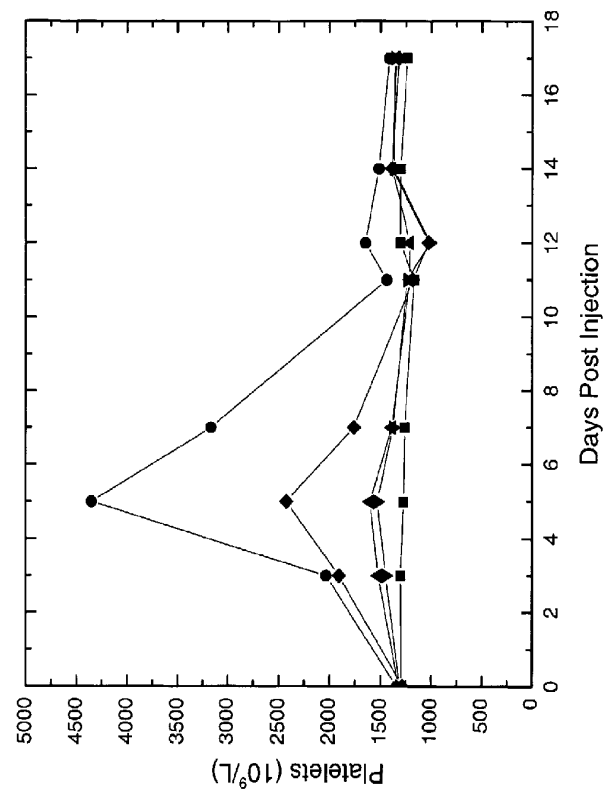


Figure 5



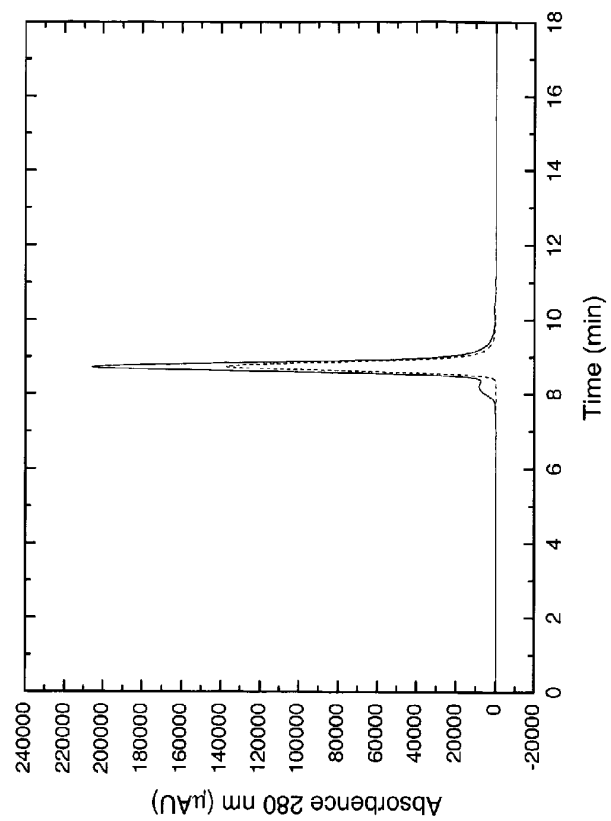
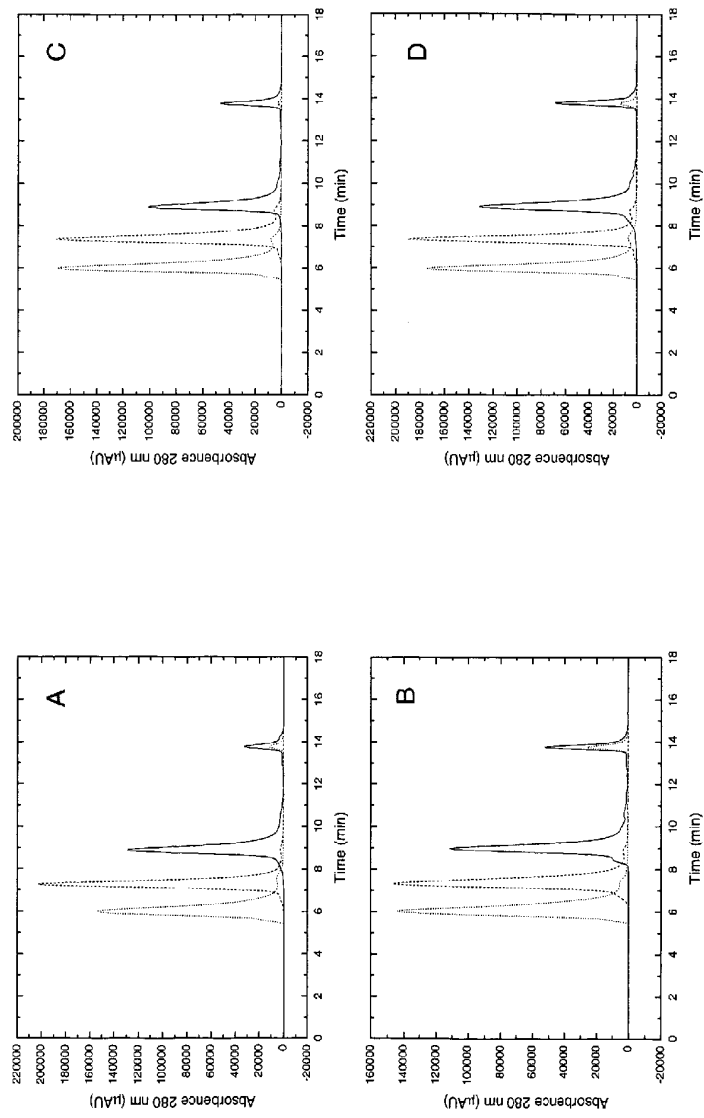
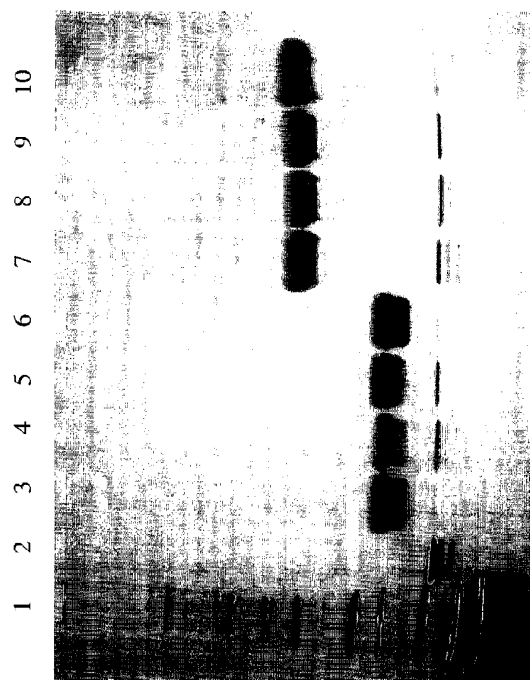
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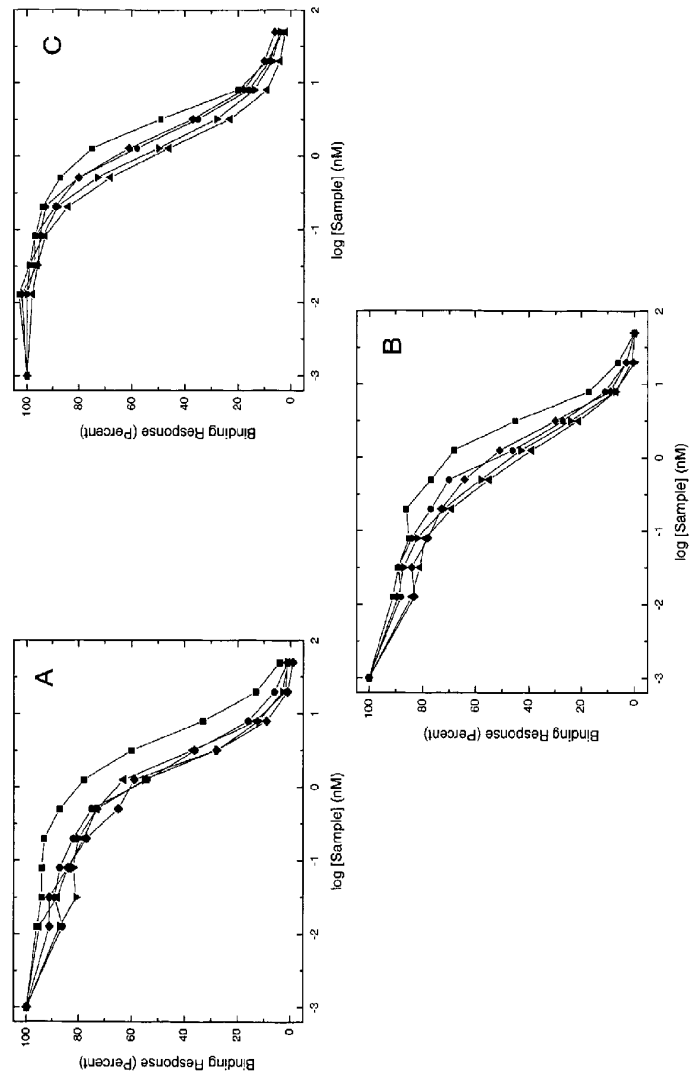


Figure 7

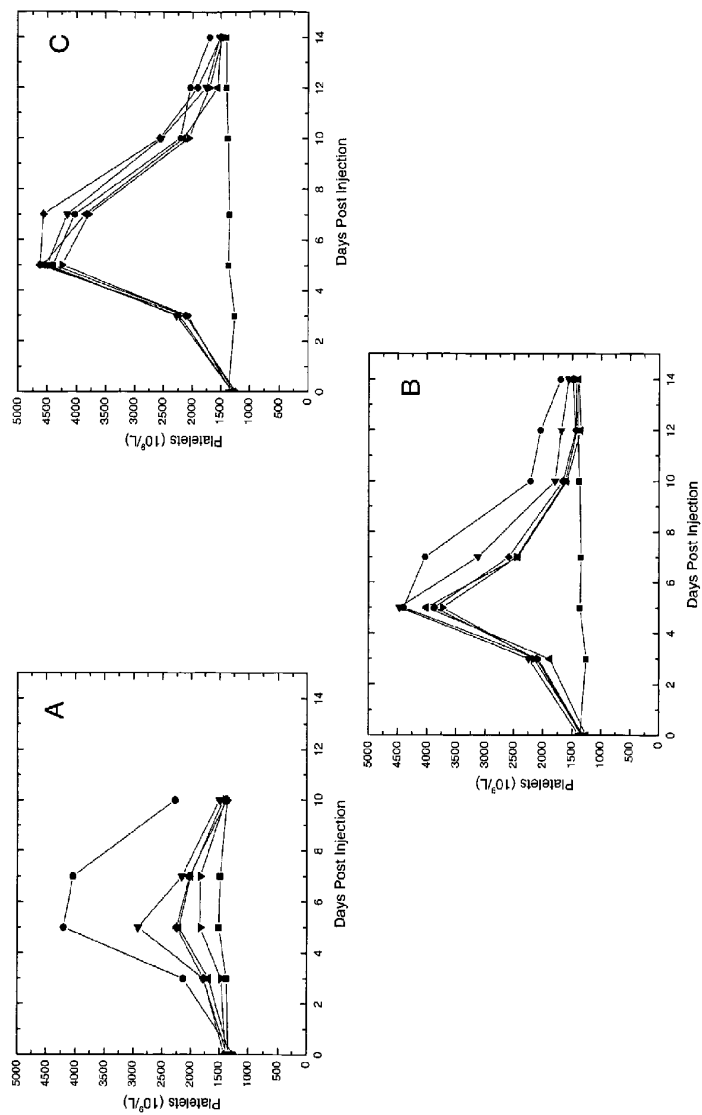


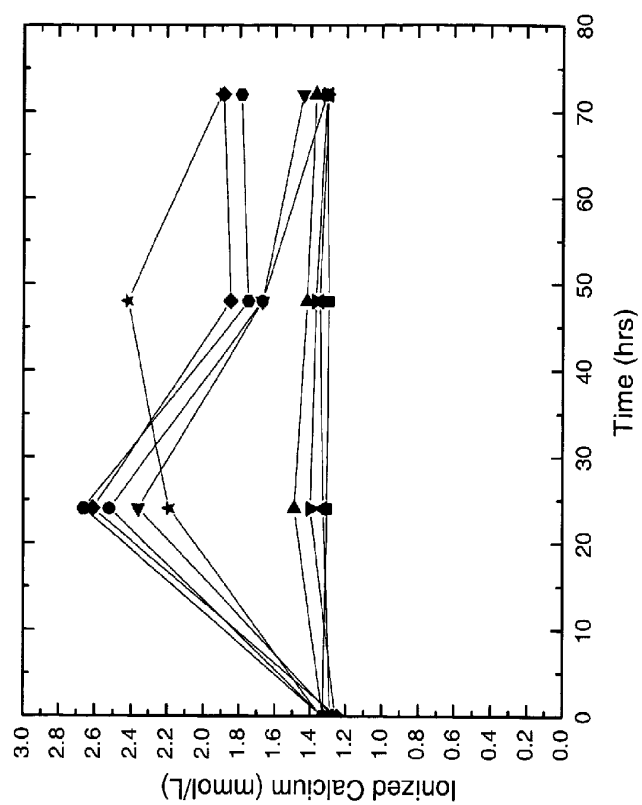
**Figure 8**

**Figure 9**

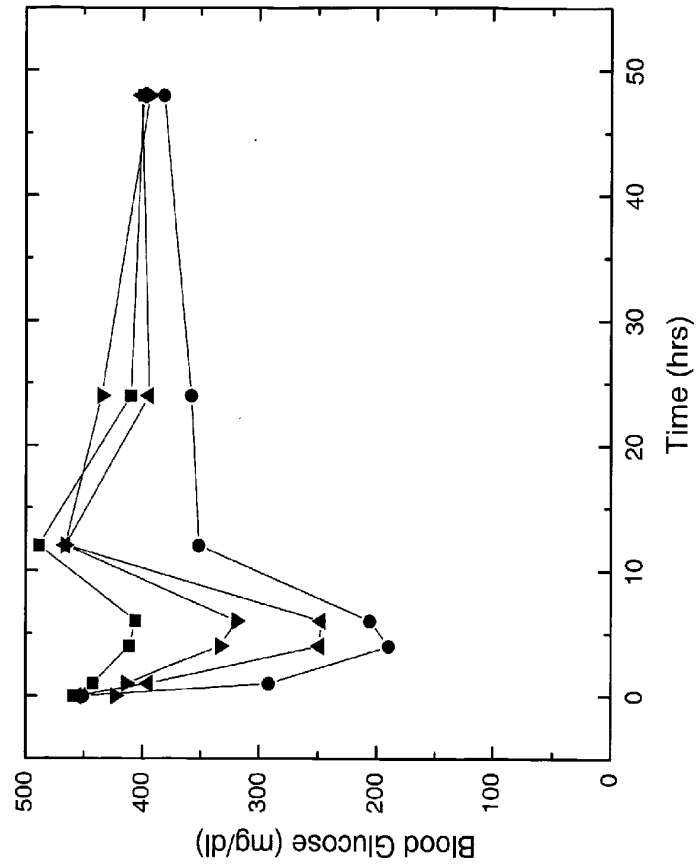


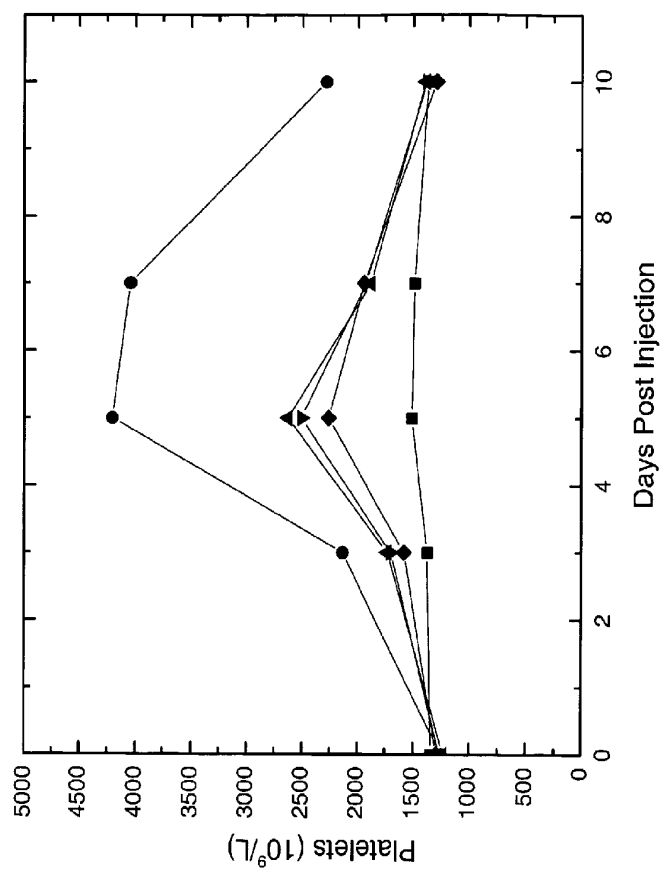
**Figure 10**



**Figure 11**

**Figure 12**



**Figure 13**

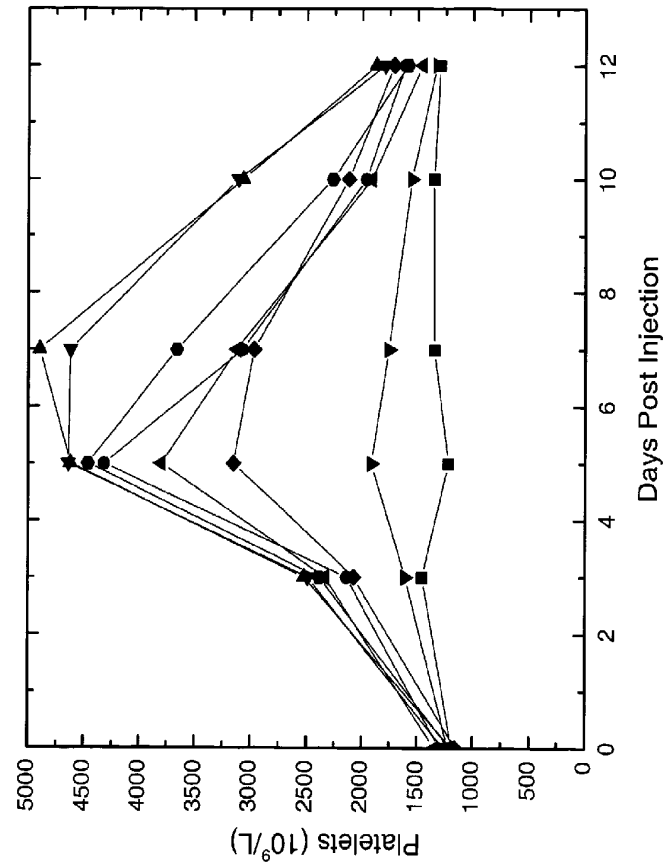
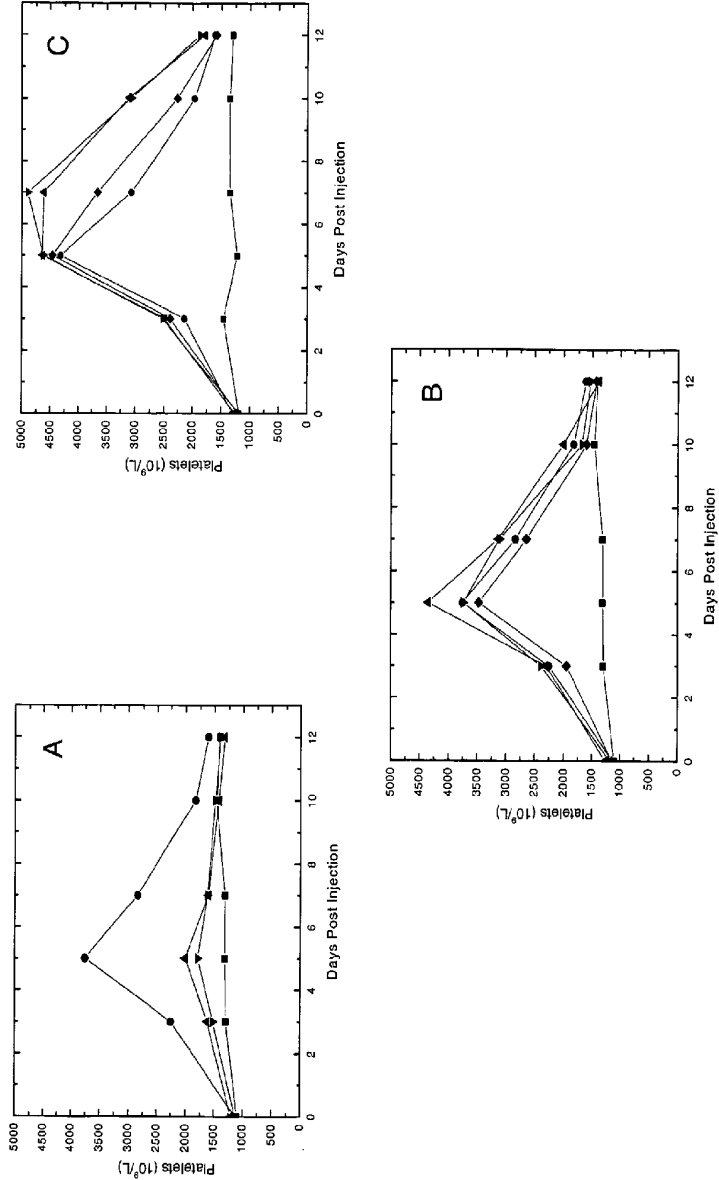
**Figure 14**



Figure 15



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 1 5 10 15  
 Leu Asp Ala Val Arg Gly Ser Pro Ala Ile Asn Val Ala Val His Val  
 20 25 30  
 Phe Arg Lys Ala Ala Asp Asp Thr Trp Glu Pro Phe Ala Ser Gly Lys  
 35 40 45  
 Thr Ser Glu Ser Gly Glu Leu His Gly Leu Thr Thr Glu Glu Glu Phe  
 50 55 60  
 Val Glu Gly Ile Tyr Lys Val Glu Ile Asp Thr Lys Ser Tyr Trp Lys  
 65 70 75 80  
 Ala Leu Gly Ile Ser Pro Phe His Glu His Ala Glu Val Val Phe Thr  
 85 90 95  
 Ala Asn Asp Ser Gly Pro Arg Arg Tyr Thr Ile Ala Ala Leu Leu Ser  
 100 105 110  
 Pro Tyr Ser Tyr Ser Thr Thr Ala Val Val Thr Asn Pro Lys Glu  
 115 120 125  
 <210> 2  
 <211> 387  
 <212> DNA  
 <213> Homo sapiens  
 <400> 2

A-813A PCT.ST25.txt

```

agtggtccaa ctggtaccgg tgaatccaag gtcctctga tggtaaagt tctagatgct 60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120
acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180
actgaggagg aattttaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240
aaggcacttg gcatctcccc attccatgag catgcagagg tggatttcac agccaacgac 300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaataa 387

<210> 3
<211> 387
<212> DNA
<213> Homo sapiens

<400> 3
atgggtccaa ctggtaccgg tgaatccaag gtcctctga tggtaaagt tctagatgct 60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120
acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180
actgaggagg aattttaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240
aaggcacttg gcatctcccc attccatgag catgcagagg tggatttcac agccaacgac 300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaataa 387

<210> 4
<211> 387
<212> DNA
<213> Homo sapiens

<400> 4
atgggtccaa ctggtaccgg tgaatccaag gtcctctga tggtaaagt tctagatgct 60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc ttgtgatgac 120
acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180
actgaggagg aattttaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240
aaggcacttg gcatctcccc attccatgag catgcagagg tggatttcac agccaacgac 300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaataa 387

<210> 5
<211> 387
<212> DNA
<213> Homo sapiens

<400> 5

```

A-813A PCT.ST25.txt

```

atgggtccaa ctggtaccgg tgaatccaag gctcctctga tgggtcaaagt tctagatgct 60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgcttgatgac 120
acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180
actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240
aaggcacttg gcatctcccc attccatgag catgcagagg tggatttcac agccaacgac 300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaataa 387

```

```

<210> 6
<211> 387
<212> DNA
<213> Homo sapiens

```

```

<400> 6
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tgggtcaaagt tctagatgct 60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgcttgatgac 120
acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180
actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240
aagtgtcttg gcatctcccc attccatgag catgcagagg tggatttcac agccaacgac 300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaataa 387

```

```

<210> 7
<211> 387
<212> DNA
<213> Homo sapiens

```

```

<400> 7
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tgggtcaaagt tctagatgct 60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgcttgatgac 120
acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180
actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240
aaggcacttt gcatctcccc attccatgag catgcagagg tggatttcac agccaacgac 300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaataa 387

```

```

<210> 8
<211> 387
<212> DNA
<213> Homo sapiens

```

```

<400> 8

```

A-813A PCT.ST25.txt

```

atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcgcagt tctagatgct    60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac    120
acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca    180
actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg    240
aaggcacttt gcatctcccc attccatgag catgcagagg tggatttcac agccaacgac    300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg    360
gctgtcgtca ccaatcccaa ggaataa                                     387

```

<210> 9  
 <211> 495  
 <212> DNA  
 <213> Homo sapiens

```

<400> 9
atgatacgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggaggcgga    60
gggggtggca ttgaggggcc aacccttcgc caatggcttg cagcacgcgc aggtccaact    120
ggtagcgggtg aatccaagtg tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt    180
cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca    240
tttgctctctg ggaaccagc tgaagtctga gagctgcatg ggctcacaac tgaggaggaa    300
ttttagaag ggatatacaa agtggaata gacaccaa atcttactggaa ggcacttggc    360
atctcccat tccatgagca tgcagagggtg gtattcacag ccaacgactc cggccccgc    420
cgctacacca ttgccccct gctgagcccc tactcctatt ccaccacggc tgcgtcacc    480
aatccaagg aataa                                     495

```

<210> 10  
 <211> 495  
 <212> DNA  
 <213> Homo sapiens

```

<400> 10
atgatacgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggaggcgga    60
gggggtggca ttgaggggcc aacccttcgc caatggcttg cagcacgcgc aggtccaact    120
ggtagcgggtg aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt    180
cctgccatca atgtggccgt gcatgtgttc agaaaggctt gtgatgacac ctgggagcca    240
tttgctctctg ggaaccagc tgaagtctga gagctgcatg ggctcacaac tgaggaggaa    300
ttttagaag ggatatacaa agtggaata gacaccaa atcttactggaa ggcacttggc    360
atctcccat tccatgagca tgcagagggtg gtattcacag ccaacgactc cggccccgc    420
cgctacacca ttgccccct gctgagcccc tactcctatt ccaccacggc tgcgtcacc    480
aatccaagg aataa                                     495

```

## A-813A PCT.ST25.txt

<210> 11  
<211> 495  
<212> DNA  
<213> Homo sapiens

<400> 11  
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggaggcgga 60  
gggggtggca ttgaggggcc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120  
ggtagcgggt aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180  
cctgccatca atgtggccgt gcatgtgttc agaaaggctg cttgtgacac ctgggagcca 240  
tttgccctctg ggaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300  
tttgtagaag ggatatacaa agtggaaata gacaccaaatt cttactggaa ggcacttggc 360  
atctcccat tccatgagca tgcagagggtg gtattcacag ccaacgactc cggccccgc 420  
cgctacacca ttgccgccct gctgagcccc tactctatt ccaccacggc tgcgtcacc 480  
aatccaagg aataa 495

<210> 12  
<211> 495  
<212> DNA  
<213> Homo sapiens

<400> 12  
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggaggcgga 60  
gggggtggca ttgaggggcc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120  
ggtagcgggt aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180  
cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240  
tttgccctctg ggaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300  
tttgtagaag ggatatacaa agtggaaata gacaccaaatt cttactggaa gtgtcttggc 360  
atctcccat tccatgagca tgcagagggtg gtattcacag ccaacgactc cggccccgc 420  
cgctacacca ttgccgccct gctgagcccc tactctatt ccaccacggc tgcgtcacc 480  
aatccaagg aataa 495

<210> 13  
<211> 495  
<212> DNA  
<213> Homo sapiens

<400> 13  
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggaggcgga 60  
gggggtggca ttgaggggcc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120  
ggtagcgggt aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180

## A-813A PCT.ST25.txt

```

cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240
tttgctcttg ggaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300
tttgtagaag ggatatacaa agtggaaata gacacaaat ctactggaa ggcactttgc 360
atctcccat tccatgagca tgcagagggt gtattcacag ccaacgactc cggccccgc 420
cgctacacca ttccgccct gctgagccc tactctatt ccaccacggc tgcgtcacc 480
aatccaagg aataa 495

```

```

<210> 14
<211> 489
<212> DNA
<213> Homo sapiens

```

```

<400> 14
atgtctgttt ctgaaatcca gctgatgcat aacctgggta aacatctgaa ctctatggaa 60
cgtgttgaat ggctgcgtaa gaaactgcag gacgttcata actttgtcc aactggtacc 120
gggtaatcca aggcctctct gatggtcgca gttctagatg ctgtccgagg cagtcctgcc 180
atcaatgtgg ccgtgcatgt gttcagaaag gctgctgatg acacctggga gccatttgcc 240
tctgggaaaa ccagtgaagtc tggagagctg catgggctca caactgagga ggaatttgta 300
gaagggatat acaaagtga aatagacacc aaatttact ggaagtgtct tggcatctcc 360
ccattccatg agcatgcaga ggtggtatc acagccaacg actccggccc cgcgcgtac 420
accattgccg cctgctgag cccctactcc tattccacca cggctgtcgt caccaatccc 480
aaggaataa 489

```

```

<210> 15
<211> 843
<212> DNA
<213> Homo sapiens

```

```

<400> 15
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cagaaaacct tctacctgcg caacaaccag ctggttgctg gctacctgca gggccgaac 120
gttaacctgg aagaaaaaat cgacgttgta ccgatcgaac cgcacgctct gttcctgggt 180
atccacgggt gtaaaatgtg cctgagctgc gtgaaatctg gtgacgaaac tcgtctgcag 240
ctggaagcag ttaacatcac tgacctgagc gaaaaccgca aacaggacaa acgtttcgca 300
ttcatccgct ctgacagcgg cccgaccacc agcttcgaat ctgctgcttg cccgggttgg 360
ttcctgtgca ctgctatgga agctgaccag ccggttaagc tgaccaacat gccggacgaa 420
ggcgtgatgg taacaaatt ctactccag gaagacgaag gtccaactgg taccggtgaa 480
tccaaggctc ctctgatggt caaagttcta gatgctgtcc gaggcagtcc tgccatcaat 540

```

A-813A PCT.ST25.txt

```

gtggccgtgc atgtgttcag aaaggctgct gatgacacct gggagccatt tgcctctggg 600
aaaaccagtg agtctggaga gctgcatggg ctcacaactg aggaggaatt tgtagaaggg 660
atatacaaa tggaataga caccaaatct tactggaagg cacttgcat ctccccattc 720
catgagcatg cagagggtgt attcacagcc aacgactccg gccccgcgct ctacaccatt 780
gccgcccctg tgagccccta ctctattcc accacggctg tcgtcaccaa tccaaggaa 840
taa 843

```

<210> 16  
 <211> 855  
 <212> DNA  
 <213> Homo sapiens

```

<400> 16
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtaaagt tctagatgct 60
gtccgaggca gtccctccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120
acctgggagc catttgcttc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180
actgaggagg aattttaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240
aaggcacttg gcatctcccc attccatgag catgcagagg tggatttcac agccaacgac 300
tccggcccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaaggtagt ggtagccgac cgtccggcgg taagagctcc 420
aaaaatcagg ctttcgctat ctgggacgtt aaccagaaaa cttctacct gcgcaacaac 480
cagctggttg ctggctacct gcagggtccg aacgttaacc tggagaagaa aatcgacgtt 540
gtaccgatcg aaccgcacgc tctgttcctg ggtatccacg gtggtaaaa gtgcctgagc 600
tgcgtgaaat ctggtgacga aactcgtctg cagctggaag cagttaacat cactgacctg 660
agcgaagacc gcaaacagga caaacgttcc gcatcatccc gctctgacag cggcccgacc 720
accagcttcg aatctgctgc ttgccgggt tggttcctgt gcaactgctat ggaagctgac 780
cagccggtaa gcctgaccaa catgccggac gaaggcgtga tggtaaccaa attctacttc 840
caggaagacg aataa 855

```

<210> 17  
 <211> 439  
 <212> DNA  
 <213> Homo sapiens

```

<400> 17
catatgggtc caactgggtc cggatgaatcc aaggctcctc tgatgggtcaa agttcttagat 60
gctgtccgag gcagtcctgc catcaatgtg gccgtgcatg tgttcagaaa ggctgctgat 120
gacacctggg agccatttgc ctctgggaaa accagtgtgt ctggagagct gcatgggctc 180
acaactgagg aggaatttgt agaagggtata tacaagtggg aaatagacac caaatcttac 240

```



## A-813A PCT.ST25.txt

tggaaggcac ttgcatctc cccattccat gagcatgcag aggtggtatt cacagccaac	300
gactccggcc ccgcccgtc caccattgcc gccctgctga gcccctactc ctattccacc	360
actgcagtcg tcaccaatcc caaggaagga tcaggatccg gaaaacgtcc gccgggtttc	420
tccccgctgt aatctcgag	439
<210> 18	
<211> 36	
<212> DNA	
<213> Homo sapiens	
<400> 18	
gaggaataac atatgggtcc aactggtacc ggtgaa	36
<210> 19	
<211> 36	
<212> DNA	
<213> Homo sapiens	
<400> 19	
ccgcggatcc tcgagattat tccttgggat tggatga	36
<210> 20	
<211> 48	
<212> DNA	
<213> Homo sapiens	
<400> 20	
gaggaataac atatgggtcc aactggtacc ggtgaatcca aggctcct	48
<210> 21	
<211> 24	
<212> DNA	
<213> Homo sapiens	
<400> 21	
agaaaggctt gtgatgacac ctgg	24
<210> 22	
<211> 24	
<212> DNA	
<213> Homo sapiens	
<400> 22	
ccaggtgtca tcacaagcct ttct	24
<210> 23	
<211> 24	
<212> DNA	
<213> Homo sapiens	
<400> 23	
agaaaggctg cttgtgacac ctgg	24

## A-813A PCT.ST25.txt

<210> 24  
<211> 24  
<212> DNA  
<213> Homo sapiens  
<400> 24  
ccaggtgtca caagcagcct ttct 24

<210> 25  
<211> 24  
<212> DNA  
<213> Homo sapiens  
<400> 25  
tactggaagt gtcttggcat ctcc 24

<210> 26  
<211> 24  
<212> DNA  
<213> Homo sapiens  
<400> 26  
ggagatgccca agacacttcc agta 24

<210> 27  
<211> 24  
<212> DNA  
<213> Homo sapiens  
<400> 27  
aaggcacttt gcatctcccc attc 24

<210> 28  
<211> 24  
<212> DNA  
<213> Homo sapiens  
<400> 28  
gaatggggag atgcaaagtg cctt 24

<210> 29  
<211> 21  
<212> DNA  
<213> Homo sapiens  
<400> 29  
ctgatgggtcg cagttctaga t 21

<210> 30  
<211> 21  
<212> DNA  
<213> Homo sapiens  
<400> 30  
atctagaact gcgaccatca g 21

## A-813A PCT.ST25.txt

<210> 31  
<211> 36  
<212> DNA  
<213> Homo sapiens  
  
<400> 31  
gaggaataac atatgatcga aggtccgact ctgcgt 36  
  
<210> 32  
<211> 41  
<212> DNA  
<213> Homo sapiens  
  
<400> 32  
ttcaccggtgta ccagttggac ctgcgcgtgc tgcaagccat t 41  
  
<210> 33  
<211> 33  
<212> DNA  
<213> Homo sapiens  
  
<400> 33  
gaggaataac atatgtctgt ttctgaaatc cag 33  
  
<210> 34  
<211> 36  
<212> DNA  
<213> Homo sapiens  
  
<400> 34  
ttcaccggtgta ccagttggac caaagttatg aacgtc 36  
  
<210> 35  
<211> 32  
<212> DNA  
<213> Homo sapiens  
  
<400> 35  
gaggaataac atatgcgacc gtccggacgt aa 32  
  
<210> 36  
<211> 36  
<212> DNA  
<213> Homo sapiens  
  
<400> 36  
ttctacttcc aggaagacga aggtccaact ggtacc 36  
  
<210> 37  
<211> 54  
<212> DNA  
<213> Homo sapiens  
  
<400> 37  
gtcgtcacca atccaagga aggtagtggg agccgaccgt ccggccgtaa gagc 54

## A-813A PCT.ST25.txt

```

<210> 38
<211> 40
<212> DNA
<213> Homo sapiens

<400> 38
ccgcgatcc tcgagattat tcgtcttcct ggaagtagaa      40

<210> 39
<211> 36
<212> DNA
<213> Homo sapiens

<400> 39
gaggaataac atatgggtcc aactgggtacc ggtgaa      36

<210> 40
<211> 26
<212> DNA
<213> Homo sapiens

<400> 40
aatatactgc agtgggtgaa taggag      26

<210> 41
<211> 68
<212> DNA
<213> Homo sapiens

<400> 41
gtcgtcacca atcccaagga aggatcagga tccgaaaac gtccgcggg tttctccccg      60
ctgtaatc      68

<210> 42
<211> 76
<212> DNA
<213> Homo sapiens

<400> 42
tcgagattac agcggggaga aaccggcgg acgttttccg gatcctgac cttccttggg      60
attggtgacg actgca      76

<210> 43
<211> 489
<212> DNA
<213> Homo sapiens

<400> 43
atgtctgttt ctgaaatcca gctgatgcat aacctgggta aacatctgaa ctctatggaa      60
cgtgttgaat ggctgcgtaa gaaactgcag gacgttcata actttggtcc aactgggtacc      120
ggtgaatcca aggcctctct gatggtcgca gttctagatg ctgtccgagg cagtcctgcc      180
atcaatgtgg ccgtgcatgt gttcagaaag gctgctgatg acacctggga gccatttgcc      240

```

## A-813A PCT.ST25.txt

```

tctgggaaaa ccagtgaagtc tggagagctg catgggctca caactgagga ggaatttgta 300
gaagggatat acaaagtga aatagacacc aaatcttact ggaagtgtct tggcatctcc 360
ccattccatg agcatgcaga ggtggtattc acagccaacg actccggccc ccgccgtac 420
accattgccg ccctgctgag cccctactcc tattccacca cggctgtcgt caccaatccc 480
aaggaataa 489

<210> 44
<211> 25
<212> DNA
<213> Homo sapiens

<400> 44
cgtacagggt tacgcaagaa aatgg 25

<210> 45
<211> 62
<212> DNA
<213> Homo sapiens

<400> 45
ggattcaccg gtaccagttg gaccaccacc accaccacca cccgcactgc ctgaaccaga 60
gc 62

<210> 46
<211> 71
<212> DNA
<213> Homo sapiens

<400> 46
tgactaagcc atatgaaaca tcaccacat caccatcatg acgaagttga tcacggtgaa 60
ggtactttca c 71

<210> 47
<211> 44
<212> DNA
<213> Homo sapiens

<400> 47
ggattcaccg gtaccagttg gaccaccacc accaccaccg ctac 44

<210> 48
<211> 594
<212> DNA
<213> Homo sapiens

<400> 48
atgaaacatc atcaccatca ccatcatgac gaagttgatc acggtgaagg tactttcact 60
tctgacgttt cttcttatct ggaaggctag gctgctaaag aattcatcgc ttggctggtt 120
aaaggctcgt gtggttctg ttctgctact ggtggttccg gctccaccgc aagctctggt 180

```

## A-813A PCT.ST25.txt

```

tcaggcagtg cgggtggttg tggtggtggt ggtccaactg gtaccggtga atccaaggct 240
cctctgatgg tcaaagttct agatgctgtc cgaggcagtc ctgccatcaa tgtggccgtg 300
catgtgttca gaaaggctgc tgatgacacc tgggagccat ttgcctcttg gaaaaccagt 360
gagtcctggag agctgcatgg gctcacaact gaggaggaat ttgtagaagg gatatacaaa 420
gtggaaatag acaccaaadc ttactggaag gcactttgca tctccccatt ccatgagcat 480
gcagagggtg tattcacagc caacgactcc ggcccccgcc gctacaccat tgccgccctg 540
ctgagccctt actcctattc caccagggt gtcgtcacca atccaagga ataa 594

```

```

<210> 49
<211> 591
<212> DNA
<213> Homo sapiens

```

```

<400> 49
atgaaacatc atcaccatca ccatcatgac gaagttgac acggtgaagg tactttcact 60
tctgacgttt cttcttatct ggaaggctcag gctgctaaag aattcatcgc ttggctggtt 120
aaaggctcgt gtggtggttg tggttctggt ggtggtggtt ctggtggttg tggttctggc 180
ggcgggtgga gcggtggttg tggtggtggt ccaactggta ccggtgaatc caaggctcct 240
ctgatggtcg cagttctaga tgcgtccga ggcagtcctg ccatcaatgt ggcggtgcat 300
gtgttcagaa aggcctgctg tgacacctgg gagccatttg cctctgggaa aaccagttag 360
tctggagagc tgcatgggct cacaactgag gaggaatttg tagaagggat atacaaagtg 420
gaaatagaca ccaaacttta ctggaaggca ctttgcattc cccattcca tgagcatgca 480
gaggtggtat tcacagccaa cgactccggc ccccgccgct acaccattgc cgccctgctg 540
agcccttact cctattccac caccgctgtc gtcaccaatc ccaaggaata a 591

```

```

<210> 50
<211> 76
<212> DNA
<213> Homo sapiens

```

```

<400> 50
agacctgtac atatgaaaca tcatcaccat caccatcatg acgaagtga tcacggtgaa 60
ggtactttca cttctg 76

```

```

<210> 51
<211> 20
<212> DNA
<213> Homo sapiens

```

```

<400> 51
gggggaagag gaaaactgac 20

```

## A-813A PCT.ST25.txt

```

<210> 52
<211> 894
<212> DNA
<213> Homo sapiens

<400> 52
atgaaacatc atcaccatca ccatcatgac gaagttgac acggtgaagg tactttcact 60
tctgacggtt cttcttatct ggaaggtag gctgctaaag aattcatcgc ttggctggtt 120
aaaggctcgtg gtggttctgg ttctgctact ggtggttccg gctccaccgc aagctctggt 180
tcaggcagtg cgactcatgg tgggtggtgg ggtgacaaaa ctacacatg tccaccgtgc 240
ccagcacctg aactcctggg gggaccgtca gttttcctct tcccccaaa acccaaggac 300
accctcatga tctcccgac ccctgaggtc acatgcgtgg tgggggacgt gagccacgaa 360
gaccctgagg tcaagtcaa ctggtacgtg gacggcgtgg aggtgcataa tgccaagaca 420
aagccgcggg aggagcagta caacagcacg taccgtgtgg tcagcgtcct caccgtcctg 480
caccagagct ggctgaatgg caaggagtac aagtgcagg tctccaaca agccctcca 540
gcccccatcg agaaaacat ctccaagcc aaagggcagc cccgagaacc acaggtgtac 600
accctgcccc catcccgga tgagctgacc aagaaccagg tcagcctgac ctgcttggtc 660
aaaggcttct atcccagca catcgccgtg gagtgggaga gcaatgggca gccggagaa 720
aactacaaga ccacgcctcc cgtgctggac tccgacggct ctttttctct ctacagcaag 780
ctcaccgtgg acaagagcag gtggcagcag gggaaactct tctcatgtc cgtgatgcat 840
gaggtctctg acaaccacta cagcagaag agcctctccc tgtctccggg taaa 894

<210> 53
<211> 60
<212> DNA
<213> Homo sapiens

<400> 53
gtcgtcacca atcccaagga aggttctggc tccgagtag ggggaccgtc agttttcctc 60

<210> 54
<211> 52
<212> DNA
<213> Homo sapiens

<400> 54
ccgcggatcc tcgagattag gatccagaac cccctttggc ttggagatg gt 52

<210> 55
<211> 42
<212> DNA
<213> Homo sapiens

<400> 55
gaggaataac atatgggtcc aactggtacc ggtgaatcca ag 42

```

## A-813A PCT.ST25.txt

<210> 56  
 <211> 735  
 <212> DNA  
 <213> Homo sapiens

<400> 56  
 atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtaaagt tctagatgct 60  
 gtccgaggca gtctgcat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120  
 acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180  
 actgaggagg aattttaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240  
 aaggcacttg gcatctcccc attccatgag catgcagagg tggattcac agccaacgac 300  
 tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360  
 gctgtcgtca ccaatcccaa ggaaggttct ggctccggat cagggggacc gtcagttttc 420  
 ctcttcccc caaaaccaa ggacaccctc atgatctccc ggaccctga ggtcacatgc 480  
 gtgggtggtg acgtgagcca cgaagaccct gaggtcaagt tcaactggtc cgtggacggc 540  
 gtggagggtc ataatccaa gacaaagccg cgggaggagc agtacaacag cacgtaccgt 600  
 gtggctcagc tcctcaccgt cctgcaccag gactggctga atggcaagga gtacaagtgc 660  
 aagggtctca acaagccct cccagcccc atcgagaaaa ccattctcaa agccaaaggg 720  
 ggttctggat cctaa 735

<210> 57  
 <211> 843  
 <212> DNA  
 <213> Homo sapiens

<400> 57  
 atgacgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggggcgga 60  
 ggggggtggc ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120  
 ggtaccggtg aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180  
 cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240  
 tttgctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300  
 tttgtagaag ggatatacaa agtggaaata gacaccaa cttactggaa ggcacttggc 360  
 atctcccat tccatgagca tgcagagggt gtattcacag ccaacgactc cggccccgc 420  
 cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgcctcacc 480  
 aatcccaagg aaggttctg ctccggatca gggggaccgt cagttttct cttccccca 540  
 aaaccaagg acacctcat gatctcccg acccctgagg tcacatgcgt ggtggtggac 600  
 gtgagccacg aagaccctga ggtcaagttc aactggtacg tggacggcgt ggaggtgcat 660  
 aatgccaaga caaagccgcg ggaggagcag tacaacagca cgtaccgtgt ggtcagcgtc 720



## A-813A PCT.ST25.txt

ctcacgtcc tgcaccagga ctggctgaat ggcaaggagt acaagtgcaa ggtctccaac 780  
 aaagccctcc cagcccccat cgagaaaacc atctccaaag ccaaaggggg ttctggatcc 840  
 taa 843

<210> 58  
 <211> 35  
 <212> DNA  
 <213> Homo sapiens

<400> 58  
 gaggaataag gatccatcga aggtccgact ctgcg 35

<210> 59  
 <211> 843  
 <212> DNA  
 <213> Homo sapiens

<400> 59  
 atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtaaagt tctagatgct 60  
 gtccgaggga gtcttgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120  
 acctgggagc catttgctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180  
 actgaggagg aattttaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240  
 aaggcacttg gcatctcccc attccatgag catgcagagg tggattcac agccaacgac 300  
 tccgcccc gccgtacac cattgccgc ctgctgagcc cctactccta ttccaccacg 360  
 gctgtcgtca ccaatcccaa ggaaggttct ggctccggat cagggggacc gtcagtttcc 420  
 ctcttcccc caaaacccaa ggacaccctc atgatctccc ggaccctga ggtcacatgc 480  
 gtgggtggtg acgtgagcca cgaagaccct gaggtcaagt tcaactggtg cgtggacggc 540  
 gtggaggtgc ataattgcaa gacaaagccg cgggaggagc agtacaacag cacgtaccgt 600  
 gtggtcagcg tcttcaccgt cctgcaccag gactggctga atggcaagga gtacaagtgc 660  
 aagggtctca acaaagccct ccagcccc atcgagaaaa ccatctcaa agccaaaggg 720  
 ggttctggat ccatcgaagg tccgactctg cgtcagtgcc tggctgctcg tgctggcggg 780  
 ggtggcggag ggggtggcat tgagggccca acccttcgcc aatggcttgc agcacgcgca 840  
 taa 843

<210> 60  
 <211> 48  
 <212> DNA  
 <213> Homo sapiens

<400> 60  
 ttcaccgta ccagttggac cagaaccccc ttggctttg gagatggt 48

## A-813A PCT.ST25.txt

<210> 61  
 <211> 48  
 <212> DNA  
 <213> Homo sapiens

<400> 61  
 gaggaataac atatgggatac cggttctggg ggaccgtcag ttttcctc 48

<210> 62  
 <211> 723  
 <212> DNA  
 <213> Homo sapiens

<400> 62  
 atgggatccg gttctggggg accgtcagtt ttcctcttcc ccccaaaacc caaggacacc 60  
 ctcatgatct cccggacccc tgaggtcaca tgcgtggtgg tggacgtgag ccacgaagac 120  
 cctgaggta agttcaactg gtacgtggac ggcgtggagg tgcataatgc caagacaaag 180  
 ccgcgggagg agcagtacaa cagcacgtac cgtgtggtca gcgtcctcac cgtcctgcac 240  
 caggactggc tgaatggcaa ggagtacaag tgcaaggctc ccaacaaagc cctcccagcc 300  
 cccatcgaga aaaccatctc caaagccaaa gggggttctg gtccaactgg taccggtgaa 360  
 tccaaggctc ctctgatggt caaagttcta gatgtgtcc gaggcagtcc tgccatcaat 420  
 gtggccgtgc atgtgttcag aaaggctgct gatgacacct gggagccatt tgcctctggg 480  
 aaaaccagtg agtctggaga gctgcatggg ctcaactg aggaggaatt tgtagaaggg 540  
 atatacaaag tggaaataga caccaaatct tactggaagg cacttgcat ctccccattc 600  
 catgagcatg cagaggtggt attcacagcc aacgactccg gccccgccg ctacaccatt 660  
 gccgccctgc tgagccccta ctctattcc accacggctg tcgtcaccaa tcccaaggaa 720  
 taa 723

<210> 63  
 <211> 33  
 <212> DNA  
 <213> Homo sapiens

<400> 63  
 gaggaataac atatgatcga aggtccgact ctg 33

<210> 64  
 <211> 36  
 <212> DNA  
 <213> Homo sapiens

<400> 64  
 taacatatgg gatcctgcgc gtgctgcaag ccattg 36

<210> 65  
 <211> 831  
 <212> DNA

## A-813A PCT.ST25.txt

&lt;213&gt; Homo sapiens

&lt;400&gt; 65

```

atgatacgaag gtccgactct gcgtcagtg ctggctgctc gtgctggcgg tggaggcgga    60
gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggatccggt    120
tctgggggac cgtcagtttt cctcttcccc ccaaaaccca aggacaccc catgatctcc    180
cggacccttg aggtcacatg cgtggtggtg gacgtgagcc acgaagaccc tgaggtaag    240
ttcaactggt acgtggacgg cgtggagggt cataatgcc agacaaagcc gcgggaggag    300
cagtacaaca gcacgtaccg tgggtcagc gtcctcaccg tcctgcacca ggactggctg    360
aatggcaagg agtacaagtg caaggctcc aacaaagccc tcccagcccc catcgagaaa    420
accatctcca aagccaaagg gggttctggt ccaactggta ccggtgaatc caaggctcct    480
ctgatggtca aagttctaga tgetgtccga ggcagtcctg ccatcaatgt gcccgatcat    540
gtgttcagaa aggtctgcta tgacacctgg gagccatttg cctctgggaa aaccagttag    600
tctggagagc tgcattgggt cacaactgag gaggaatttg tagaagggat atacaaagtg    660
gaaatagaca ccaaatctta ctggaaggca ctggcatct cccattcca tgagcatgca    720
gaggtggtat tcacagccaa cgactccggc ccccgccgct acaccattgc cgccctgctg    780
agccccact cctattccac cagggtgtc gtcaccaatc ccaaggaata a    831

```

&lt;210&gt; 66

&lt;211&gt; 42

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 66

```

gtcgtcacca atcccaagga aggttctggt tctggtatcg aa    42

```

&lt;210&gt; 67

&lt;211&gt; 40

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 67

```

ccgcggatcc tcgagattat gcgcgtgctg caagccattg    40

```

&lt;210&gt; 68

&lt;211&gt; 510

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 68

```

atgggtccaa ctggtaccgg tgaatccaag tgcctctga tggtaaaagt tctagatgct    60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac    120
acctgggagc catttgcttc tgggaaaacc agtgagtctg gagagctgca tgggctcaca    180
actgaggagg aattttaga agggatatac aaagtggaaa tagacaccaa atcttactgg    240

```

## A-813A PCT.ST25.txt

```

aaggcacttg gcattctccc attccatgag catgcagagg tggattcac agccaacgac 300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaagggttct ggttctggta tcgaagggtcc gactctgcgt 420
cagtggctgg ctgctcgtgc tggcgggtggg ggcggagggg gtggcattga gggcccaacc 480
cttcgccaat ggcttgagc acgcgcataa 510

```

```

<210> 69
<211> 510
<212> DNA
<213> Homo sapiens

```

```

<400> 69
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggctgcagt tctagatgct 60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgcctgatgac 120
acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180
actgaggagg aattttaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240
aaggcacttg gcattctccc attccatgag catgcagagg tggattcac agccaacgac 300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaagggttct ggttctggta tcgaagggtcc gactctgcgt 420
cagtggctgg ctgctcgtgc tggcgggtggg ggcggagggg gtggcattga gggcccaacc 480
cttcgccaat ggcttgagc acgcgcataa 510

```

```

<210> 70
<211> 510
<212> DNA
<213> Homo sapiens

```

```

<400> 70
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggctgcagt tctagatgct 60
gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc ttgtgatgac 120
acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180
actgaggagg aattttaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240
aaggcacttg gcattctccc attccatgag catgcagagg tggattcac agccaacgac 300
tccggccccc gccgtacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360
gctgtcgtca ccaatcccaa ggaagggttct ggttctggta tcgaagggtcc gactctgcgt 420
cagtggctgg ctgctcgtgc tggcgggtggg ggcggagggg gtggcattga gggcccaacc 480
cttcgccaat ggcttgagc acgcgcataa 510

```

```

<210> 71

```

## A-813A PCT.ST25.txt

<211> 510  
 <212> DNA  
 <213> Homo sapiens

<400> 71  
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 gtccgaggca gtccctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgcctgatgac 120  
 acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180  
 actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240  
 aagtgtcttg gcatctcccc attccatgag catgcagagg tggattcac agccaacgac 300  
 tccggcccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360  
 gctgtcgtca ccaatcccaa ggaaggttct ggttctggta tcgaaggctc gactctgcgt 420  
 cagtggctgg ctgctcgtgc tggcgggtgt ggcggagggg gtggcattga gggccaacc 480  
 cttcgccaat ggcttcgagc acgcgcataa 510

<210> 72  
 <211> 510  
 <212> DNA  
 <213> Homo sapiens

<400> 72  
 atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggctgcagt tctagatgct 60  
 gtccgaggca gtccctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgcctgatgac 120  
 acctgggagc catttgccctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180  
 actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240  
 aaggcacttt gcatctcccc attccatgag catgcagagg tggattcac agccaacgac 300  
 tccggcccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360  
 gctgtcgtca ccaatcccaa ggaaggttct ggttctggta tcgaaggctc gactctgcgt 420  
 cagtggctgg ctgctcgtgc tggcgggtgt ggcggagggg gtggcattga gggccaacc 480  
 cttcgccagt ggcttcgagc acgcgcataa 510

<210> 73  
 <211> 495  
 <212> DNA  
 <213> Homo sapiens

<400> 73  
 atgatcgaag gtccgactct gcgtcagtg ctggctgctc gtgctggcgg tggggcgga 60  
 ggggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120  
 ggtaccgggtg aatccaaggc tcctctgatg gtcgcagttc tagatgctgt ccgaggcagt 180  
 cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240

A-813A PCT.ST25.txt

```

tttgctctg ggaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300
tttgtagaag ggatatacaa agtggaata gacaccaaatt cttactggaa ggcactttgc 360
atctcccat tccatgagca tgcagagggtg gtattcacag ccaacgactc cggccccgc 420
cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480
aatccaagg aataa 495

<210> 74
<211> 495
<212> DNA
<213> Homo sapiens

<400> 74
atgacgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggaggcggg 60
gggggtggca ttgagggcc aaccttcgc caatggcttg cagcagcgc aggtccaact 120
ggtaccggtg aatccaaggc tcctctgatg gtcgcagttc tagatgctgt ccgaggcagt 180
cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240
tttgctctg ggaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300
tttgtagaag ggatatacaa agtggaata gacaccaaatt cttactggaa gtgtcttggc 360
atctcccat tccatgagca tgcagagggtg gtattcacag ccaacgactc cggccccgc 420
cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480
aatccaagg aataa 495

<210> 75
<211> 495
<212> DNA
<213> Homo sapiens

<400> 75
atgacgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggaggcggg 60
gggggtggca ttgagggcc aaccttcgc caatggcttg cagcagcgc aggtccaact 120
ggtaccggtg aatccaaggc tcctctgatg gtcgcagttc tagatgctgt ccgaggcagt 180
cctgccatca atgtggccgt gcatgtgttc agaaaggctt gtgatgacac ctgggagcca 240
tttgctctg ggaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300
tttgtagaag ggatatacaa agtggaata gacaccaaatt cttactggaa ggcacttggc 360
atctcccat tccatgagca tgcagagggtg gtattcacag ccaacgactc cggccccgc 420
cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480
aatccaagg aataa 495

```