(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[7]: 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

(71) Applicant: **AMGEN INC.** [US/US]; One Amgen Center Drive, Thousand Oaks, CA 91320-1799 (US).

(72) Inventors: **WALKER, Kenneth**; 175 Mesa Avenue, Newbury Park, CA 91320 (US). **XIONG, Fei**; 2757 Autumn Ridge Drive, Thousand Oaks, CA 91362 (US).

(74) Agents: **ODRE, Steven, M.** et al.; Amgen Inc., One Amgen Center Drive, M/S27-4-A, Thousand Oaks, CA 91320-1789 (US).

(81) Designated States *(national)*: AE, 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 TRANSTHYRETIN 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 transthyretin (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, filed 04 April 2002, which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

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 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 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) 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

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

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 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 enough to be orally absorbed.

SUMMARY OF THE INVENTION

It has been found, surprisingly and 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-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 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.

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

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

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an SDS gel that depicts the purification of an *E. coli* expressed, 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

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

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 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).

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 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 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-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.

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-

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

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 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 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 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 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).

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, ▲

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).

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).

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).

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-α and TGF-β, respectively), and growth hormones. The terms "-mimetic peptide" and "-agonist peptide" refer to a peptide having biological

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.

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 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, 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 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 having TNF-antagonistic subject matter; see, foe 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 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. 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 any two or three components of the complex. Exemplary IL-1 antagonist or IL-1ra-mimetic peptides can be

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 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, 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.

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 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 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. 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., *The Proteins*, 3rd ed., 2:105-253 (1976); and Erickson et al., *The Proteins*, 3rd ed., 2:257-527 (1976). Solid

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 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). 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 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 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 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 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 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 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. 91/05795, 92/17505 and 95/17206, hereby incorporated by reference including drawings), NESP (PCT Publication No. US94/02957, 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 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, adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), human 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),

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 (PGDF), colony simulating 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
GPTGTGESKCPLMVKVLDAVRGSPAINVAVHVFRKAADDTWEPFASGKTSESGEL
HGLTTEEEFVEGIYKVEIDTKSYWKALGISPFHEHAEVVFTANDSGPRRYTIAAL
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

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-

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, 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 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 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 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 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 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 interest, protein of interest, TTR or TTR variant can be prepared using well known recombinant DNA technology

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 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, 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 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 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 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 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 100 nucleotides can be synthesized as several fragments using these methods. The fragments can then be ligated

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 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 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 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 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 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 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 as well such as an enhancer(s), an origin of replication element, a transcriptional termination

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. 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 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 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 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 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.

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 thus be isolated from the proper tissue source using the appropriate restriction endonucleases. In some

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.

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 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, 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 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. 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 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. Usually, the transcription termination element in prokaryotic cells is a G-C rich fragment followed by a

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 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 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 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 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 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.

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 comparable to the methods set forth above (*i.e.*, synthesis of the DNA, library screening, and the like).

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, DH5α, 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

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

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

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 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 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 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 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. 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 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 compounds of the present invention may also be employed, alone or in combination with other cytokines,

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.

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

administration of glucagon-like peptide-1 (GLP-1) has antidiabetic properties. However, GLP-1 is rapidly degraded by dipeptidyl peptidase IV (DPPIV) 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^{9}-GLP-1(7-37), D-Gln^{9}-GLP-1(7-37), Thr^{16}-Lys^{18}-GLP-1(7-37), and Lys^{18}-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 it's 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 pharmokinetic profile of the

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

The therapeutic methods, compositions and compounds of the present invention may also be 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. 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. 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, 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 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 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; additives such as detergents and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80, etc.), anti-oxidants

(*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 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 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 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 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 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 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 based on the Oros therapeutic system (Alza Corp.), i.e., the drug is enclosed in a semipermeable membrane

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 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., 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 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 (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).

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 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 nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler,

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 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 in therapy.

The inventive compound should most advantageously be prepared in particulate form with an average particle size of less than 10μm (or microns), most preferably 0.5 to 5μm, for most effective delivery 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 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 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 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 μg to 100 mg of the inventive compound per kilogram of body weight per day, preferably 0.1 to 1000 μg/kg; and more preferably

0.1 to 150 μg/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.

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 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 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, 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.

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 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 clinical factors. As studies are conducted, further information will emerge regarding the appropriate

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

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).

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 $lacI^{q}$ 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 $luxP_R$. 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

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 renamed F'tet/GM101. F'tet/GM101 was then modified by the delivery of a $lacI^{q}$ 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 construct was delivered to the chromosome using a recombinant phage called AGebg-$lacI^{q}$ #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 was cured from the strain using acridine orange at a concentration of 25 μg/ml in LB. The cured strain was identified as tetracyline sensitive and was stored as GM221.

Oligonucleotides (1.0 nm each) were 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 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 Nde1 and Xho1 (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 England Biolabs). Ligation was done at 4°C for 2 hrs.

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 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 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 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 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 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 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 resulting plasmid (strain #5316) was shown to have the

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 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 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. 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 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 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) 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 plasmids are described as follows:
TTR(C10A/A37C)(strain 5641) had the DNA sequence

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) 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. The resulting strain, TTR(C10A/K15A/G83C)(strain 5895) had the DNA sequence identified in SEQ ID NO:8.

Table 1

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

Example 2

This example describes the preparation of 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 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

the first 7 codons of the TMP plus a 12 nucleotide 5' extension including a Nde1 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 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 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 Xba1, the resulting Xba1/Xba1 insert containing the TMP and the first 18 codons of TTR(C10A) was gel purified and ligated with Xba1 restricted, phosphatase treated and gel purified vector derived from 5641, 5642, 5643 and 5651. DNA sequencing was performed to select the correct 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 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.

Table 2

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

Example 3

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	GAGGAATAACATATGTCTGTTTCTGAAATCCAG	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

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	TTCTACTTCCAGGAAGACGAAGGTCCAACTGGTACC	36
2787-32	GTCGTCACCAATCCCAAGGAAGGTAGTGGTAGCCGA CCGTCCGGCCGTAAGAGC	37
2787-33	CCGCGGATCCTCGAGATTATTCGTCTTCCTGGAAGT AGAA	38

Example 5

This example describes the preparation of 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 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 and XhoI, and the annealed oligonucleotide linkers 2943-48, which encodes the GSGSG linker, and oligonucleotide 2943-49, which encodes the Bradykinin antagonist peptide KRPPGFSPL with PstI 5' and XhoI 3' overlapping ends. GM121 was transformed with this 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. The new strain was designated TTR(C10A/G83C)-Bradykinin (strain 5914) and had the DNA sequence identified in SEQ ID NO:17.

Table 5

Oligo	Sequence	SEQ ID Number
2693-79	GAGGAATAACATATGGGTCCAACTGGTACCGGTGAA	39
2943-47	AATATACTGCAGTGGTGGAATAGGAG	40
2943-48	GTCGTCACCAATCCCAAGGAAGGATCAGGATCCGGA AAACGTCCGCCGGGTTTCTCCCCGCTGTAATC	41
2943-49	TCGAGATTACAGCGGGGAGAAACCCGGCGGACGTTTT CCGGATCCTGATCCTTCCTTGGGATTGGTGACGACTGCA	42

EXAMPLE 6

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

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

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 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 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

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 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 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, 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 NaH_2PO_4, 1 M ammonium 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 NaH_2PO_4, pH 7.0). The remaining half of the Q-pool was then processed using the same protocol as the first 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 NaH_2PO_4, pH 7.0) for 2 hours at room temperature (about 23 °C) using 20.4 mm 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 µl of 1 M DTT was added followed by incubation at room

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 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 NaH_2PO_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 (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 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 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 μm cellulose acetate filter and the protein concentration was determined to be 16.9 mg/ml using a calculated extinction coefficient of 18,450 M^{-1} cm^{-1}. The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Ameboycyte 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 0.52.

EXAMPLE 8

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-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 300 mm) (Phenomenex, Torrance, CA) in SEC-Buffer (50 mM NaH_2PO_4, 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 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 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.

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 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-Buffer at 1 ml/min. Bio-Rad molecular weight standards

(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 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 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 shape of the molecule, since size exclusion chromatography is shape dependant and the standards are calibrated for globular proteins.

EXAMPLE 10

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 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.

For this example, human MPL receptor was covalently bound to a BIAcore CM5 chip at R_L = 1300 R_U using the EDC/NHS chemistry as per the manufacturer's instructions (BIAcore, Uppsula, Sweden). All samples were passed over the chip at 50 µl/min in Dulbecco's 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

This example shows the effect of injecting TMP(m)-TTR into mice on blood platelet count. For this example 50 BDF1 mice (Charles River Laboratories, Wilmington, Massachusetts) were split into 5 groups and injected (day 0) subcutaneously with either diluting agent (Dulbecco's PBS with 0.1% bovine serum albumin) or diluting agent with 50 μg test protein per kg animal. Each group was divided in half and bled (140 μl) on alternate time points (day 0, 3, 5, 7, 11, 12, 14, and 17). 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 5, Fc-TMP showed the greatest response with platelet count peaking at 4.3 X 10^{12} platelets L^{-1} on day 5, which is over 3.4 times baseline at 1.2 X 10^{12} platelets L^{-1}. TMP(m)-TTR-PEG 5K was a moderate responder peaking at

2.3×10^{12} platelets L^{-1} which is just under twice the baseline level. The non-pegylated form of TMP(m)-TTR shows very little response at 1.5×10^{12} platelets L^{-1} 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

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^{-1} 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

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.

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, Uppsula, 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.

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).

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 X 10^{12} platelets L^{-1} on day 5 which is 3 times baseline at 1.4 X 10^{12} platelets L^{-1}. All 4 of the non-pegylated TMP-TTR constructs preformed better than the control, but not as well as Fc-TMP with platelet counts between 1.8 and 2.9 X 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 X 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 X 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)

control). Each group was bled (75 µl) 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++/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:

ATGTCTGTTTCTGAAATCCAGCTGATGCATAACCTGGGTAAACATCTGAACTCTA
TGGAACGTGTTGAATGGCTGCGTAAGAAACTGCAGGACGTTCATAACTTTGGTCC
AACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAGATGCTGTC
CGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTGCTGATG

ACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCTGCATGG
GCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATAGACACC
AAATCTTACTGGAAGTGTCTTGGCATCTCCCCATTCCATGAGCATGCAGAGGTGG
TATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCTGCTGAG
CCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

EXAMPLE 18

This example describes the preparation of a 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.

The bacterial host GM121 is an *E. coli* K-12 strain that has been modified to contain the lacI^{Q} 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 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^{Q} 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 construct was delivered to the chromosome using a recombinant phage called AGebg- lacI^{Q} #5.

After recombination and resolution only the 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 tetracyline sensitive and was stored as GM121 (ATCC #202174).

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

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 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).

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 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 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 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 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. The PCR product was cloned and sequenced as described

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) fusion, two oligonucleotides, oligonucleotide 3183-83, which contains and 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 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 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. 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.

Table 6

Oligo	Sequence	SEQ ID Number
1209-85	CGTACAGGTTTACGCAAGAAAATGG	44
3131-63	GGATTCACCGGTACCAGTTGGACCACCACCACCAC CACCACCCGCACTGCCTGAACCAGAGC	45
3183-83	TGACTAAGCCATATGAAACATCATCACCATCACCAT CATGACGAAGTTGATCACGGTGAAGGTACTTTCAC	46
3183-84	GGATTCACCGGTACCAGTTGGACCACCACCACCAC CACCGCTAC	47

SEQ ID NO:48

ATGAAACATCATCACCATCACCATCATGACGAAGTTGATCACGGTGAAGGTACTT
TCACTTCTGACGTTTCTTCTTATCTGGAAGGTCAGGCTGCTAAAGAATTCATCGC
TTGGCTGGTTAAAGGTCGTGGTGGTTCTGGTTCTGCTACTGGTGGTTCCGGCTCC
ACCGCAAGCTCTGGTTCAGGCAGTGCGGGTGGTGGTGGTGGTGGTGGTCCAACTG
GTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAGATGCTGTCCGAGG

CAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTGCTGATGACACC
TGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCTGCATGGGCTCA
CAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATAGACACCAAATC
TTACTGGAAGGCACTTTGCATCTCCCCATTCCATGAGCATGCAGAGGTGGTATTC
ACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCTGCTGAGCCCCT
ACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

SEQ ID NO:49

ATGAAACATCATCACCATCACCATCATGACGAAGTTGATCACGGTGAAGGTACTT
TCACTTCTGACGTTTCTTCTTATCTGGAAGGTCAGGCTGCTAAAGAATTCATCGC
TTGGCTGGTTAAAGGTCGTGGTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGT
GGTGGTGGTTCTGGCGGCGGTGGTAGCGGTGGTGGTGGTGGTGGTCCAACTGGTA
CCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAGATGCTGTCCGAGGCAG
TCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTGCTGATGACACCTGG
GAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCTGCATGGGCTCACAA
CTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATAGACACCAAATCTTA
CTGGAAGGCACTTTGCATCTCCCCATTCCATGAGCATGCAGAGGTGGTATTCACA
GCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCTGCTGAGCCCCTACT
CCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

EXAMPLE 19

This example describes the preparation of a 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 sequence between the AatII and ClaI recognition sites is replaced with AATTGTGAGCGGATAACAATTGAC AAATGCTAAAATTCTTGATTAATTGTGAGCGGATAACAATTTATCGATTTGATTC TAGAAGGAGGAATAA) and some of the sequence after the SacII recognition site was deleted (leaving ATAAATAAGTAACGATCCGGTCCAGTAATGACCTCAGAAC TCCATCTGGATTTGTTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATTGGTGA GAATCGCAGCAACTTGTCGCGCCAATCGAGCCATGTCGTCGTCAACGACCCCCCA TTCAAGAACAGCAAGCAGCATTGAGAACTTTGGAATCCAGTCCCTCTTCCACCTG CTGACCG). Each of the oligonucleotides utilized in this example are listed in Table 7.

To prepare the GLP-1(A2G)-Fc fusion, two oligonucleotides, oligonucleotide 2985-92, which

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

Oligo	Sequence	SEQ ID Number
2985-92	AGACCTGTACATATGAAACATCATCACCATCACCAT CATGACGAAGTTGATCACGGTGAAGGTACTTTCAC TTCTG	50
2687-50	GGGGGAAGAGGAAAACTGAC	51

SEQ ID NO: 52

ATGAAACATCATCACCATCACCATCATGACGAAGTTGATCACGGTGAAGGTACTT
TCACTTCTGACGTTTCTTCTTATCTGGAAGGTCAGGCTGCTAAAGAATTCATCGC
TTGGCTGGTTAAAGGTCGTGGTGGTTCTGGTTCTGCTACTGGTGGTTCCGGCTCC
ACCGCAAGCTCTGGTTCAGGCAGTGCGACTCATGGTGGTGGTGGTGGTGACAAAA
CTCACACATGTCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTTTT
CCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTC
ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGT
ACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTA
CAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTG
AATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCG
AGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCT
GCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTC
AAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGG
AGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCT
CTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCA
TGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCC
TGTCTCCGGGTAAA

EXAMPLE 20

This example describes the cloning of the CH2 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 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 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 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 similarly restricted pAMG21. The resulting plasmid is described as 6017 (TTRC10A-CH2):

SEQ ID NO:56:

ATGGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAG
ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGC
TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
CTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC
AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
CTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
GTTCTGGCTCCGGATCAGGGGGACCGTCAGTTTTCCTCTTCCCCCCAAAACCCAA
GGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTG
AGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGC
ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGT
CAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGC

AAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCA
AAGGGGGTTCTGGATCCTAA

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

SEQ ID NO:57:

ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC
AGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAGAT
GCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTG
CTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCT
GCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATA
GACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGCAG
AGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT
GCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAGGT
TCTGGCTCCGGATCAGGGGGACCGTCAGTTTTCCTCTTCCCCCCAAAACCCAAGG
ACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAG
CCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCAT
AATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCA
GCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAA
GGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAA
GGGGGTTCTGGATCCTAA

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

2974-70:

GAG GAA TAA GGA TCC ATC GAA GGT CCG ACT CTG CG

(SEQ ID NO:58).

The amplified fragment was digested with BamH1 and Xho1 and was subsequently ligated with similarly restricted 6017. The resulting clone is described as strain 6104 (TTRC10A-CH2-TMP).

SEQ ID NO:59:

ATGGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAG
ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGC
TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
CTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA

TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC
AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
CTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
GTTCTGGCTCCGGATCAGGGGGACCGTCAGTTTTCCTCTTCCCCCCAAAACCCAA
GGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTG
AGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGC
ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGT
CAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGC
AAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCA
AAGGGGGTTCTGGATCCATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCG
TGCTGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGG
CTTGCAGCACGCGCATAA

Another configuration of this fusion was made as 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

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:

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 similarly restricted pAMG21. The resulting clone is described as 6016 (CH2-TTRC10A):

SEQ ID NO:62:

ATGGGATCCGGTTCTGGGGGACCGTCAGTTTTCCTCTTCCCCCCAAAACCCAAGG
ACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAG
CCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCAT
AATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCA
GCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAA
GGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAA
GGGGGTTCTGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAG
TTCTAGATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAG
AAAGGCTGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCT
GGAGAGCTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAG

TGGAAATAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGA
GCATGCAGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATT
GCCGCCCTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCA
AGGAATAA

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

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

SEQ ID NO:65:

ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC
AGGATCCGGTTCTGGGGGACCGTCAGTTTTCCTCTTCCCCCCAAAACCCAAGGAC
ACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCC
ACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAA
TGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGC
GTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGG
TCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGG
GGGTTCTGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTT
CTAGATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAA
AGGCTGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGG
AGAGCTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTG
GAAATAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGC
ATGCAGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGC
CGCCCTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAG
GAATAA

EXAMPLE 21

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

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 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).

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):

SEQ ID NO:68:

ATGGGTCCAACTGGTACCGGTGAATCCAAGTGTCCTCTGATGGTCAAAGTTCTAG
ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGC
TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
CTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC
AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
CTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT
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:

SEQ ID NO:69:

ATGGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAG
ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGC
TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
CTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC

AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
CTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT
GCAGCACGCGCATAA

Plasmid 5921 was subsequently modified by replacing the amino acids at the following positions: A37, A81 and G83, with the amino acid Cysteine as 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), and Strain 5984 containing TTRC10A/K15A/G83C-TMP (SEQ ID NO:72).

SEQ ID NO:70:

ATGGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAG
ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGC
TTGTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
CTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC
AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
CTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT
GCAGCACGCGCATAA

SEQ ID NO:71:

ATGGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAG
ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGC
TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
CTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
TAGACACCAAATCTTACTGGAAGTGTCTTGGCATCTCCCCATTCCATGAGCATGC
AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
CTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT
GCAGCACGCGCATAA

SEQ ID NO:72:

ATGGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAG
ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGC
TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
CTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
TAGACACCAAATCTTACTGGAAGGCACTTTGCATCTCCCCATTCCATGAGCATGC

AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
CTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAGTGGCTT
GCAGCACGCGCATAA

EXAMPLE 22

This example describes the construction of TMP-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 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 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 described as 5918 (TMP-TTRC10A/K15A/A81C) and 6023 (TMP-TTRC10A/K15A/A37C).

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

ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC
AGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAGAT
GCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTG
CTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCT
GCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATA
GACACCAAATCTTACTGGAAGGCACTTTGCATCTCCCCATTCCATGAGCATGCAG
AGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT
GCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

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

ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC

AGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAGAT
GCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTG
CTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCT
GCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATA
GACACCAAATCTTACTGGAAGTGTCTTGGCATCTCCCCATTCCATGAGCATGCAG
AGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT
GCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

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

ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC
AGGTCCAACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAGAT
GCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTT
GTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCT
GCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATA
GACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGCAG
AGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT
GCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

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 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 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*. The incubation was continued for an additional 2-4 hours and the cells were collected by centrifugation.

EXAMPLE 24

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

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 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 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 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). 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 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 sulfate soluble fraction and discarded, and the pellet was resuspended in 450 ml 10 mM NaH_2PO_4, 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

soluble fraction and filtered through a 0.45 μm cellulose acetate filter. Added 240 μl 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 NaH_2PO_4, pH 7.0). The fractions from the 50% elution were pooled as HA-pool (725 ml) and filtered through a 0.22 μm 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 μm cellulose acetate filter. The protein concentration was determined to be 3.7 mg/ml using a calculated extinction coefficient of 23,950 M^{-1} cm^{-1}. The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Ameboycyte 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

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. 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 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) 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, 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 solublized pellet was 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

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 µm 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_2PO_4, pH 7.0). The fractions from the 2.5% elution were pooled as HA-pool (80 ml) and filtered through a 0.22 µm cellulose acetate filter. The protein concentration was determined to be 6.8 mg/ml using a calculated extinction coefficient of 29,450 M^{-1} cm^{-1}. The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Ameboycyte 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

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 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 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. 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 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 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 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 Millipore CENTRIPREP™ 10 and then passed through a Pall

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^{-1} cm^{-1}. The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Ameboycyte 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.

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_2PO_4, 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.

Sample was then loaded on to a 50 ml chelating sepharose fast flow column (Amersham Pharmacia Biotech, Piscataway, NJ), loaded with $NiCl_2$, at 5 ml/min. After washing with about 150 ml Ni-Buffer A (6 M guanidine 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 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 M^{-1}. Added 800 µl 500 mM EDTA pH 8.0 and removed 80 mg of protein for the PEGylation reaction. Added 230 µl 1 M DTT and incubated for 30 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 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 M^{-1}. Reacted 45% of the buffer exchanged material with 950 µl of 5 mM methoxy-PEG-maleimide 5K (Shearwater Corporation, Huntsville, AL) for 140 min at 30 °C. Added 100 µl 1 M 2-mercaptoethanol to each reaction to quench. Dialyzed reaction against 1 L 25 mM NaH_2PO_4, 3 M urea, pH 7.25 using a Pierce 10 kDa Slidealyzer for 2 hour room temperature. Changed the dialysis buffer for 25 mM NaH_2PO_4, 10% sucrose, 2 mM EDTA, pH 7.25 and incubated for about 16 hours room temperature. Added 140 µl 5% CHAPS and 7.28 µl 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

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 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 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_4 column (Vydac, Hisperia, CA) in 95% RP-Buffer A (0.1% trifluoroacetic acid) with 5% RP-Buffer B (95% 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 ml using 20 mM tris HCl pH 8.0. Repeated buffer exchange 3 more times then passed though 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^{-1} cm^{-1}. The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Ameboycyte 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 28

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

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 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 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.

EXAMPLE 29

This example shows the effect of injecting TMP-TTR constructs with fused antibody CH2 domains into 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 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 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 X 1012 platelets L-1 on day 5 which is 3 times baseline at 1.4 X 1012 platelets L-1. All three of the CH2 fused TMP-TTR constructs preformed better than the control, but not as well as Fc-TMP with

platelet counts between 2.3 X 1012 and 2.6 X 1012 platelets L-1 on day 5, which is between a 64% and 86% improvement over baseline.

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 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 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 14, Fc-TMP and the three amino terminal (TMP-TTR) fusions showed the greatest response with platelet count rising between 4.3 X 10^{12} and 4.6 X 10^{12} platelets L-1 on day 5 which is over three times baseline at 1.3 X 10^{12} platelets L-1. All three of the carboxy terminal (TTR-TMP) constructs performed better than the control.

EXAMPLE 31

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 per kg animal (TMP fusion constructs, Fc-TMP, or a TTR(C10A) control) (this study was split into two

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 X 10^{12} platelets L-1) with platelet responses at day 5 rising between 1.8 X 10^{12} and 2.0 X 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 X 10^{12} and 4.4 X 10^{12} platelets L-1 which is at least 2.7 times baseline (1.3 X 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 X 10^{12} and 4.6 X 10^{12} platelets L-1 which is over three times baseline at 1.3 X 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 X 10^{12} to 4.9 X 10^{12} platelets L-1 compared to Fc-TMP at 3.1 X 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 X 10^{12} to 3.1 X 10^{12} platelets L-1 compared to Fc-TMP at 2.0 X 10^{12} platelets L-1.

WHAT IS CLAIMED IS:

1. A method for increasing the serum half-life of a biologically active agent comprising fusing the biologically active agent to transthyretin (TTR) or a TTR variant.

2. The method of claim 1 where said TTR or TTR variant is chemically modified with a chemical selected from the group consisting of dextran, poly(n-vinyl pyurrolidone), polyethylene glycols, propropylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohols.

3. The method of claim 2 where said TTR or TTR variant is chemically modified with polyethylene glycol.

4. The method of claim 3 wherein said polyethylene glycol has a molecular weight of between about 1kD and 100kD.

5. The method of claim 4 wherein said polyethylene glycol has a molecular weight of between about 5kD and 30kD.

6. The method of claim 1 wherein said TTR is encoded by the nucleic acid of SEQ ID NO:2.

7. The method of claim 1 wherein the TTR variant is encoded by the nucleic acid of SEQ ID NO:8.

8. The method of claim 1 wherein the biologically active agent is a protein.

9. The method of claim 1 wherein the biologically active agent is a peptide.

10. The method of claim 9 wherein the peptide is a TPO mimetic peptide (TMP).

11. The method of claim 9 wherein the biologically active agent is a Glucagon-like Peptide-1 (GLP-1).

12. A substantially homogenous preparation of a TTR-biologically active agent fusion, optionally in a pharmaceutically acceptable diluent, carrier or adjuvant.

13. A substantially homogenous preparation of a PEG-TTR-biologically active agent fusion, optionally in a pharmaceutically acceptable diluent, carrier or adjuvant.

14. The preparation of claim 13 wherein the biologically active agent is a protein.

15. The preparation of claim 13 wherein the biologically active agent is a peptide.

16. The preparation of claim 15 wherein the peptide is a TMP.

17. The preparation of claim 15 wherein the peptide is a GLP-1.

18. A substantially homogenous preparation of a TTR variant-biologically active agent fusion, optionally in a pharmaceutically acceptable diluent, carrier or adjuvant.

19. A substantially homogenous preparation of a PEG-TTR variant-biologically active agent fusion, optionally in a pharmaceutically acceptable diluent, carrier or adjuvant.

20. The preparation of claim 19 wherein the biologically active agent is a protein.

21. The preparation of claim 19 wherein the biologically active agent is a peptide.

22. The preparation of claim 21 wherein the peptide is a TMP.

23. The preparation of claim 21, wherein the peptide is GLP-1.

24. The preparation of any of claims 10-23 wherein the fusion contains a linker peptide.

25. A process for preparing a substantially homogenous preparation of a TTR-biologically active agent fusion comprising: (a) fusing said TTR to a biologically active agent to provide a TTR-biologically active agent fusion; and (b) isolating said TTR-biologically active agent fusion.

26. A process for preparing a substantially homogenous preparation of a TTR variant-biologically active agent fusion comprising: (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) fusing said TTR variant to a biologically active agent to provide a TTR variant-

biologically active agent fusion; and (c) isolating said TTR variant-biologically active agent fusion.

27. A process for preparing a substantially homogenous preparation of a PEG-TTR-biologically active agent fusion comprising: (a) conjugating a polyethylene glycol to said TTR to provide a PEG-TTR; (b) fusing said PEG-TTR to a biologically active agent to provide a PEG-TTR-biologically active agent fusion; and (c) isolating said PEG-TTR-biologically active agent fusion.

28. A process for preparing a substantially homogenous preparation of a PEG-TTR variant-biologically active agent fusion comprising: (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 variant; (c) fusing said PEG-TTR variant to a biologically active agent to provide a PEG-TTR-biologically active agent fusion; and (d) isolating said PEG-TTR-biologically active agent fusion.

29. A method of treating thrombocytopenia comprising administering a therapeutically effective dose of a preparation of claim 16.

30. A method of treating thrombocytopenia comprising administering a therapeutically effective dose of a preparation of claim 22.

31. A method of treating non-insulin dependent diabetes comprising administering a

therapeutically effective dose of a preparation of claim 17.

32. A method of treating non-insulin dependent diabetes comprising administering a therapeutically effective dose of a preparation of claim 23.

33. A fusion protein comprising a TTR protein fused to a heterologous sequence.

34. A fusion protein of claim 33 wherein the heterologous sequence is a TMP.

35. A fusion protein of claim 33 wherein the heterologous sequence is a GLP-1.

36. A fusion protein of any one of claims 33, 34 or 35 further comprising a linker sequence between the TTR protein and the heterologous sequence.

37. A nucleic acid encoding the fusion protein of any one of claims 33, 34 or 35.

Figure 1

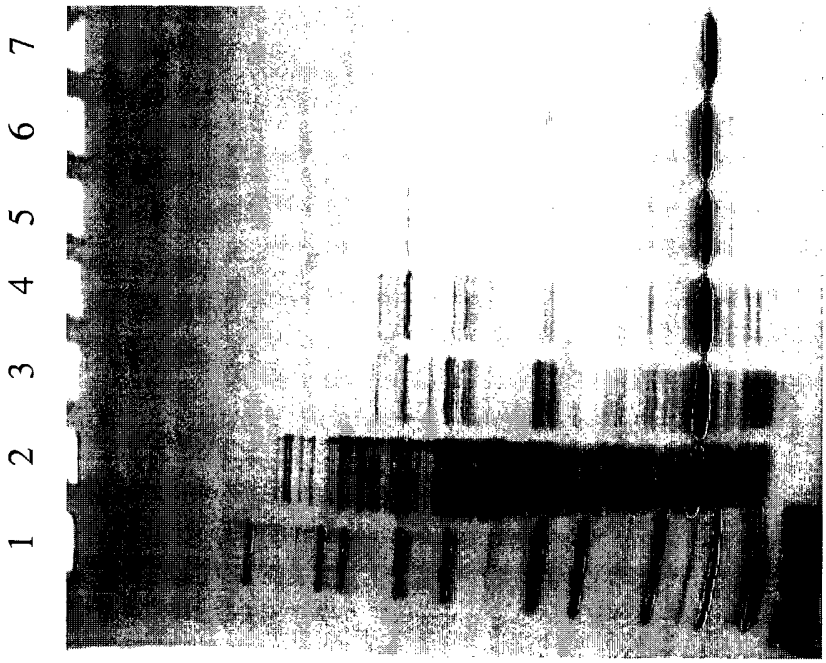


Figure 2

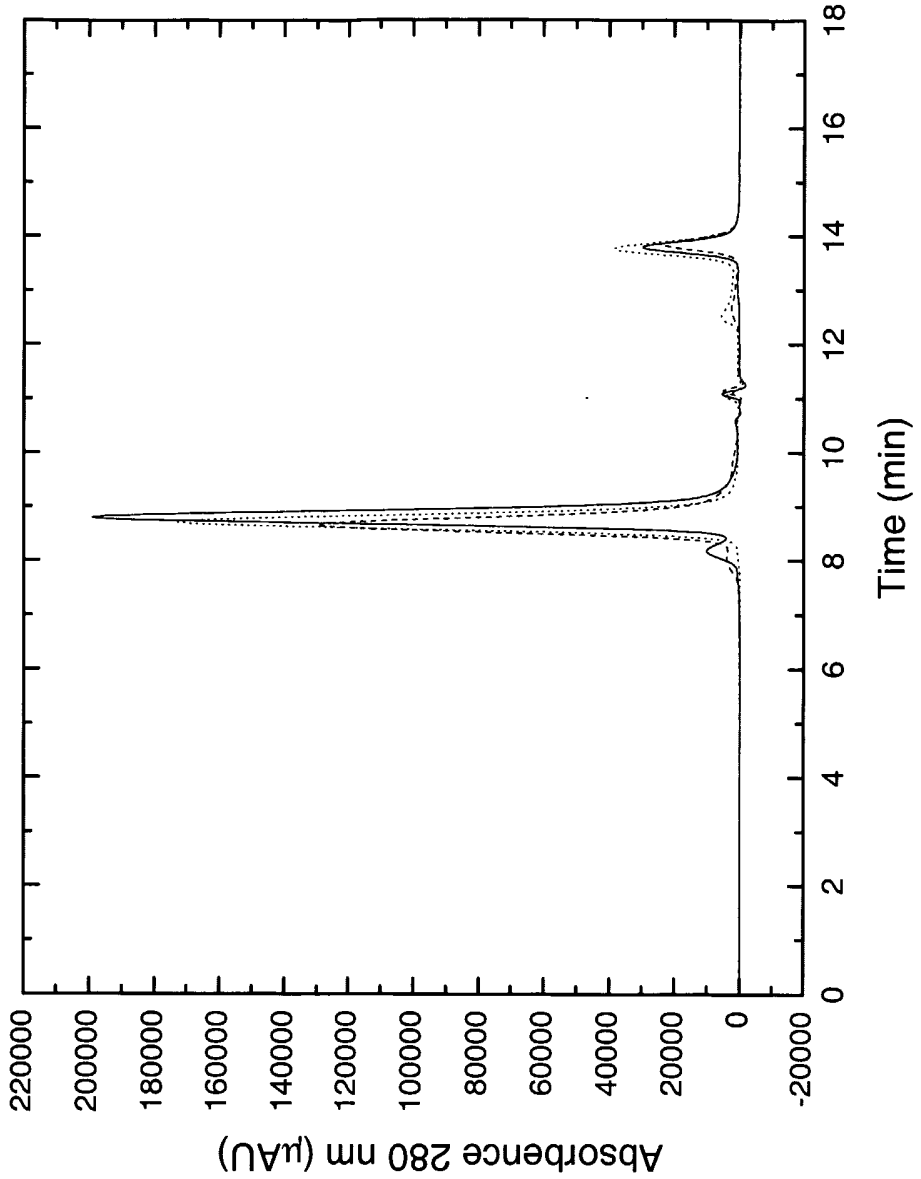

Absorbence 280 nm (μAU)
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
-20000
0
2
4
6
8
10
12
14
16
18
Time (min)

Figure 3

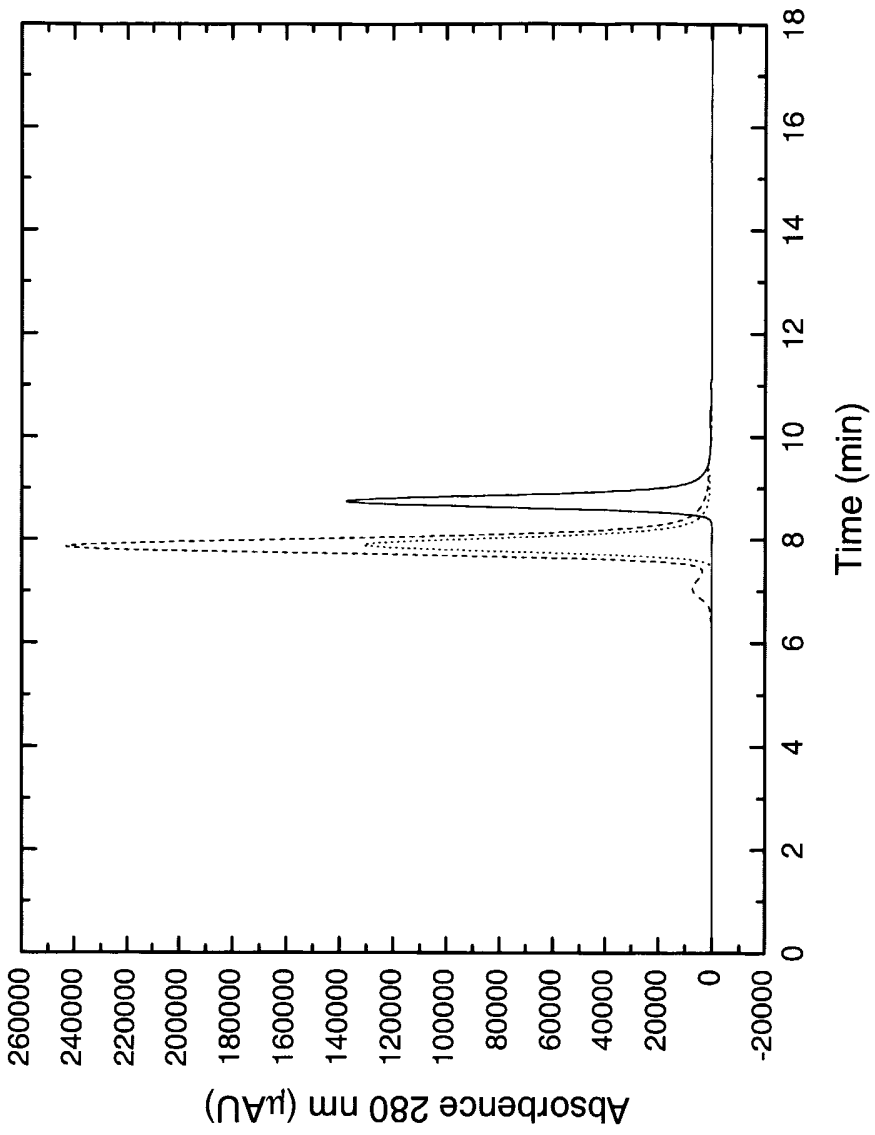

Absorbence 280 nm (μAU)
260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
-20000
0
2
4
6
8
10
12
14
16
18
Time (min)

Figure 4

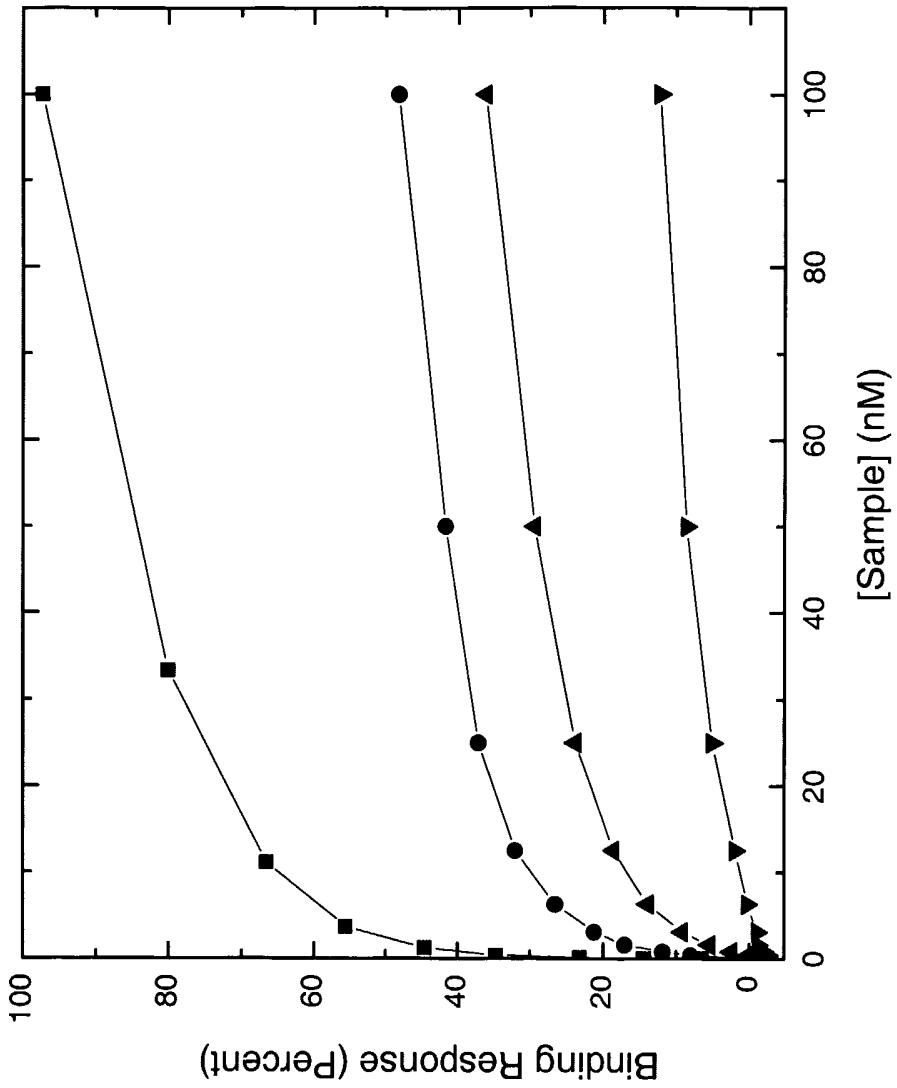

Binding Response (Percent)
0
20
40
60
80
100
[Sample] (nM)
0
20
40
60
80
100

Figure 5

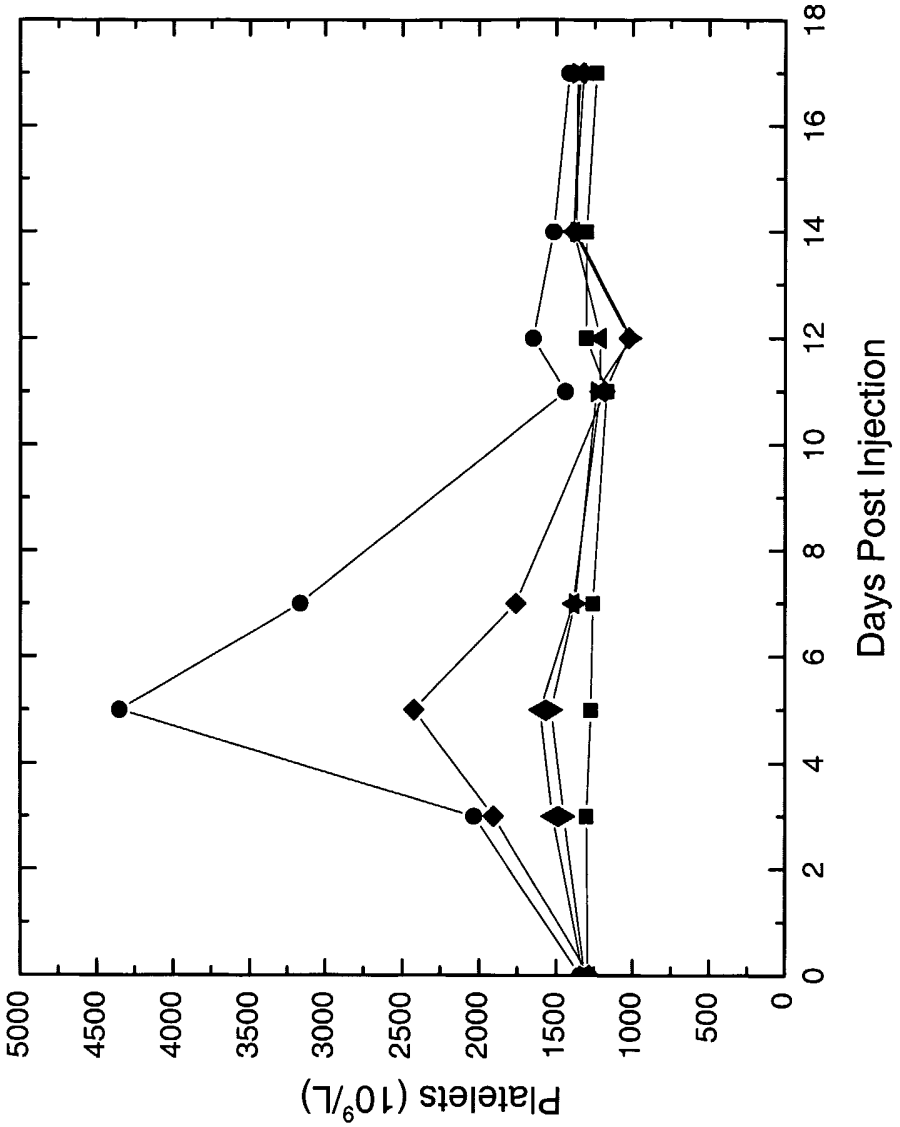

Platelets (10^9/L)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
16
18
Days Post Injection

Figure 6

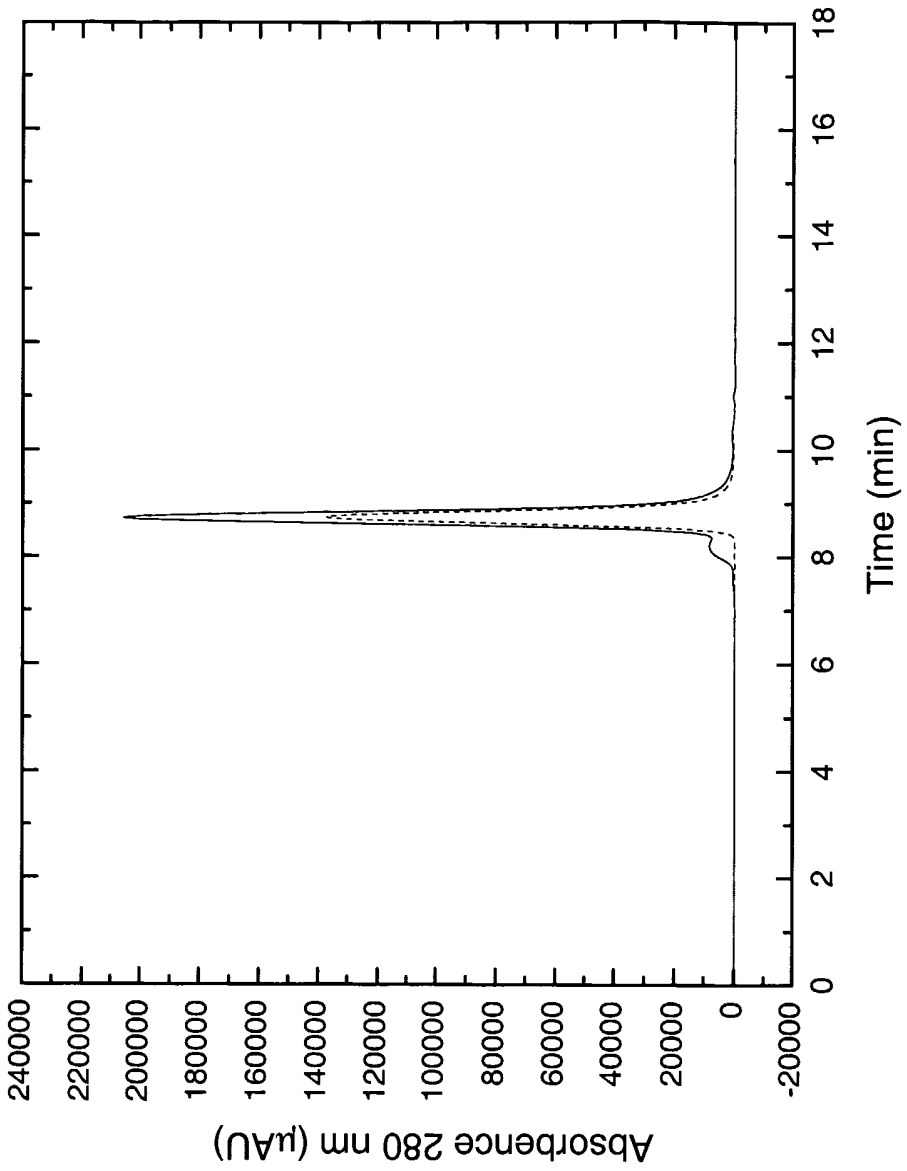

Absorbence 280 nm (μAU)
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
-20000
0
2
4
6
8
10
12
14
16
18
Time (min)

Figure 7

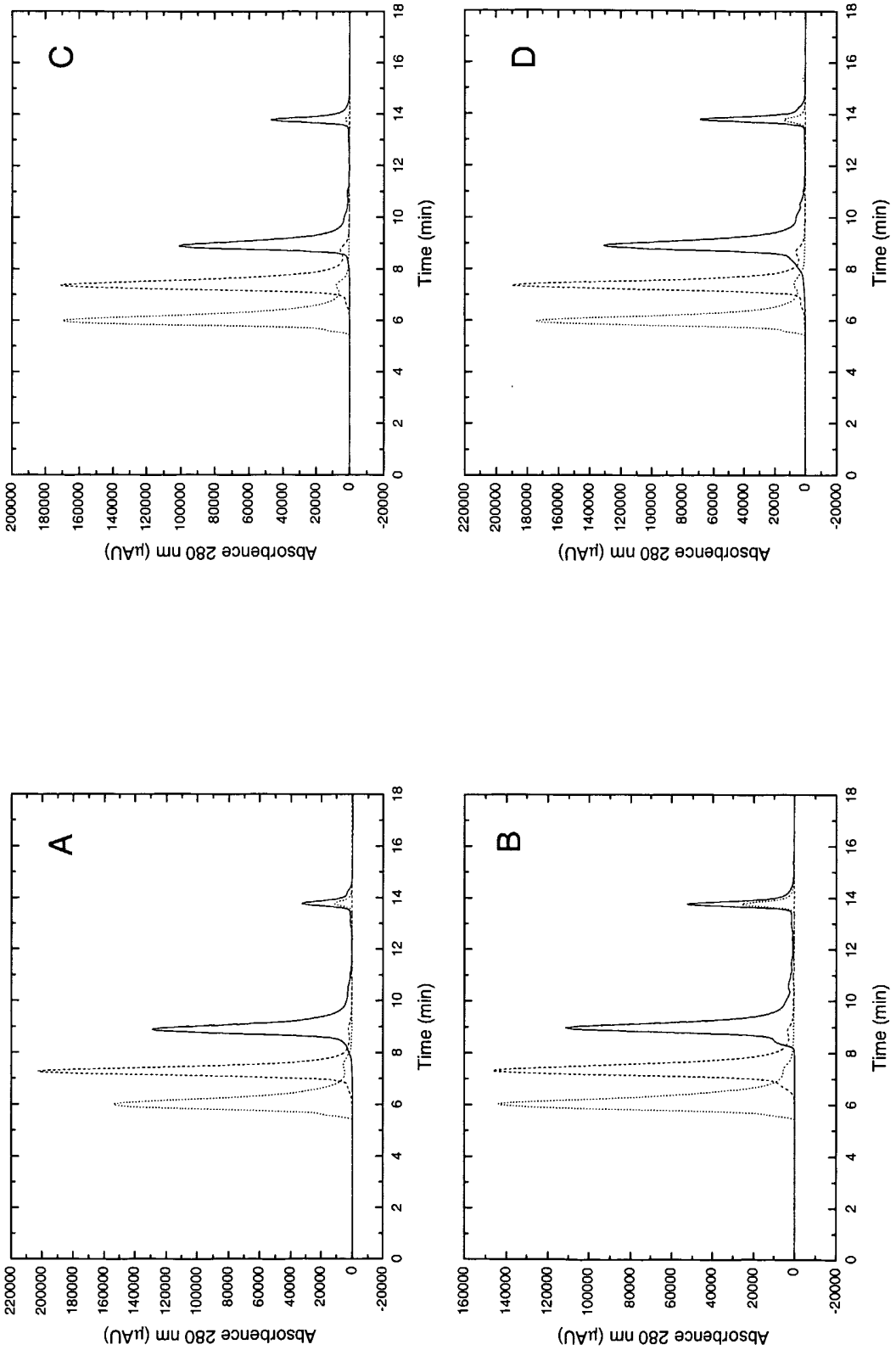


Figure 8

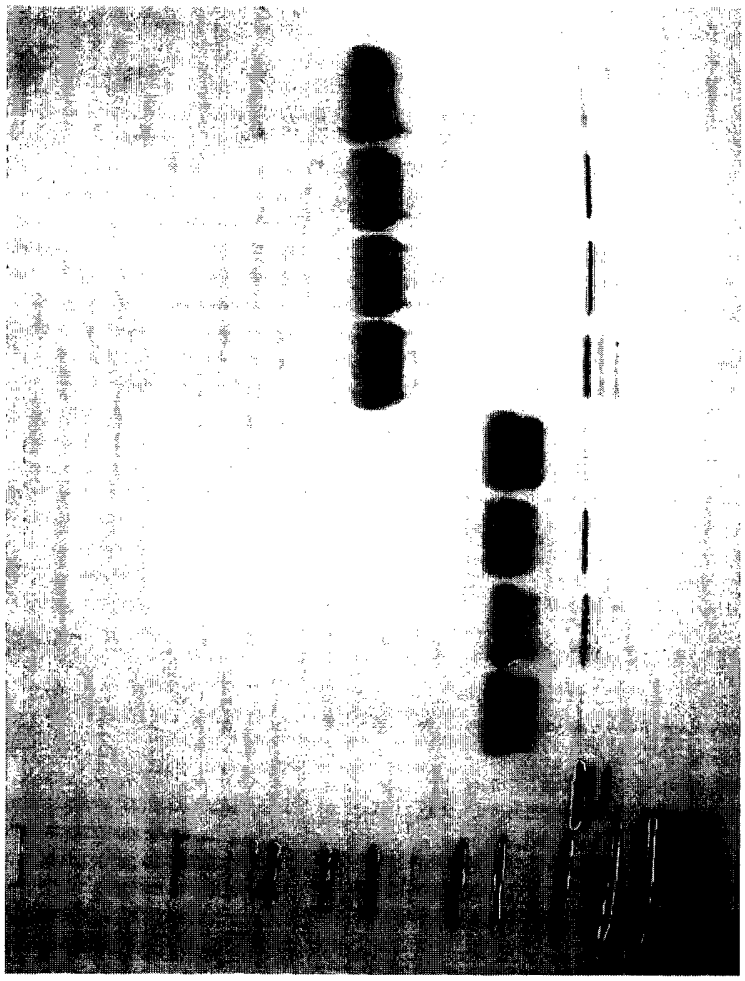


Figure 9

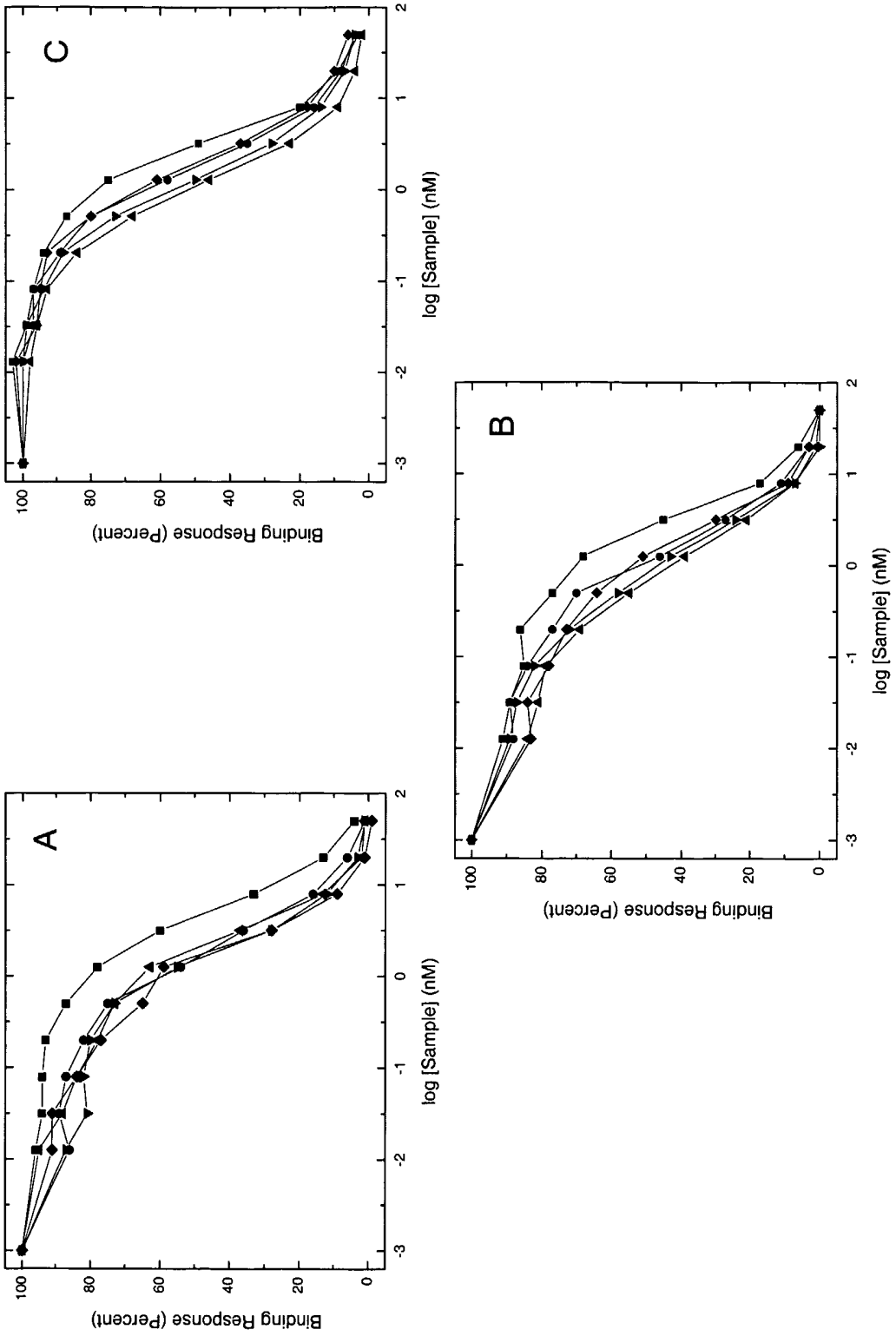

A
B
C
Binding Response (Percent)
log [Sample] (nM)
100
80
60
40
20
0
-3
-2
-1
0
1
2

Figure 10

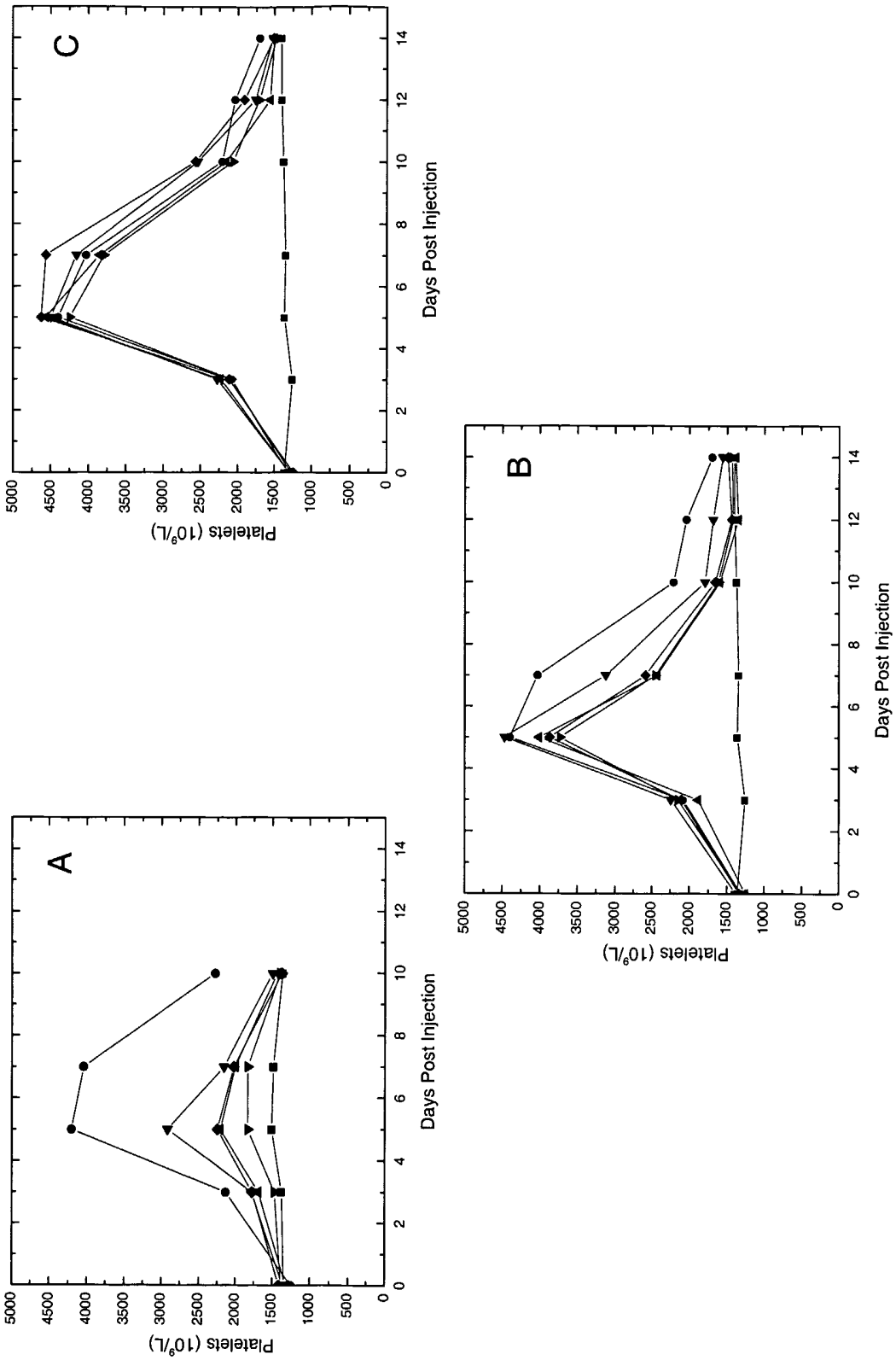

A
B
C
Platelets (10^9/L)
Days Post Injection
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
0
2
4
6
8
10
12
14

Figure 11

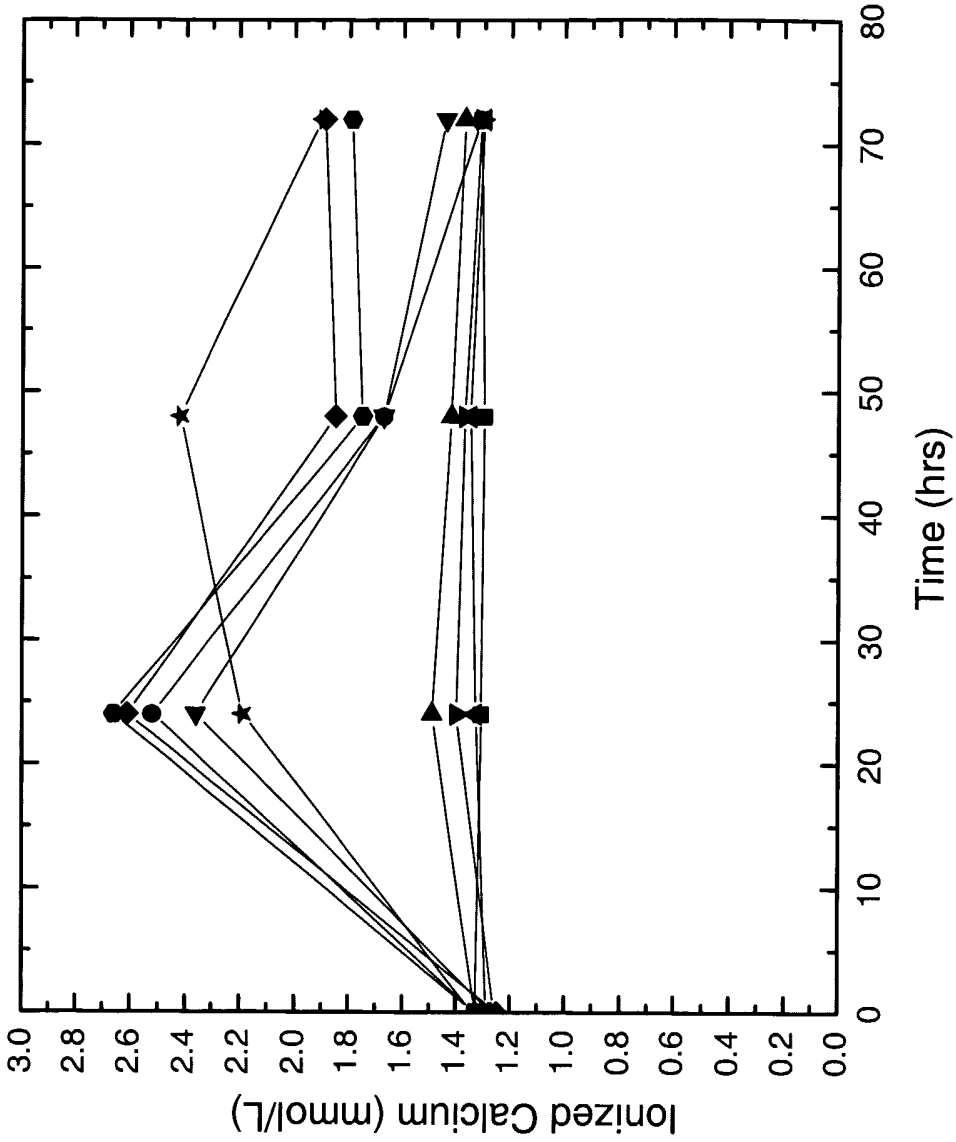

Ionized Calcium (mmol/L)
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
Time (hrs)

Figure 12

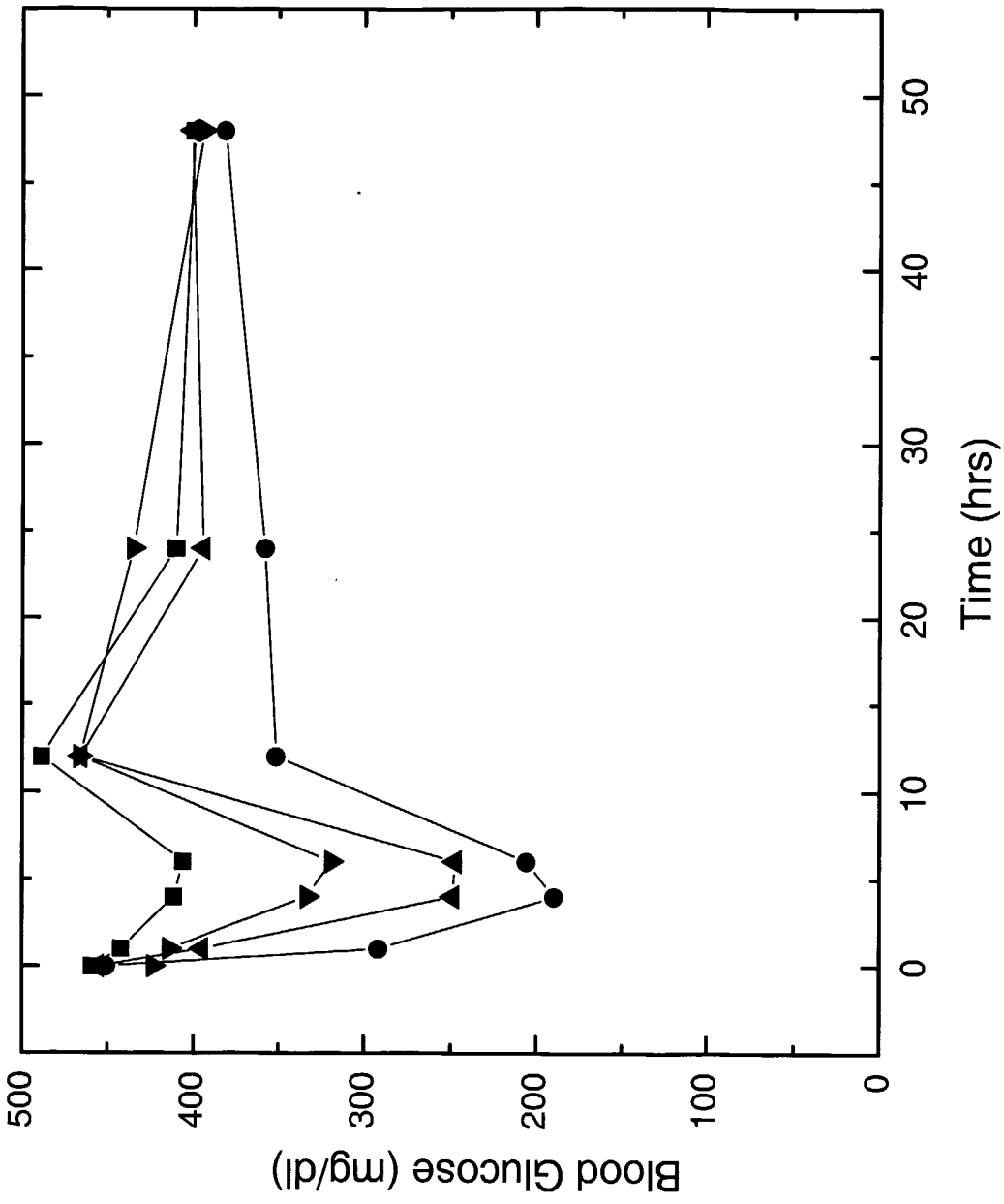

Blood Glucose (mg/dl)
0
100
200
300
400
500
Time (hrs)
0
10
20
30
40
50

Figure 13

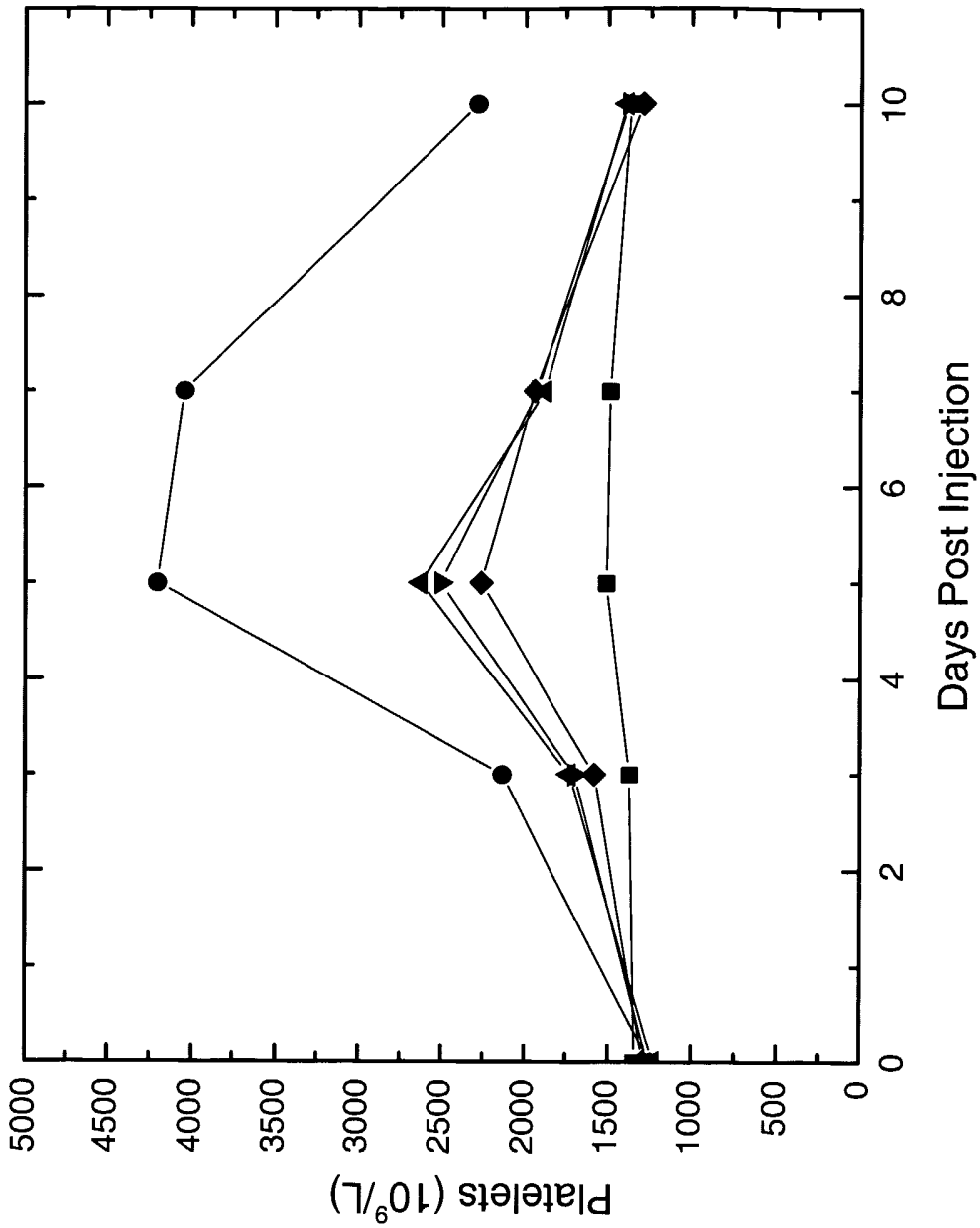

Platelets (10^9/L)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
Days Post Injection

Figure 14

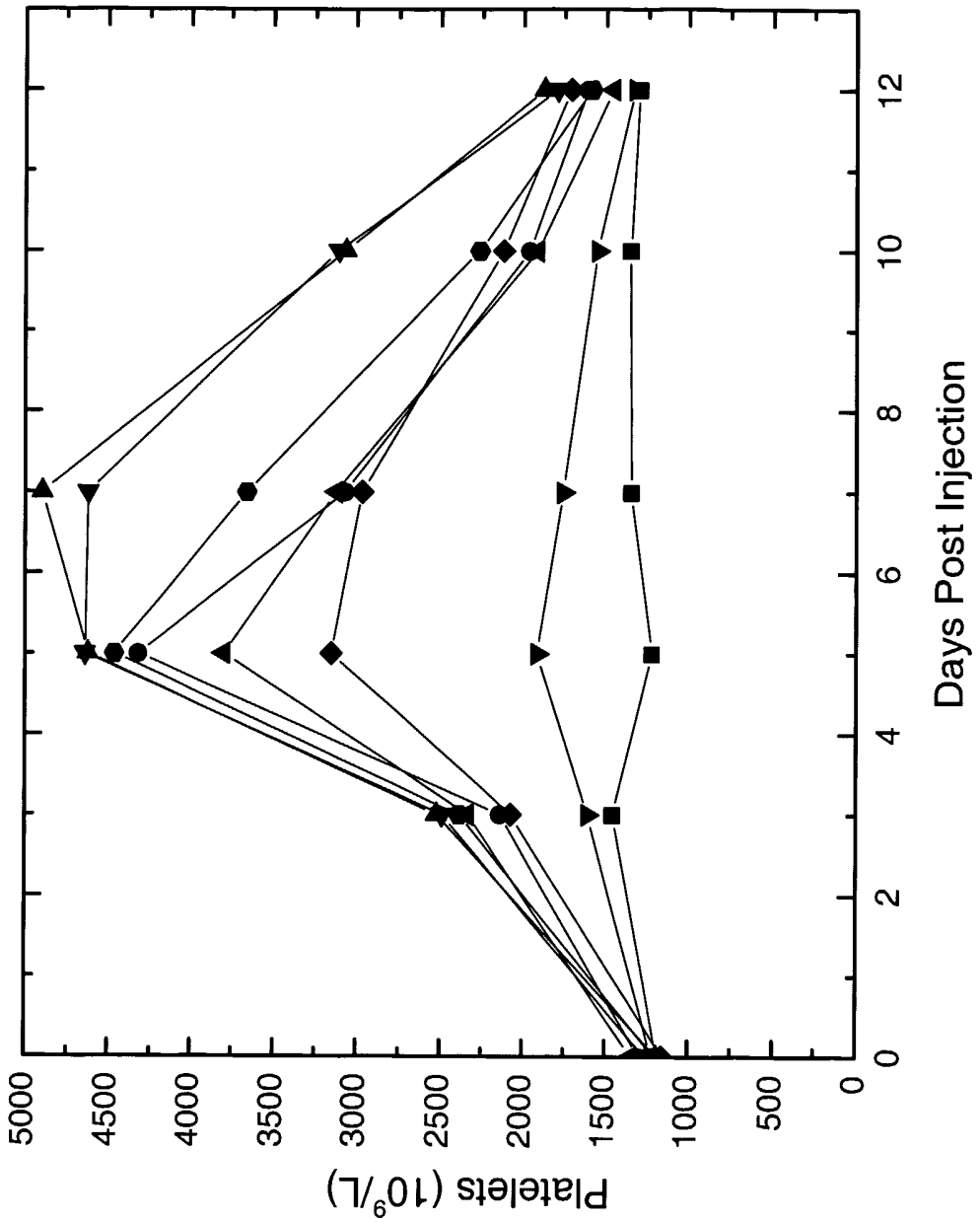
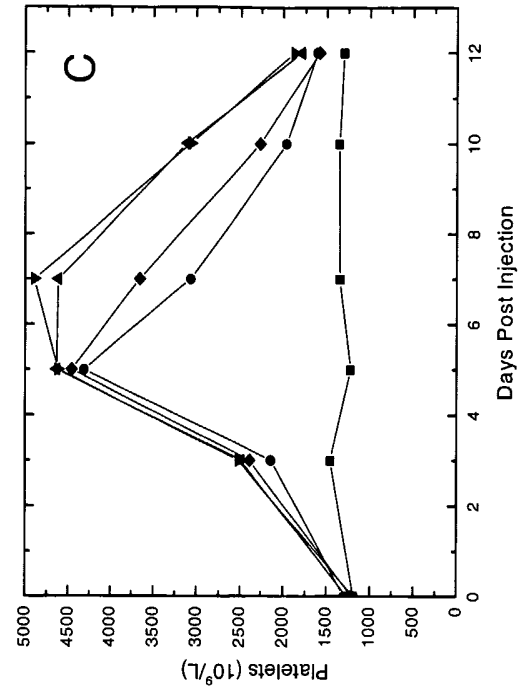
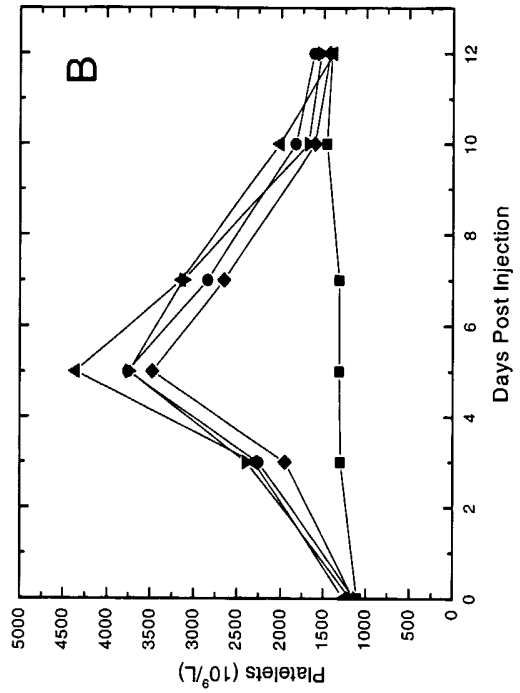
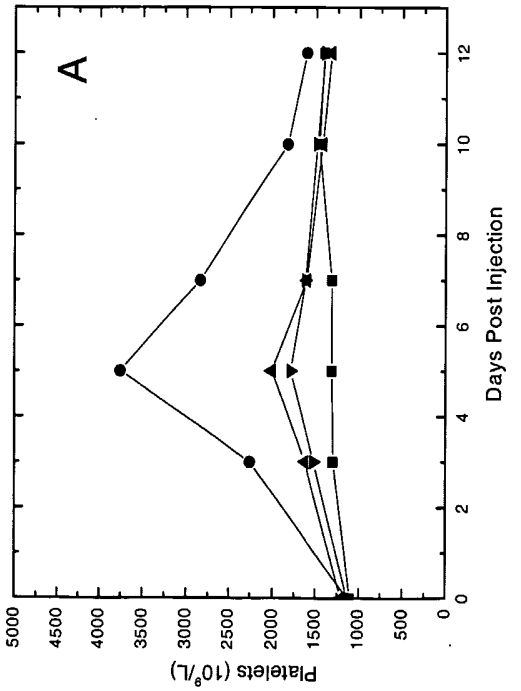

Platelets (10^9/L)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
Days Post Injection

Figure 15


C
Platelets (10^9/L)
Days Post Injection


B
Platelets (10^9/L)
Days Post Injection


A
Platelets (10^9/L)
Days Post Injection

A-813A PCT.ST25.txt
SEQUENCE LISTING

<110> Amgen Inc.

<120> Use of Transthyretin Peptide/Protein Fusions to Increase the Serum ...

<130> A-813A (PCT)

<140> TBA
<141> 2003-04-03

<150> A-813
<151> 2002-04-04

<160> 75

<170> PatentIn version 3.1

<210> 1
<211> 127
<212> PRT
<213> Homo sapiens

<400> 1

Gly Pro Thr Gly Thr Gly Glu Ser Lys Cys Pro Leu Met Val Lys Val
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

agtggtccaa ctggtaccgg tgaatccaag tgtcctctga tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaataa 387

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

<400> 3
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaataa 387

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

<400> 4
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc ttgtgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaataa 387

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

<400> 5

atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgcttgtgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaataa 387

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

<400> 6
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aagtgtcttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaataa 387

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

<400> 7
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttt gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaataa 387

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

<400> 8

atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcgcagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttt gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaataa 387

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

<400> 9
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120

ggtaccggtg aatccaagtg tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180

cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240

tttgcctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300

tttgtagaag ggatatacaa agtggaaata gacaccaaat cttactggaa ggcacttggc 360

atctccccat tccatgagca tgcagaggtg gtattcacag ccaacgactc cggccccgc 420

cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480

aatcccaagg aataa 495

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

<400> 10
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120

ggtaccggtg aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180

cctgccatca atgtggccgt gcatgtgttc agaaaggctt gtgatgacac ctgggagcca 240

tttgcctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300

tttgtagaag ggatatacaa agtggaaata gacaccaaat cttactggaa ggcacttggc 360

atctccccat tccatgagca tgcagaggtg gtattcacag ccaacgactc cggccccgc 420

cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480

aatcccaagg aataa 495

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

<400> 11
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120

ggtaccggtg aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180

cctgccatca atgtggccgt gcatgtgttc agaaaggctg cttgtgacac ctgggagcca 240

tttgcctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300

tttgtagaag ggatatacaa agtggaaata gacaccaaat cttactggaa ggcacttggc 360

atctccccat tccatgagca tgcagaggtg gtattcacag ccaacgactc cggcccccgc 420

cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480

aatcccaagg aataa 495

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

<400> 12
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120

ggtaccggtg aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180

cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240

tttgcctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300

tttgtagaag ggatatacaa agtggaaata gacaccaaat cttactggaa gtgtcttggc 360

atctccccat tccatgagca tgcagaggtg gtattcacag ccaacgactc cggcccccgc 420

cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480

aatcccaagg aataa 495

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

<400> 13
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120

ggtaccggtg aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180

cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240

tttgcctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300

tttgtagaag ggatatacaa agtggaaata gacaccaaat cttactggaa ggcactttgc 360

atctccccat tccatgagca tgcagaggtg gtattcacag ccaacgactc cggcccccgc 420

cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480

aatcccaagg aataa 495

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

<400> 14
atgtctgttt ctgaaatcca gctgatgcat aacctgggta aacatctgaa ctctatggaa 60

cgtgttgaat ggctgcgtaa gaaactgcag gacgttcata actttggtcc aactggtacc 120

ggtgaatcca aggctcctct gatggtcgca gttctagatg ctgtccgagg cagtcctgcc 180

atcaatgtgg ccgtgcatgt gttcagaaag gctgctgatg acacctggga gccatttgcc 240

tctgggaaaa ccagtgagtc tggagagctg catgggctca caactgagga ggaatttgta 300

gaagggatat acaaagtgga aatagacacc aaatcttact ggaagtgtct tggcatctcc 360

ccattccatg agcatgcaga ggtggtattc acagccaacg actccggccc ccgccgctac 420

accattgccg ccctgctgag cccctactcc tattccacca cggctgtcgt caccaatccc 480

aaggaataa 489

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

<400> 15
atgcgaccgt ccggccgtaa gagctccaaa atgcaggctt tccgtatctg ggacgttaac 60

cagaaaacct tctacctgcg caacaaccag ctggttgctg gctacctgca gggtccgaac 120

gttaacctgg aagaaaaaat cgacgttgta ccgatcgaac cgcacgctct gttcctgggt 180

atccacggtg gtaaaatgtg cctgagctgc gtgaaatctg gtgacgaaac tcgtctgcag 240

ctggaagcag ttaacatcac tgacctgagc gaaaaccgca aacaggacaa acgtttcgca 300

ttcatccgct ctgacagcgg cccgaccacc agcttcgaat ctgctgcttg cccgggttgg 360

ttcctgtgca ctgctatgga agctgaccag ccggtaagcc tgaccaacat gccggacgaa 420

ggcgtgatgg taaccaaatt ctacttccag gaagacgaag gtccaactgg taccggtgaa 480

tccaaggctc ctctgatggt caaagttcta gatgctgtcc gaggcagtcc tgccatcaat 540

gtggccgtgc atgtgttcag aaaggctgct gatgacacct gggagccatt tgcctctggg 600

aaaaccagtg agtctggaga gctgcatggg ctcacaactg aggaggaatt tgtagaaggg 660

atatacaaag tggaaataga caccaaatct tactggaagg cacttggcat ctccccattc 720

catgagcatg cagaggtggt attcacagcc aacgactccg gcccccgccg ctacaccatt 780

gccgccctgc tgagccccta ctcctattcc accacggctg tcgtcaccaa tcccaaggaa 840

taa 843

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

<400> 16
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaaggtagt ggtagccgac cgtccggccg taagagctcc 420

aaaatgcagg ctttccgtat ctgggacgtt aaccagaaaa ccttctacct gcgcaacaac 480

cagctggttg ctggctacct gcagggtccg aacgttaacc tggaagaaaa aatcgacgtt 540

gtaccgatcg aaccgcacgc tctgttcctg ggtatccacg gtggtaaaat gtgcctgagc 600

tgcgtgaaat ctggtgacga aactcgtctg cagctggaag cagttaacat cactgacctg 660

agcgaaaacc gcaaacagga caaacgtttc gcattcatcc gctctgacag cggcccgacc 720

accagcttcg aatctgctgc ttgcccgggt tggttcctgt gcactgctat ggaagctgac 780

cagccggtaa gcctgaccaa catgccggac gaaggcgtga tggtaaccaa attctacttc 840

caggaagacg aataa 855

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

<400> 17
catatgggtc caactggtac cggtgaatcc aaggctcctc tgatggtcaa agttctagat 60

gctgtccgag gcagtcctgc catcaatgtg gccgtgcatg tgttcagaaa ggctgctgat 120

gacacctggg agccatttgc ctctgggaaa accagtgagt ctggagagct gcatgggctc 180

acaactgagg aggaatttgt agaagggata tacaaagtgg aaatagacac caaatcttac 240

tggaaggcac tttgcatctc cccattccat gagcatgcag aggtggtatt cacagccaac 300

gactccggcc cccgccgcta 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 tggtga 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

<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
ggagatgcca 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
ctgatggtcg cagttctaga t 21

<210> 30
<211> 21
<212> DNA
<213> Homo sapiens

<400> 30
atctagaact gcgaccatca g 21

<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
ttcaccggta 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
ttcaccggta 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 atcccaagga aggtagtggt agccgaccgt ccggccgtaa gagc 54

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

<400> 38
ccgcggatcc tcgagattat tcgtcttcct ggaagtagaa 40

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

<400> 39
gaggaataac atatgggtcc aactggtacc ggtgaa 36

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

<400> 40
aatatactgc agtggtggaa taggag 26

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

<400> 41
gtcgtcacca atcccaagga aggatcagga tccggaaaac gtccgccggg tttctccccg 60

ctgtaatc 68

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

<400> 42
tcgagattac agcggggaga aacccggcgg acgttttccg gatcctgatc 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 aactggtacc 120

ggtgaatcca aggctcctct gatggtcgca gttctagatg ctgtccgagg cagtcctgcc 180

atcaatgtgg ccgtgcatgt gttcagaaag gctgctgatg acacctggga gccatttgcc 240

tctgggaaaa ccagtgagtc tggagagctg catgggctca caactgagga ggaatttgta 300

gaagggatat acaaagtgga aatagacacc aaatcttact ggaagtgtct tggcatctcc 360

ccattccatg agcatgcaga ggtggtattc acagccaacg actccggccc ccgccgctac 420

accattgccg ccctgctgag ccccctactcc tattccacca cggctgtcgt caccaatccc 480

aaggaataa 489

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

<400> 44
cgtacaggtt 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 tcatcaccat 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 ggaaggtcag gctgctaaag aattcatcgc ttggctggtt 120

aaaggtcgtg gtggttctgg ttctgctact ggtggttccg gctccaccgc aagctctggt 180

tcaggcagtg cgggtggtgg tggtggtggt ggtccaactg gtaccggtga atccaaggct 240

cctctgatgg tcaaagttct agatgctgtc cgaggcagtc ctgccatcaa tgtggccgtg 300

catgtgttca gaaaggctgc tgatgacacc tgggagccat ttgcctctgg gaaaaccagt 360

gagtctggag agctgcatgg gctcacaact gaggaggaat ttgtagaagg gatatacaaa 420

gtggaaatag acaccaaatc ttactggaag gcactttgca tctccccatt ccatgagcat 480

gcagaggtgg tattcacagc caacgactcc ggcccccgcc gctacaccat tgccgccctg 540

ctgagcccct actcctattc caccacggct gtcgtcacca atcccaagga ataa 594

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

<400> 49
atgaaacatc atcaccatca ccatcatgac gaagttgatc acggtgaagg tactttcact 60

tctgacgttt cttcttatct ggaaggtcag gctgctaaag aattcatcgc ttggctggtt 120

aaaggtcgtg gtggtggtgg tggttctggt ggtggtggtt ctggtggtgg tggttctggc 180

ggcggtggta gcggtggtgg tggtggtggt ccaactggta ccggtgaatc caaggctcct 240

ctgatggtcg cagttctaga tgctgtccga ggcagtcctg ccatcaatgt ggccgtgcat 300

gtgttcagaa aggctgctga tgacacctgg gagccatttg cctctgggaa aaccagtgag 360

tctggagagc tgcatgggct cacaactgag gaggaatttg tagaagggat atacaaagtg 420

gaaatagaca ccaaatctta ctggaaggca ctttgcatct ccccattcca tgagcatgca 480

gaggtggtat tcacagccaa cgactccggc ccccgccgct acaccattgc cgccctgctg 540

agcccctact cctattccac cacggctgtc gtcaccaatc ccaaggaata a 591

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

<400> 50
agacctgtac atatgaaaca tcatcaccat caccatcatg acgaagttga tcacggtgaa 60

ggtactttca cttctg 76

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

<400> 51
gggggaagag gaaaactgac 20

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

<400> 52
atgaaacatc atcaccatca ccatcatgac gaagttgatc acggtgaagg tactttcact 60

tctgacgttt cttcttatct ggaaggtcag gctgctaaag aattcatcgc ttggctggtt 120

aaaggtcgtg gtggttctgg ttctgctact ggtggttccg gctccaccgc aagctctggt 180

tcaggcagtg cgactcatgg tggtggtggt ggtgacaaaa ctcacacatg tccaccgtgc 240

ccagcacctg aactcctggg gggaccgtca gttttcctct tccccccaaa acccaaggac 300

accctcatga tctcccggac ccctgaggtc acatgcgtgg tggtggacgt gagccacgaa 360

gaccctgagg tcaagttcaa ctggtacgtg gacggcgtgg aggtgcataa tgccaagaca 420

aagccgcggg aggagcagta caacagcacg taccgtgtgg tcagcgtcct caccgtcctg 480

caccaggact ggctgaatgg caaggagtac aagtgcaagg tctccaacaa agccctccca 540

gcccccatcg agaaaaccat ctccaaagcc aaagggcagc cccgagaacc acaggtgtac 600

accctgcccc catcccggga tgagctgacc aagaaccagg tcagcctgac ctgcctggtc 660

aaaggcttct atcccagcga catcgccgtg gagtgggaga gcaatgggca gccggagaac 720

aactacaaga ccacgcctcc cgtgctggac tccgacggct ccttcttcct ctacagcaag 780

ctcaccgtgg acaagagcag gtggcagcag gggaacgtct tctcatgctc cgtgatgcat 840

gaggctctgc acaaccacta cacgcagaag agcctctccc tgtctccggg taaa 894

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

<400> 53
gtcgtcacca atcccaagga aggttctggc tccggatcag ggggaccgtc agttttcctc 60

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

<400> 54
ccgcggatcc tcgagattag gatccagaac ccccttttggc tttggagatg gt 52

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

<400> 55
gaggaataac atatgggtcc aactggtacc ggtgaatcca ag 42

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

<400> 56
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaaggttct ggctccggat caggggggacc gtcagttttc 420

ctcttccccc caaaacccaa ggacaccctc atgatctccc ggacccctga ggtcacatgc 480

gtggtggtgg acgtgagcca cgaagaccct gaggtcaagt tcaactggta cgtggacggc 540

gtggaggtgc ataatgccaa gacaaagccg cgggaggagc agtacaacag cacgtaccgt 600

gtggtcagcg tcctcaccgt cctgcaccag gactggctga atggcaagga gtacaagtgc 660

aaggtctcca acaaagccct cccagccccc atcgagaaaa ccatctccaa agccaaaggg 720

ggttctggat cctaa 735

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

<400> 57
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120

ggtaccggtg aatccaaggc tcctctgatg gtcaaagttc tagatgctgt ccgaggcagt 180

cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240

tttgcctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300

tttgtagaag ggatatacaa agtggaaata gacaccaaat cttactggaa ggcacttggc 360

atctccccat tccatgagca tgcagaggtg gtattcacag ccaacgactc cggcccccgc 420

cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480

aatcccaagg aaggttctgg ctccggatca gggggaccgt cagttttcct cttcccccca 540

aaacccaagg acaccctcat gatctcccgg acccctgagg tcacatgcgt ggtggtggac 600

gtgagccacg aagaccctga ggtcaagttc aactggtacg tggacggcgt ggaggtgcat 660

aatgccaaga caaagccgcg ggaggagcag tacaacagca cgtaccgtgt ggtcagcgtc 720

ctcaccgtcc tgcaccagga ctggctgaat ggcaaggagt acaagtgcaa ggtctccaac 780

aaagccctcc cagccccat 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 tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaaggttct ggctccggat caggggggacc gtcagttttc 420

ctcttccccc caaaacccaa ggacaccctc atgatctccc ggacccctga ggtcacatgc 480

gtggtggtgg acgtgagcca cgaagaccct gaggtcaagt tcaactggta cgtggacggc 540

gtggaggtgc ataatgccaa gacaaagccg cgggaggagc agtacaacag cacgtaccgt 600

gtggtcagcg tcctcaccgt cctgcaccag gactggctga atggcaagga gtacaagtgc 660

aaggtctcca acaaagccct cccagccccc atcgagaaaa ccatctccaa agccaaaggg 720

ggttctggat ccatcgaagg tccgactctg cgtcagtggc tggctgctcg tgctggcggt 780

ggtggcggag ggggtggcat tgagggccca acccttcgcc aatggcttgc agcacgcgca 840

taa 843

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

<400> 60
ttcaccggta ccagttggac cagaaccccc tttggctttg gagatggt 48

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

<400> 61
gaggaataac atatgggatc 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

cctgaggtca agttcaactg gtacgtggac ggcgtggagg tgcataatgc caagacaaag 180

ccgcgggagg agcagtacaa cagcacgtac cgtgtggtca gcgtcctcac cgtcctgcac 240

caggactggc tgaatggcaa ggagtacaag tgcaaggtct ccaacaaagc cctcccagcc 300

cccatcgaga aaaccatctc caaagccaaa gggggttctg gtccaactgg taccggtgaa 360

tccaaggctc ctctgatggt caaagttcta gatgctgtcc gaggcagtcc tgccatcaat 420

gtggccgtgc atgtgttcag aaaggctgct gatgacacct gggagccatt tgcctctggg 480

aaaaccagtg agtctggaga gctgcatggg ctcacaactg aggaggaatt tgtagaaggg 540

atatacaaag tggaaataga caccaaatct tactggaagg cacttggcat ctccccattc 600

catgagcatg cagaggtggt attcacagcc aacgactccg gcccccgccg ctacaccatt 660

gccgccctgc tgagccccta ctcctattcc 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

<213> Homo sapiens

<400> 65
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggatccggt 120

tctgggggac cgtcagtttt cctcttcccc ccaaaaccca aggacaccct catgatctcc 180

cggacccctg aggtcacatg cgtggtggtg gacgtgagcc acgaagaccc tgaggtcaag 240

ttcaactggt acgtggacgg cgtggaggtg cataatgcca agacaaagcc gcgggaggag 300

cagtacaaca gcacgtaccg tgtggtcagc gtcctcaccg tcctgcacca ggactggctg 360

aatggcaagg agtacaagtg caaggtctcc aacaaagccc tcccagcccc catcgagaaa 420

accatctcca aagccaaagg gggttctggt ccaactggta ccggtgaatc caaggctcct 480

ctgatggtca aagttctaga tgctgtccga ggcagtcctg ccatcaatgt ggccgtgcat 540

gtgttcagaa aggctgctga tgacacctgg gagccatttg cctctgggaa aaccagtgag 600

tctggagagc tgcatgggct cacaactgag gaggaatttg tagaagggat atacaaagtg 660

gaaatagaca ccaaatctta ctggaaggca cttggcatct ccccattcca tgagcatgca 720

gaggtggtat tcacagccaa cgactccggc ccccgccgct acaccattgc cgccctgctg 780

agcccctact cctattccac cacggctgtc gtcaccaatc ccaaggaata a 831

<210> 66
<211> 42
<212> DNA
<213> Homo sapiens

<400> 66
gtcgtcacca atcccaagga aggttctggt tctggtatcg aa 42

<210> 67
<211> 40
<212> DNA
<213> Homo sapiens

<400> 67
ccgcggatcc tcgagattat gcgcgtgctg caagccattg 40

<210> 68
<211> 510
<212> DNA
<213> Homo sapiens

<400> 68
atgggtccaa ctggtaccgg tgaatccaag tgtcctctga tggtcaaagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaaggttct ggttctggta tcgaaggtcc gactctgcgt 420

cagtggctgg ctgctcgtgc tggcggtggt ggcggagggg gtggcattga gggcccaacc 480

cttcgccaat ggcttgcagc acgcgcataa 510

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

<400> 69
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcgcagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaaggttct ggttctggta tcgaaggtcc gactctgcgt 420

cagtggctgg ctgctcgtgc tggcggtggt ggcggagggg gtggcattga gggcccaacc 480

cttcgccaat ggcttgcagc acgcgcataa 510

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

<400> 70
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcgcagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc ttgtgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaaggttct ggttctggta tcgaaggtcc gactctgcgt 420

cagtggctgg ctgctcgtgc tggcggtggt ggcggagggg gtggcattga gggcccaacc 480

cttcgccaat ggcttgcagc acgcgcataa 510

<210> 71

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

<400> 71
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcgcagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aagtgtcttg gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaaggttct ggttctggta tcgaaggtcc gactctgcgt 420

cagtggctgg ctgctcgtgc tggcggtggt ggcggagggg gtggcattga gggcccaacc 480

cttcgccaat ggcttgcagc acgcgcataa 510

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

<400> 72
atgggtccaa ctggtaccgg tgaatccaag gctcctctga tggtcgcagt tctagatgct 60

gtccgaggca gtcctgccat caatgtggcc gtgcatgtgt tcagaaaggc tgctgatgac 120

acctgggagc catttgcctc tgggaaaacc agtgagtctg gagagctgca tgggctcaca 180

actgaggagg aatttgtaga agggatatac aaagtggaaa tagacaccaa atcttactgg 240

aaggcacttt gcatctcccc attccatgag catgcagagg tggtattcac agccaacgac 300

tccggccccc gccgctacac cattgccgcc ctgctgagcc cctactccta ttccaccacg 360

gctgtcgtca ccaatcccaa ggaaggttct ggttctggta tcgaaggtcc gactctgcgt 420

cagtggctgg ctgctcgtgc tggcggtggt ggcggagggg gtggcattga gggcccaacc 480

cttcgccagt ggcttgcagc acgcgcataa 510

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

<400> 73
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120

ggtaccggtg aatccaaggc tcctctgatg gtcgcagttc tagatgctgt ccgaggcagt 180

cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240

tttgcctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300

tttgtagaag ggatatacaa agtggaaata gacaccaaat cttactggaa ggcactttgc 360

atctccccat tccatgagca tgcagaggtg gtattcacag ccaacgactc cggcccccgc 420

cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480

aatcccaagg aataa 495

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

<400> 74
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120

ggtaccggtg aatccaaggc tcctctgatg gtcgcagttc tagatgctgt ccgaggcagt 180

cctgccatca atgtggccgt gcatgtgttc agaaaggctg ctgatgacac ctgggagcca 240

tttgcctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300

tttgtagaag ggatatacaa agtggaaata gacaccaaat cttactggaa gtgtcttggc 360

atctccccat tccatgagca tgcagaggtg gtattcacag ccaacgactc cggcccccgc 420

cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480

aatcccaagg aataa 495

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

<400> 75
atgatcgaag gtccgactct gcgtcagtgg ctggctgctc gtgctggcgg tggtggcgga 60

gggggtggca ttgagggccc aacccttcgc caatggcttg cagcacgcgc aggtccaact 120

ggtaccggtg aatccaaggc tcctctgatg gtcgcagttc tagatgctgt ccgaggcagt 180

cctgccatca atgtggccgt gcatgtgttc agaaaggctt gtgatgacac ctgggagcca 240

tttgcctctg ggaaaaccag tgagtctgga gagctgcatg ggctcacaac tgaggaggaa 300

tttgtagaag ggatatacaa agtggaaata gacaccaaat cttactggaa ggcacttggc 360

atctccccat tccatgagca tgcagaggtg gtattcacag ccaacgactc cggcccccgc 420

cgctacacca ttgccgccct gctgagcccc tactcctatt ccaccacggc tgtcgtcacc 480

aatcccaagg aataa 495

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/10443

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/00
US CL : 514/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/12, 11, 16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A ✓	US 5,714,142 A (BLANEY et al.) 03 February 1998(03.03.1998), see entire document.	1-37
A ✓	SCHMIDT et al. Familial Amyloidotic Polyneuropathy: Domino Liver Transplantation. Journal of Hepatology. 1999, Vol. 30, pp 293-298, see entire document.	1-37

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2003 (14.06.2003)	30 JUN 2003
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer B. Dell Chism Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

Continuation of B. FIELDS SEARCHED Item 3:
STN MEDLINE SCISEARCH CAPLUS USPATFULL BIOSIS
search terms:transthyretin, TTR, prealbumin, PEG, variant, half-life, serum