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

(57) **Abrégé/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.



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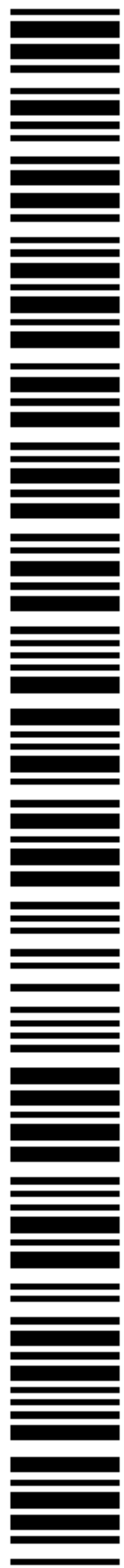
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(54) Title: USE OF TRANSTHYRETIN PEPTIDE/PROTEIN FUSIONS TO INCREASE THE SERUM HALF-LIFE OF PHARMACOLOGICALLY ACTIVE PEPTIDES/PROTEINS

(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.



WO 03/086444 A1

- 1 -

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

5 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

10

 Proteins, peptides and other drug molecules
for therapeutic use are currently available in suitable
forms in adequate quantities largely as a result of the
advances in recombinant DNA technologies. The
15 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
20 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.

25

 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
30 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)
35 disclose PEG:protein conjugates involving, for example,
enzymes and insulin. Shaw (U.S. Patent No. 4,904,584)
disclose the modification of the number of lysine

- 2 -

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

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

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

- 3 -

the protein. Specifically, Blaney et al. demonstrate that covalent attachment of a peptide, protein, nucleotide, oligonucleotide, oligosaccharide or other drug to a transthyretin-selective ligand will
5 reversibly bind the drug to TTR and thereby increase the serum half-life of the agent based on the affinity of the ligand for TTR. It is stated that the intrinsic activity of the drug is not adversely affected and the resulting drug-TTR ligand conjugate will still be small
10 enough to be orally absorbed.

SUMMARY OF THE INVENTION

It has been found, surprisingly and
15 importantly, that TTR (or a TTR variant), and in particular, a TTR or TTR variant which has been chemically modified via conjugation to a water soluble polymer, e.g., can be used as a fusion partner with a biologically active agent to increase the serum half-
20 life of the biologically active agent. Accordingly, the present invention provides a means for increasing the serum half-life of a selected biologically active agent.

The present invention thus relates to
25 substantially homogenous preparations of TTR (or a TTR variant)-biologically active agent fusions and PEG-TTR (PEG-TTR variant)-biologically active agent fusions. As compared to the biologically active agent alone, the TTR-biologically active agent fusion and/or PEG-TTR-
30 biologically active agent fusion has substantially increased serum half-life.

The present invention further relates to
TTR-biologically active agent fusions and PEG-TTR-
biologically active agent fusions, in a
35 pharmaceutically acceptable carrier, to provide a pharmacologically active compound.

- 4 -

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.

10 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

- 5 -

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

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

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

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

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

- 6 -

Figure 6 demonstrates by size exclusion chromatography that native TTR and TTR(C10A) maintain a similar oligomeric configuration (an apparent tetramer). Solid line is native TTR and the dashed
5 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.
10 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).

15 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
20 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
25 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
30 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-
35

- 7 -

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

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

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

- 8 -

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

Figure 13 shows that injection of TMP-TTR
 5 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).

10

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:
 15 ■ 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-
 20 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
 25 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
 30 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).

- 9 -

DETAILED DESCRIPTION OF THE INVENTION

For purposes of describing the present
5 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.
10 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
15 "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
20 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
25 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-
30 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
35 "-agonist peptide" refer to a peptide having biological

- 10 -

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

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

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

- 11 -

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

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

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

- 12 -

identified or derived as described in U.S. Pat. Nos. 5,608,035, 5,786,331, 5,880,096. Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides 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

- 13 -

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

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

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

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

- 14 -

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
5 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
10 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
15 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
20 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
25 reference including figures).

In addition, biologically active agents can also include but are not limited to insulin, gastrin, prolactin, adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), luteinizing hormone (LH),
30 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),

- 15 -

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

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

SEQ ID NO:1

GPTGTGESKCPLMVKVLDVAVRGSPAINVAVHVFRKAADDTWEPFASGKTSESSEL
HGLTTEEEFVEGIYKVEIDTKSYWKALGISPFHEHAEEVFTANDSGPRRYTIAAL
LSPYSYSTTAVVTNPKE

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

- 16 -

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

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

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

- 17 -

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

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

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

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

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

- 18 -

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

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

- 19 -

together to form a gene coding for the full length protein of interest. Usually, the DNA fragment encoding the amino terminus of the polypeptide will have an ATG, which encodes a methionine residue. This 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

- 20 -

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

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

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

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

- 21 -

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

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

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

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

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

- 22 -

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

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

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

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

- 23 -

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

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

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

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

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

- 24 -

expression vector to be used are compatible. Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in practicing this invention in conjunction
5 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
10 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*.

15 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
20 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
25 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
30 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,
35 and that construct can then be ligated to the other fusion partner gene. Ligations are typically

- 25 -

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

A separate procedure provides for first ligating one fusion partner gene into the selected
5 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
10 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
15 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
20 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
25 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
30 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-
35 Hegglin anomaly, Wiskott-Aldrich syndrome, and paroxysmal nocturnal hemoglobinuria. Also, certain

- 26 -

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

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

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

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

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

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

- 27 -

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

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

- 28 -

administration of glucagon-like peptide-1 (GLP-1) has antidiabetic properties. However, GLP-1 is rapidly degraded by dipeptidyl peptidase IV (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 insulintropic effects. Particular examples of such variations known in the art include, for example, GLP-1(7-34), (7-35), (7-36) or (7-37), Gln⁹-GLP-1(7-37), D-Gln⁹-GLP-1(7-37), Thr¹⁶-Lys¹⁸-GLP-1(7-37), and Lys¹⁸-GLP-1(7-37). Additional examples of GLP-1 variants are described in U.S. Patent Nos. 5,118,666, 5,545,618, 5,977,071, and WO 02/46227 and in Adelhorst et al., J. Biol. Chem. 269:6275 (1994), which are incorporated by reference. Accordingly, any GLP-1 peptide can be used to generate fusion proteins of the invention, as long as the GLP-1 fusion protein is capable of binding and inducing a signal through its cognate receptor. Receptor binding and activation can be measured by standard assays (U.S. Patent No. 5,120,712).

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

- 29 -

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

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

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

- 30 -

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

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

- 31 -

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

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

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

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

- 32 -

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

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

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

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

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

- 33 -

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

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

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

- 34 -

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

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

Example 1

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

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

20 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^0 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 .

25 The untransformed host has no antibiotic resistances. The ribosomal binding site of the cI857s7 gene has been modified to include an enhanced RBS. It has been inserted into the *ebg* operon between nucleotide position 1170 and 1411 as numbered in Genbank accession number M64441Gb_Ba with deletion of the intervening *ebg* sequence. The construct was delivered to the

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- 35 -

chromosome using a recombinant phage called MMeBg-cI857s7 enhanced RBS #4 into F'tet/393. After recombination and resolution only the chromosomal insert described above remains in the cell. It was
5 renamed F'tet/GM101. F'tet/GM101 was then modified by the delivery of a lacI⁰ construct into the *ebg* operon between nucleotide position 2493 and 2937 as numbered in the Genbank accession number M64441Gb_Ba with the deletion of the intervening *ebg* sequence. The
10 construct was delivered to the chromosome using a recombinant phage called AGebg-lacI⁰ #5 into F'tet/GM101. After recombination and resolution only the chromosomal insert described above remains in the cell. It was renamed F'tet/GM221. The F'tet episome
15 was cured from the strain using acridine orange at a concentration of 25 µg/ml in LB. The cured strain was identified as tetracycline sensitive and was stored as GM221.

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

PCR was performed with the Expand Long Polymerase according to the manufacturer's protocol (Boehringer Mannheim). PCR products were verified by agarose gel electrophoresis, purified and digested with
30 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
35 England Biolabs). Ligation was done at 4°C for 2 hrs.

- 36 -

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

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

- 37 -

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

Mutant TTR(C10A) was made by using
5 oligonucleotide 2693-80 above and oligonucleotide 2820-88 (encompasses the first 11 codons of native TTR in which the codon Cys at the tenth position was changed to Ala). The PCR procedure and the process for selecting the expression strain were similar to that
10 described above. The resulting strain (strain #5619) had the DNA sequence identified in SEQ ID NO:3.

Plasmid 5619 was further modified by replacing the amino acids at the following positions: A37, D38, A81 and G83, with the amino acid Cysteine.
15 As described below, each pair of the complementary oligonucleotides harboring the desired mutations was used in conjunction with TTR 5' and 3' primers described above in a standard two-step PCR procedure designed for site-specific mutagenesis. Each of the
20 forward primers were used with a TTR 3' primer and each of the reverse primers were used with a TTR 5' primer in a 20-cycle PCR in which plasmid derived from strain 5619 was used as the template. The resulting PCR amplified 5' and 3' fragments were mixed and used as
25 the template for the second step PCR to generate the full-length mutants. Subsequent cloning and sequencing procedures were similar to those already described. The following oligonucleotides were utilized: TTR(A37C) forward (Oligo 2823-91); TTR(A37C) reverse (Oligo 2823-92); TTR(D38C) forward (Oligo 2823-93); TTR(D38C) reverse (Oligo 2823-94); TTR(A81C) forward (Oligo 2823-95); TTR(A81C) reverse (Oligo 2823-96); TTR(G83C) forward (Oligo 2823-97); TTR(G83C) reverse (Oligo 2823-98). The resulting *E. coli* strains containing the
35 plasmids are described as follows:
TTR(C10A/A37C) (strain 5641) had the DNA sequence

- 38 -

identified in SEQ ID NO:4. TTR(C10A/D38C) (strain 5642) had the DNA sequence identified in SEQ ID NO:5. TTR(C10A/A81C) (strain 5643) had the DNA sequence identified in SEQ ID NO:6. TTR(C10A/G83C) (strain 5651) 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
15	2693-79	GAGGAATAACATATGGGTCCAACCTGGTACCGGTGAA	18
	2693-80	CCGCGGATCCTCGAGATTATTCCTTGGGATTGGTGA	19
	2820-88	GAGGAATAACATATGGGTCCAACCTGGTACCGGTGAA	
20		TCCAAGGCTCCT	20
	2823-91	AGAAAGGCTTGTGATGACACCTGG	21
	2823-92	CCAGGTGTCATCACAAGCCTTTCT	22
	2823-93	AGAAAGGCTGCTTGTGACACCTGG	23
	2823-94	CCAGGTGTCACAAGCAGCCTTTCT	24
25	2823-95	TACTGGAAGTGTCTTGGCATCTCC	25
	2823-96	GGAGATGCCAAGACACTTCCAGTA	26
	2823-97	AAGGCACTTTGCATCTCCCCATTC	27
	2823-98	GAATGGGGAGATGCAAAGTGCCTT	28
	2953-67	CTGATGGTTCGAGTTCTAGAT	29
30	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

- 39 -

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
 5 fragment was mixed with plasmid derived from strain 5619 and the mixture was used as a template for oligonucleotide primers 2743-96 and 2693-80 to amplify full-length TMP-TTR. Similar procedures described above were used for cloning and expression. The
 10 resulting strain, TMP-TTR (strain 5513) had the DNA sequence identified in SEQ ID NO:9.

The TMP was then introduced to the N-terminus of strains 5641, 5642, 5643 and 5651, respectively. Plasmid 5513 was digested with Xba1, the resulting
 15 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
 20 orientation for each fusion. The resulting *E. coli* strains containing the plasmids are described as follows: TMP-TTR(C10A/A37C) (strain 5704) had the DNA sequence identified in SEQ ID NO:10. TMP-
 25 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.

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Table 2

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

Example 3

- 40 -

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

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

Table 3

Oligo	Sequence	SEQ ID Number
2694-01	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

- 41 -

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

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

Table 4

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

- 42 -

Example 5

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

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

Table 5

30	Oligo	Sequence	SEQ ID Number
	2693-79	GAGGAATAACATATGGGTCCAACCTGGTACCGGTGAA	39
	2943-47	AATATACTGCAGTGGTGGGAATAGGAG	40
	2943-48	GTCGTCACCAATCCCAAGGAAGGATCAGGATCCGGA	
35		AAACGTCCGCCGGGTTTCTCCCCGCTGTAATC	41
	2943-49	TCGAGATTACAGCGGGGAGAAACCCGGCGGACGTTTT	
		CCGGATCCTGATCCTTCCTTGGGATTGGTGACGACTGCA	42

EXAMPLE 6

- 43 -

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

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

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

- 44 -

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

EXAMPLE 7

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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

- 45 -

Q-sepharose fast flow (5 cm ID) column (Amersham Pharmacia Biotech, Piscataway, NJ) at 20 ml/min equilibrated in Q-Buffer A (20 mM tris HCl, 2.5 mM EDTA, pH 8.0) at room temperature (about 23°C). Column
5 was washed with about 2300 ml Q-Buffer A at 20 ml/min. Q-column was eluted with a 15 column volume linear gradient to 60% Q-Buffer B (20 mM tris HCl, 1 M NaCl, 2.5 mM EDTA, pH 8.0) followed by a 2 column volume step to 100% Q-Buffer B. Fractions containing the TTR
10 fusion as determined by SDS-PAGE were pooled into a single Q-pool (1150 ml) (Figure 1, Lane 4) and 1.77 g of DTT was added. The Q-pool was gently stirred for 30 min at room temperature (about 23°C). To the Q-pool, 410 ml of 3.8 M ammonium sulfate pH 7.0 was slowly
15 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
20 sulfate, pH 7.0) at 10 ml/min. The column was washed with about 170 ml P-Buffer A followed by three step elutions using 50%, 80%, and 100% P-Buffer B (50 mM NaH_2PO_4 , pH 7.0). The remaining half of the Q-pool was then processed using the same protocol as the first
25 half. Fractions containing the TTR fusion as determined by SDS-PAGE were pooled into a single P-pool (260 ml) (Figure 1, Lane 5) and the P-pool was dialyzed against 4 L of HA-Buffer A (10 mM NaH_2PO_4 , pH 7.0) for 2 hours at room temperature (about 23 °C) using 20.4 mm
30 diameter 8 kDa cutoff dialysis tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA). The dialysis buffer was changed with a fresh 4 L of HA-Buffer A and dialysis was continued for approximately an additional 15 hours. The P-pool was removed from dialysis and 600
35 μl of 1 M DTT was added followed by incubation at room

- 46 -

temperature (about 23 °C) for about 1 hour. P-pool was loaded on to a 105 ml (2.6 cm) type 1 ceramic hydroxyapatite column (Bio-Rad Inc., Hercules, CA) at 10 ml/min in HA-Buffer A. Column was washed with 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 NaH₂PO₄, 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* Ameboyocyte 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

- 47 -

This example demonstrates that fusing a peptide to either the C-terminus or N-terminus of TTR(C10A/G83C) does not have a significant impact on its oligomeric structure. TTR(C10A/G83C), PTH-
5 TTR(C10A/K15A/G83C), and TTR(C10A/G83C)-Bradykinin in 20 mM tris pH 8.0 and about 250 mM NaCl were reduced with 9 mM DTT for about 1 hour at room temperature (about 23 °C). About 50 µg of the reduced TTR was injected on to a Biosep-Sec-S 3000 column (7.8 mm ID X
10 300 mm) (Phenomenex, Torrance, CA) in SEC-Buffer (50 mM NaH₂PO₄, 500 mM NaCl, pH 6.7) at 1 ml/min. Bio-Rad molecular weight standards (151-1901) were used to calibrate the column and calculate the approximate molecular size of the injected samples. As can be seen
15 in Figure 2, TTR(C10A/G83C) eluted at approximately 8.8 min corresponding to a molecular size of 49 kDa, which is comparable to the calculated molecular weight of the tetramer at 55 kDa. PTH-TTR(C10A/K15A/G83C) eluted at about 8.6 min corresponding to a molecular size of 67
20 kDa, which is close to the calculated 71 kDa for the tetramer. TTR(C10A/G83C)-Bradykinin eluted at about 8.7 min corresponding to a molecular size of 57 kDa, which is also close to the calculated 60 kDa for the tetramer.

25

EXAMPLE 9

This example demonstrates that fusing a protein containing disulfide bonds to either the C-
30 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-
35 Buffer at 1 ml/min. Bio-Rad molecular weight standards

- 48 -

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

EXAMPLE 10

20

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

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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 μ l/min in Dulbecco's
35 PBS (Gibco BRL, Gaithersburg, MD) with 0.1 mg/ml bovine serum albumin and 0.005% P20 (polyoxyethylenesorbitan).

- 49 -

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
5 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
10 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.

15

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,
20 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
25 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
30 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 4.3×10^{12} platelets L^{-1} on day 5, which is over 3.4 times baseline at 1.2×10^{12} platelets L^{-1} .
35 TMP(m)-TTR-PEG 5K was a moderate responder peaking at

- 50 -

2.3 X 10¹² 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 X 10¹² 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

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- 51 -

This example demonstrates that mutation of alanine 37 to cysteine TMP-TTR(C10A/A37C), aspartate 38 to cysteine TMP-TTR(C10A/D38C), alanine 81 to cysteine TMP-TTR(C10A/A81C), or glycine 83 to cysteine TMP-TTR(C10A/G83C) in a cysteine 10 to alanine background does not have a significant impact on the oligomeric structure of TTR. In addition, this example demonstrates that pegylation of these mutant forms of TTR with a 5K or 20K PEG produces two distinct species of TTR with significantly greater molecular size than the unpegylated form. The pegylation of TTR was carried out by first reducing about 8 ml of the TTR (7.28 mg/ml) with 10 mM DTT for 30 minutes at 30 °C in the presence of 50 mM tris HCl, pH 8.5. The reduced TTR was then desalted using a 26 ml SEPHADEX™ G25 medium column (2.6 cm ID) (Amersham Pharmacia Biotech) at 2.5 ml/min in 20 mM tris HCl, pH 8.5. The concentration was then determined by measuring the absorbance of the reduced TTR at 280 nm and using the calculated extinction coefficient ($29,450 \text{ M}^{-1}$ 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

- 52 -

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

30 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 35 very little unmodified monomer.

- 53 -

EXAMPLE 14

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

25

EXAMPLE 15

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

- 54 -

Mice were anesthetized with isoflurane prior to collection.

The collected blood was analyzed for a complete and differential count using an ADVIA 120 automated blood analyzer with murine software (Bayer Diagnostics, New York, NY). As seen in Figure 10A, Fc-TMP showed the greatest response with platelet count rising to over 4.2×10^{12} platelets L^{-1} on day 5 which is 3 times baseline at 1.4×10^{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 2.9×10^{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 4.4×10^{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 4.6×10^{12} platelets L^{-1} (3.2 to 3.5 times baseline). Since all of the TMP fusion constructs had similar binding affinities for MPL *in vitro*, this difference is likely due to the effect of PEG conjugation increasing the effective biological half-life of the construct.

EXAMPLE 16

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

- 55 -

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

The collected blood was analyzed for ionized calcium using a Ciba*Corning 634 Ca⁺⁺/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
TGGAACGTGTTGAATGGCTGCGTAAGAACTGCAGGACGTTTCATAACTTTGGTCC
AACTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAGATGCTGTC
CGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGCTGCTGATG

- 56 -

ACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCTGCATGG
 GCTCACAAGTGAAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATAGACACC
 AAATCTTACTGGAAGTGTCTTGGCATCTCCCCATTCCATGAGCATGCAGAGGTGG
 TATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCTGCTGAG
 5 CCCCTACTCCTATTCCACCACGGCTGTCTGTCACCAATCCCAAGGAATAA

EXAMPLE 18

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

15 The bacterial host GM121 is an *E. coli* K-12 strain
 that has been modified to contain the *lacI*⁰ repressor
 in the late *ebg* region (68 minutes). The presence of
 this repressor gene allows the use of this host with a
 variety of expression systems, however this repressor
 20 is irrelevant to the expression from *luxPR*. The
 untransformed host has no antibiotic resistances.
 Specifically, F'tet/393 was modified by the delivery of
 a *lacI*⁰ construct into the *ebg* operon between
 nucleotide position 2493 and 2937 as numbered in the
 25 Genbank accession number M64441Gb_Ba with the deletion
 of the intervening *ebg* sequence. The construct was
 delivered to the chromosome using a recombinant phage
 called AGebg-*lacI*⁰ #5.

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

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

- 57 -

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

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

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

- 58 -

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

To prepare the GLP-1-TTR(C10A/K15A/G83C) fusion, two oligonucleotides, oligonucleotide 3183-83, which contains an NdeI site and encodes the purification and cleavage sequence described above plus the first six amino acids of GLP-1(A2G), and 3183-84, which encodes the last 6 amino acids of the fusion 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.

25 Table 6

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

SEQ ID NO:48

ATGAAACATCATCACCATCACCATCATGACGAAGTTGATCACGGTGAAGGTACTT
TCACTTCTGACGTTTCTTCTTATCTGGAAGGTCAGGCTGCTAAAGAATTCATCGC
TTGGCTGGTTAAAGGTCGTGGTGGTTCTGGTTCTGCTACTGGTGGTTCGGCTCC
40 ACCGCAAGCTCTGGTTCAGGCAGTGCGGGTGGTGGTGGTGGTGGTCCAAGT
GTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAGATGCTGTCCGAGG

5 CAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGCTGCTGATGACACC
TGGGAGCCATTTGCCCTCTGGGAAAACCAAGTGAGTCTGGAGAGCTGCATGGGCTCA
CAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAATAGACACCAAATC
TTACTGGAAGGCACTTTGCATCTCCCCATTCCATGAGCATGCAGAGGTGGTATTC
ACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCTGCTGAGCCCCT
ACTCCTATTCCACCACGGCTGTTCGTCACCAATCCCAAGGAATAA

EXAMPLE 19

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

- 60 -

contains and NdeI site and encodes the purification and cleavage sequence described above plus the first eight amino acids of GLP-1(A2G) , and 2687-50, which encodes the amino acids 18 through 23 of the Fc, were

5 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

10 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

15 insert introduced. The resultant strain containing the new plasmid was designated GLP-1(A2G)-K-Fc (strain 5945) and had the DNA sequence identified in SEQ ID NO:51.

Table 7

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

SEQ ID NO: 52

ATGAAACATCATCACCATCACCATCATGACGAAGTTGATCACGGTGAAGGTACTT
TCACTTCTGACGTTTCTTCTTATCTGGAAGGTCAGGCTGCTAAAGAATTCATCGC

30 TTGGCTGGTTAAAGGTCGTGGTGGTTCTGGTTCTGCTACTGGTGGTTCCGGCTCC
ACCGCAAGCTCTGGTTCAGGCAGTGCGACTCATGGTGGTGGTGGTGGTGACAAAA
CTCACACATGTCCACCGTGCCCGAGCACCTGAACTCCTGGGGGGACCGTCAGTTTT
CCTCTTCCCCCCTAAACCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTC
ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGT

35 ACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTA
CAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCTGCACCAGGACTGGCTG
AATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCG
AGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCT
GCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTC

40 AAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGG
AGAACAACACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCT
CTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCA
TGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCC
TGTCTCCGGGTAAA

- 61 -

EXAMPLE 20

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

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

2973-77:

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

2973-78:

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

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

2973-79:

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

followed by Nde1/XhoI digest and cloning into
25 similarly restricted pAMG21. The resulting plasmid is
described as 6017 (TTRC10A-CH2):

SEQ ID NO:56:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAG
30 ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC
TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCATGAGCATGC
AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
35 CTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
GTTCTGGCTCCGGATCAGGGGGACCGTCAGTTTTCCTCTTCCCCCAAACCCAA
GGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTG
AGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGC
ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGT
40 CAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGC

- 62 -

AAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCA
AAGGGGGTTCTGGATCCTAA

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

SEQ ID NO:57:

10 ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC
AGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAGAT
GCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGCTG
CTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAAGTGAGTCTGGAGAGCT
GCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATA
15 GACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGCAG
AGGTGGTATTACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT
GCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAGGT
TCTGGCTCCGGATCAGGGGGACCGTCAGTTTTCTCTTCCCCCCTTCCCCCAAAACCCAAGG
ACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAG
20 CCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCAT
AATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCA
GCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAA
GGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAA
GGGGGTTCTGGATCCTAA
25

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

30 2974-70:

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

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

SEQ ID NO:59:

40 ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTTCTAG
ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC
TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAAGTGAGTCTGGAGAG
CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA

- 63 -

TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC
 AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
 CTGCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
 GTTCTGGCTCCGGATCAGGGGGACCGTCAGTTTTCCTCTTCCCCCCTTCCCAA
 5 GGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTG
 AGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGC
 ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGT
 CAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGC
 AAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCA
 10 AAGGGGGTTCTGGATCCATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCG
 TGCTGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGG
 CTTGCAGCACGCGCATAA

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

20

2974-65:

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

2974-66:

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

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

SEQ ID NO:62:

35 ATGGGATCCGGTTCTGGGGGACCGTCAGTTTTCCTCTTCCCCCCTTCCCAAAGG
 ACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAG
 CCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCAT
 AATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCA
 GCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAA
 GGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAA
 40 GGGGGTTCTGGTCCAAGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAG
 TTCTAGATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCA
 AAAGGCTGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAAGTGA
 GGAGAGCTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAG

- 64 -

TGGAAATAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGA
GCATGCAGAGGTGGTATTACAGCCAACGACTCCGGCCCCCGCCGCTACACCATT
GCCGCCCTGCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCA
AGGAATAA

5

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

10

2974-68:

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

2974-69:

15 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):

20

SEQ ID NO:65:

ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC
AGGATCCGGTTCTGGGGGACCGTCAGTTTTCCTCTTCCCCCAAAACCCAAGGAC
25 ACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCC
ACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAA
TGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGC
GTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGG
TCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAGCCAAAGG
30 GGGTTCTGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCAAAGTT
CTAGATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAA
AGGCTGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAAGTGAGTCTGG
AGAGCTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTG
GAAATAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGC
35 ATGCAGAGGTGGTATTACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGC
CGCCCTGCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAG
GAATAA

EXAMPLE 21

40

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

- 65 -

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

2694-18:

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

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

2694 - 19:

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

ATT G (SEQ ID NO:67).

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

20 SEQ ID NO:68:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGTGTCTCTGATGGTCAAAGTTCTAG
 ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC
 TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAAGTGAGTCTGGAGAG
 CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
 25 TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC
 AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
 CTGCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
 GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
 TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT
 30 GCAGCACGCGCATAA

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

SEQ ID NO:69:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAG
 ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC
 TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAAGTGAGTCTGGAGAG
 40 CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
 TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC

- 66 -

AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTTGCCGCC
 CTGCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
 GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
 TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT
 5 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
 10 described in example 1, except that the TTR 3' oligo
 utilized with the mutation oligos (2693-80) in example
 1 was replaced with 2694-19, resulting in Strain 5982,
 containing TTRC10A/K15A/A37C-TMP (SEQ ID NO:70), Strain
 5983 containing TTRC10A/K15A/A81C-TMP (SEQ ID NO:71),
 15 and Strain 5984 containing TTRC10A/K15A/G83C-TMP (SEQ
 ID NO:72).

SEQ ID NO:70:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAG
 ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC
 20 TTGTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
 CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
 TAGACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGC
 AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTTGCCGCC
 CTGCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
 25 GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
 TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT
 GCAGCACGCGCATAA

SEQ ID NO:71:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAG
 ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC
 TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
 CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
 TAGACACCAAATCTTACTGGAAGTGTCTTGGCATCTCCCCATTCCATGAGCATGC
 35 AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTTGCCGCC
 CTGCTGAGCCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
 GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
 TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTT
 GCAGCACGCGCATAA
 40

SEQ ID NO:72:

ATGGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTCGCAGTTCTAG
 ATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGC
 TGCTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAG
 45 CTGCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAA
 TAGACACCAAATCTTACTGGAAGGCACTTTGCATCTCCCCATTCCATGAGCATGC

- 67 -

AGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCC
CTGCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAAG
GTTCTGGTTCTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGC
TGGCGGTGGTGGCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAGTGGCTT
5 GCAGCACGCGCATAA

EXAMPLE 22

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

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

ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
30 GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC
AGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTTCGAGTTCTAGAT
GCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGCTG
CTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCT
GCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATA
35 GACACCAAATCTTACTGGAAGGCACTTTGCATCTCCCATTCATGAGCATGCAG
AGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT
GCTGAGCCCCTACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATAA

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

40 ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
GCGGAGGGGGTGGCATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCACGCGC

- 68 -

AGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTTCGCAGTTCTAGAT
 GCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGCTG
 CTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCT
 GCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAATA
 5 GACACCAAATCTTACTGGAAGTGTCTTGGCATCTCCCCATTCCATGAGCATGCAG
 AGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT
 GCTGAGCCCCTACTCCTATTCCACCACGGCTGTTCGTACCAATCCCAAGGAATAA

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

10 ATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTGGCGGTGGTG
 GCGGAGGGGGTGGCATTTGAGGGCCCAACCCCTTCGCCAATGGCTTGCAGCACGCGC
 AGGTCCAACCTGGTACCGGTGAATCCAAGGCTCCTCTGATGGTTCGCAGTTCTAGAT
 GCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTTCAGAAAGGCTT
 GTGATGACACCTGGGAGCCATTTGCCTCTGGGAAAACCAGTGAGTCTGGAGAGCT
 15 GCATGGGCTCACAACCTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAATA
 GACACCAAATCTTACTGGAAGGCACTTGGCATCTCCCCATTCCATGAGCATGCAG
 AGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCT
 GCTGAGCCCCTACTCCTATTCCACCACGGCTGTTCGTACCAATCCCAAGGAATAA

20 EXAMPLE 23

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

EXAMPLE 24

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

- 69 -

clone 5933 stored at -80°C was defrosted in 1480 ml of 50 mM tris HCl, 5 mM EDTA, pH 8.0. 60 tablets of Sigma protease inhibitor cocktail 1-873-580 (Saint Louis, MO) was dissolved in the cell suspension and the suspension
5 was passed through a model 110-Y microfluidizer (Microfluidics, Newton, MA) twice at 14,000 PSI. The lysate was centrifuged at $11,325 \times g$ for 50 min 4°C . The supernatant was removed as the soluble fraction. The soluble fraction was heated in a 65°C water bath
10 for 30 minutes in polypropylene bottles, at which time the temperature of the contents was 63°C . The soluble fraction was centrifuged at $11,325 \times g$ for 50 minutes 4°C . The supernatant was removed as Heat Soluble. The heat soluble fraction was filtered through a $0.45 \mu\text{m}$
15 cellulose acetate filter with two prefilters and then loaded on to a 240 ml Q-sepharose fast flow (5 cm ID) column (Amersham Pharmacia Biotech, Piscataway, NJ) at 25 ml/min equilibrated in Q-Buffer A (20 mM tris HCl, 2.5 mM EDTA, pH 8.0) at room temperature (about 23°C).
20 Column was washed with about 2200 ml Q-Buffer A at 30 ml/min. Q-column was eluted with a 15 column volume linear gradient to 60% Q-Buffer B (20 mM tris HCl, 1 M NaCl, 2.5 mM EDTA, pH 8.0) followed by a 2 column volume step to 100% Q-Buffer B. Fractions containing
25 the TTR fusion as determined by SDS-PAGE were pooled into a single Q-pool (1300 ml). To the Q-pool, 464 ml of 3.8 M ammonium sulfate pH 7.2 was slowly added. The solution was centrifuged at $11,325 \times g$ for 50 min 4°C . The supernatant was removed as the ammonium
30 sulfate soluble fraction and discarded, and the pellet was resuspended in 450 ml 10 mM NaH_2PO_4 , pH 7.0 by gentle agitation at room temperature for about 30 min. The solution was centrifuged at $11,325 \times g$ for 50 min 4°C . Supernatant was removed as phosphate buffer

- 70 -

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 $\text{M}^{-1}\text{cm}^{-1}$. The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Falmouth, MA). The nucleic acid content was determined to be negligible, since the ratio of the absorbance at 260 nm over 280 nm was determined to be 0.61.

EXAMPLE 25

- 71 -

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

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

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

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

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

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

- 72 -

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

EXAMPLE 26

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

- 73 -

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

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

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

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

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

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

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

35 Millipore CENTRIPREP™ 10 and then passed through a Pall

- 74 -

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

10

EXAMPLE 27

This example describes the purification of GLP1-TTR (C10A/K15A/G83C). About 30 g of *E. coli* paste from clone 6450 stored at -80°C was defrosted in 250 ml of 50 mM NaH₂PO₄, pH 7.0. Cell suspension was passed through a microfluidizer (Microfluidics, Newton, MA) twice at 12,000 PSI. The lysate was centrifuged at 15,344 x g for 50 min 4°C. The supernatant was discarded as the soluble fraction, and the pellet was resuspended in 200 ml deoxycholate using a tissue grinder. The suspension was centrifuged at 15,344 x g for 50 min 4°C. The supernatant was discarded as the wash, and the pellet was resuspended in 200 ml water using a tissue grinder. The suspension was centrifuged at 15,344 x g for 50 min 4°C. The supernatant was discarded as the wash, and the pellet was resuspended in 100 ml water using a tissue grinder. The suspension was centrifuged at 27,216 x g for 30 min room temperature. The supernatant was discarded as the wash, and about 2/3 of the pellets were dissolved in 75 ml 8 M guanidine HCl, 50 mM tris HCl, pH 8.0 by agitation for about 15 min. The suspension was centrifuged at 27,216 x g for 30 min room temperature, and the supernatant was diluted with 18 ml water.

- 75 -

Sample was then loaded on to a 50 ml chelating
sepharose fast flow column (Amersham Pharmacia Biotech,
Piscataway, NJ), loaded with NiCl_2 , at 5 ml/min. After
washing with about 150 ml Ni-Buffer A (6 M guanidine
5 HCl, 37.5 mM tris HCl, pH 8.0) at 10 ml/min, eluted
with two step of 10% and 100% Ni-Buffer B (6 M
guanidine HCl, 37.5 mM tris HCl, 400 mM imidazole, pH
8.0). Combined the peak containing the fusion
construct as Ni-Pool (40 ml) and determined the protein
10 content to be 6.4 mg/ml by observing the absorbance at
280 nm in 8 M guanidine HCl using an extinction
coefficient of $25,440 \text{ M}^{-1}$. Added 800 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
15 min at 30 °C. Loaded on to a 130 ml SEPHADEX™ G25
medium column (2.6 cm ID) (Amersham Pharmacia Biotech,
Piscataway, NJ) at 8 ml/min in 20 mM tris HCl, 6 M
urea, pH 8.5. Pooled the protein peak as determined by
absorbance at 280 nm (22 ml) and determined the
20 concentration to be 3.2 mg/ml by observing the
absorbance at 280 nm in 20 mM tris HCl, 6 M urea, pH
8.5 using an extinction coefficient of $25,440 \text{ M}^{-1}$.
Reacted 45% of the buffer exchanged material with 950
 μl of 5 mM methoxy-PEG-maleimide 5K (Shearwater
25 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
30 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

- 76 -

a 5 ml HiTrap Q-sepharose HP column (Amersham Pharmacia Biotech, Piscataway, NJ) at 1 ml/min in 20 mM tris HCl pH 8.0 followed by about a 15 ml wash in the same buffer. Column was then developed at 5 ml/min using a
5 linear gradient to 60% 20 mM tris HCl, 1 M NaCl, pH 8.0 followed by a step to 100% of the elution buffer. Fractions containing the TTR fusion as determined by SDS-PAGE were pooled into a single Q-pool (9.5 ml). Concentrated Q-Pool to 3.2 ml using a Millipore
10 CENTRIPREP™ 30 kDa and filtered through a Pall MUSTANG™ E membrane at about 1 ml/min. Diluted Q-Pool to 6.5 ml with water and added 375 µl acetonitrile. Injected on to a Vydac Protein/Peptide 10 X 250 mm C₄ column (Vydac, Hisperia, CA) in 95% RP-Buffer A (0.1%
15 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
20 ml using 20 mM tris HCl pH 8.0. Repeated buffer exchange 3 more times then passed through a Pall MUSTANG™ E membrane at about 1 ml/min. The protein concentration was determined to be 7.7 mg/ml using a calculated extinction coefficient of 25,440 M⁻¹ cm⁻¹.
25 The pyrogen level was determined to be <1 EU/mg of protein using the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Falmouth, MA). The nucleic acid content was determined to be negligible, since the ratio of the absorbance at 260 nm over 280 nm was
30 determined to be 0.54.

EXAMPLE 28

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

- 77 -

week-old mice were split into 4 groups and injected (hour 0) intraperitoneal with 7.4 - 16.6 mg test protein per animal (538 pmol monomers for all groups) (5K pegylated GLP1-TTR fusion construct 10 mg, 20K
5 pegylated GLP1-TTR fusion construct 10 mg, GLP1-Fc 16.6 mg, and a TTR(C10A) control 7.4 mg). Each group was bled at time points 0(baseline measurement), 1, 4, 6, 12, 24, and 48 hours post injection. Food was withheld
10 and replaced after the bleed at the 6 hour time point.

Each collected drop of blood per time point was analyzed for glucose content using a One Touch Profile glucose meter and the results are depicted in Figure 12.

15

EXAMPLE 29

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

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

- 78 -

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

5

EXAMPLE 30

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

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

EXAMPLE 31

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

- 79 -

batches (PEG 20K in one and the PEG 5K and non-pegylated samples in the other) completed at separate times with repeated controls). Each group was divided in half and bled (140 ml) on alternate time points (day 5 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 10 Diagnostics, New York, NY). As seen in Figure 15A, the two non-pegylated constructs outperformed the baseline (1.3×10^{12} platelets L-1) with platelet responses at day 5 rising between 1.8×10^{12} and 2.0×10^{12} platelets L-1. As seen in Figure 15B, Fc-TMP and the three 5K 15 pegylated fusions showed equivalent responses at day 5 with platelet counts rising between 3.5×10^{12} and 4.4×10^{12} platelets L-1 which is at least 2.7 times baseline (1.3×10^{12} platelets L-1). As seen in Figure 15C, Fc-TMP and the three 20K pegylated fusions showed 20 equivalent responses at day 5 with platelet count rising between 4.3×10^{12} and 4.6×10^{12} platelets L-1 which is over three times baseline at 1.3×10^{12} platelets L-1.

In addition, the 20K pegylated TTR constructs 25 appear to have an improved sustained response with platelets at day 7 ranging from 3.7×10^{12} to 4.9×10^{12} platelets L-1 compared to Fc-TMP at 3.1×10^{12} platelets L-1. This sustained response is maintained at day 10 for the three 20K pegylated TTR constructs with 30 platelets ranging from 2.3×10^{12} to 3.1×10^{12} platelets L-1 compared to Fc-TMP at 2.0×10^{12} platelets L-1.

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- 80 -

WHAT IS CLAIMED IS:

1. A method for increasing the serum half-life of a biologically active agent comprising fusing
5 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
10 selected from the group consisting of dextran, poly(n-vinyl pyrrolidone), polyethylene glycols, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohols.

15

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

20

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
25 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.

30

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
35 biologically active agent is a protein.

- 81 -

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).

10

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

15

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

20

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.

25

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.

30

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

35

- 82 -

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

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

10 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.
15

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

24. The preparation of any of claims 10-23
20 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
25 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
30 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
35 biologically active agent to provide a TTR variant-

- 83 -

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.

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

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

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

- 84 -

therapeutically effective dose of a preparation of claim 17.

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

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

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

15 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
20 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.

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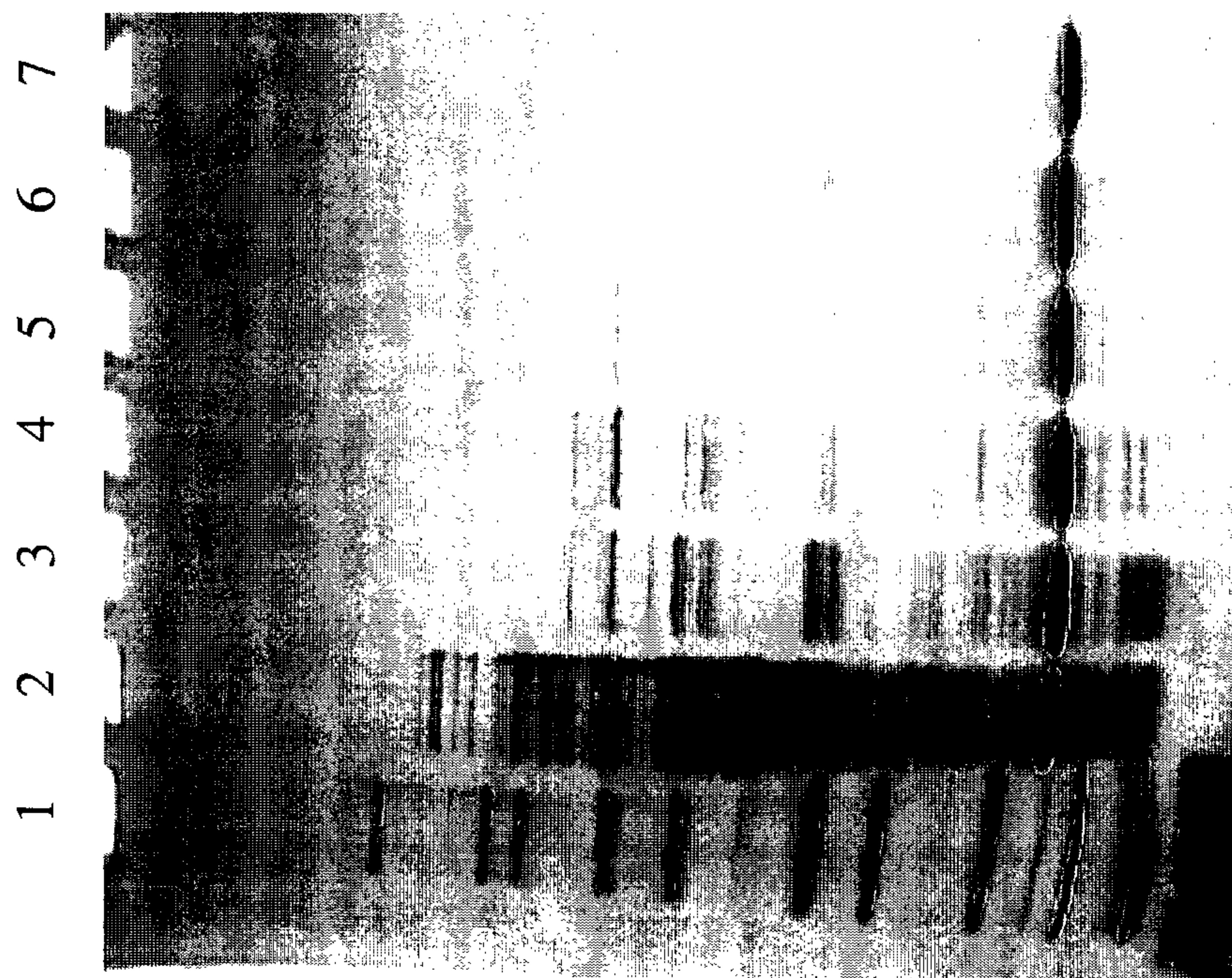
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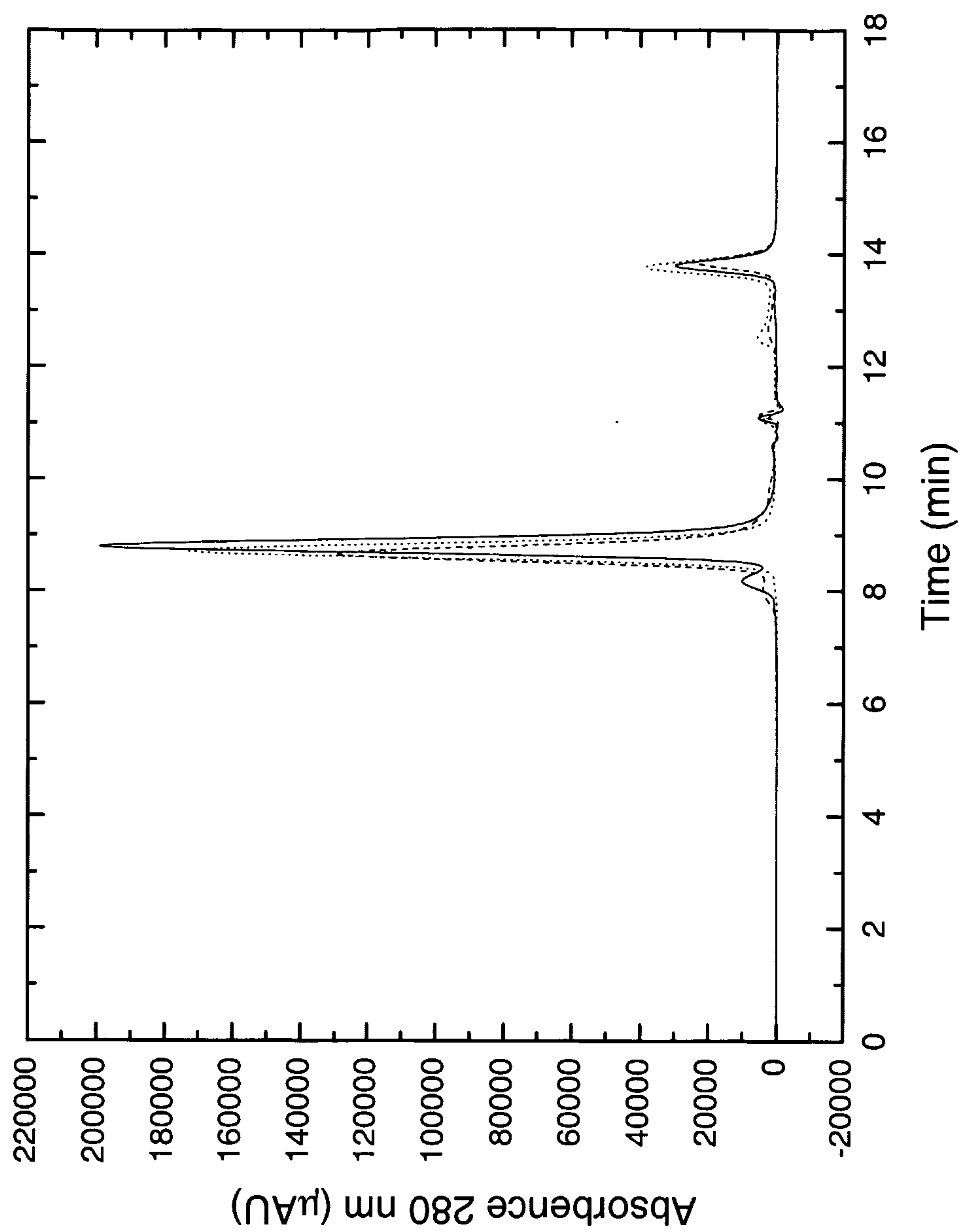
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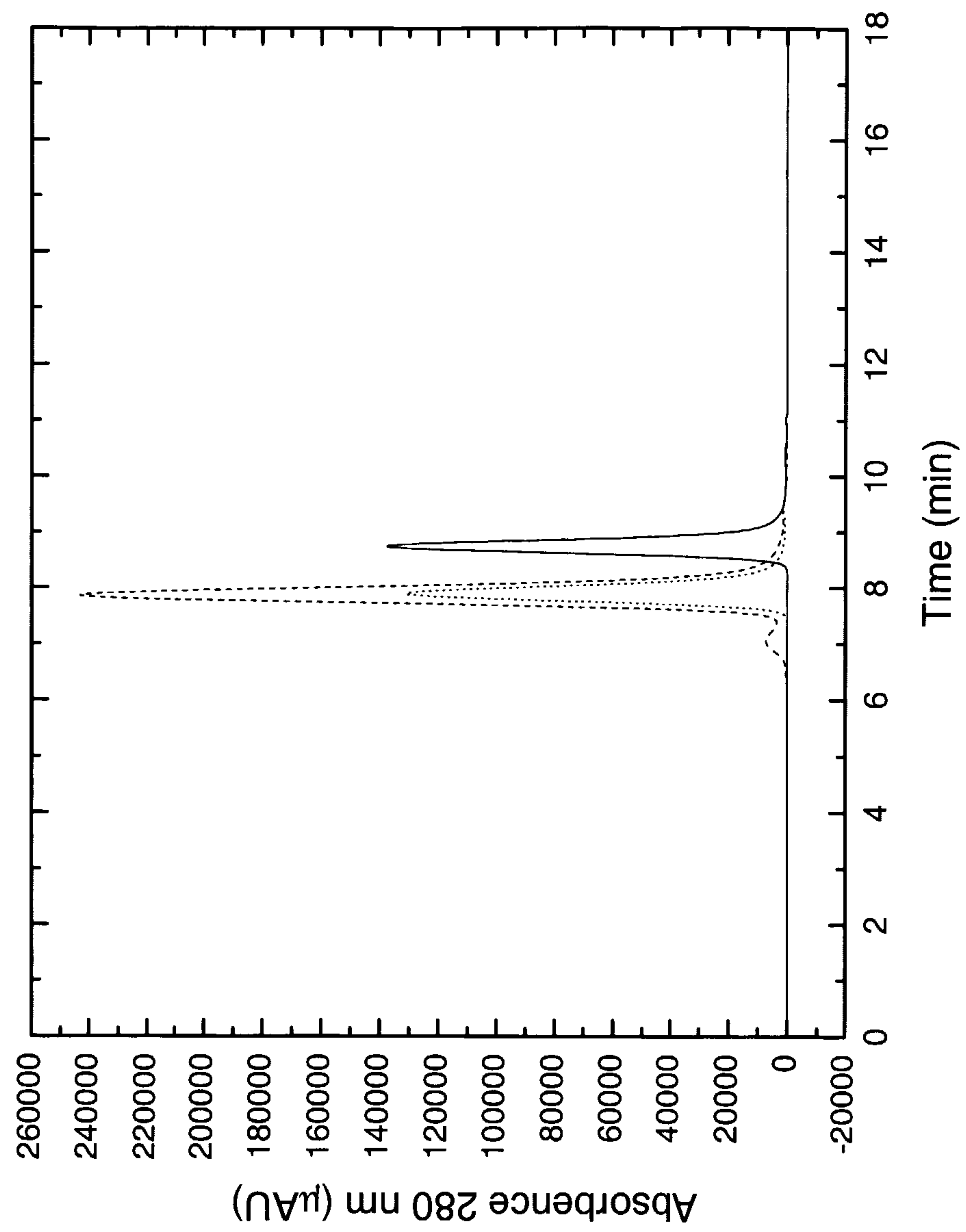
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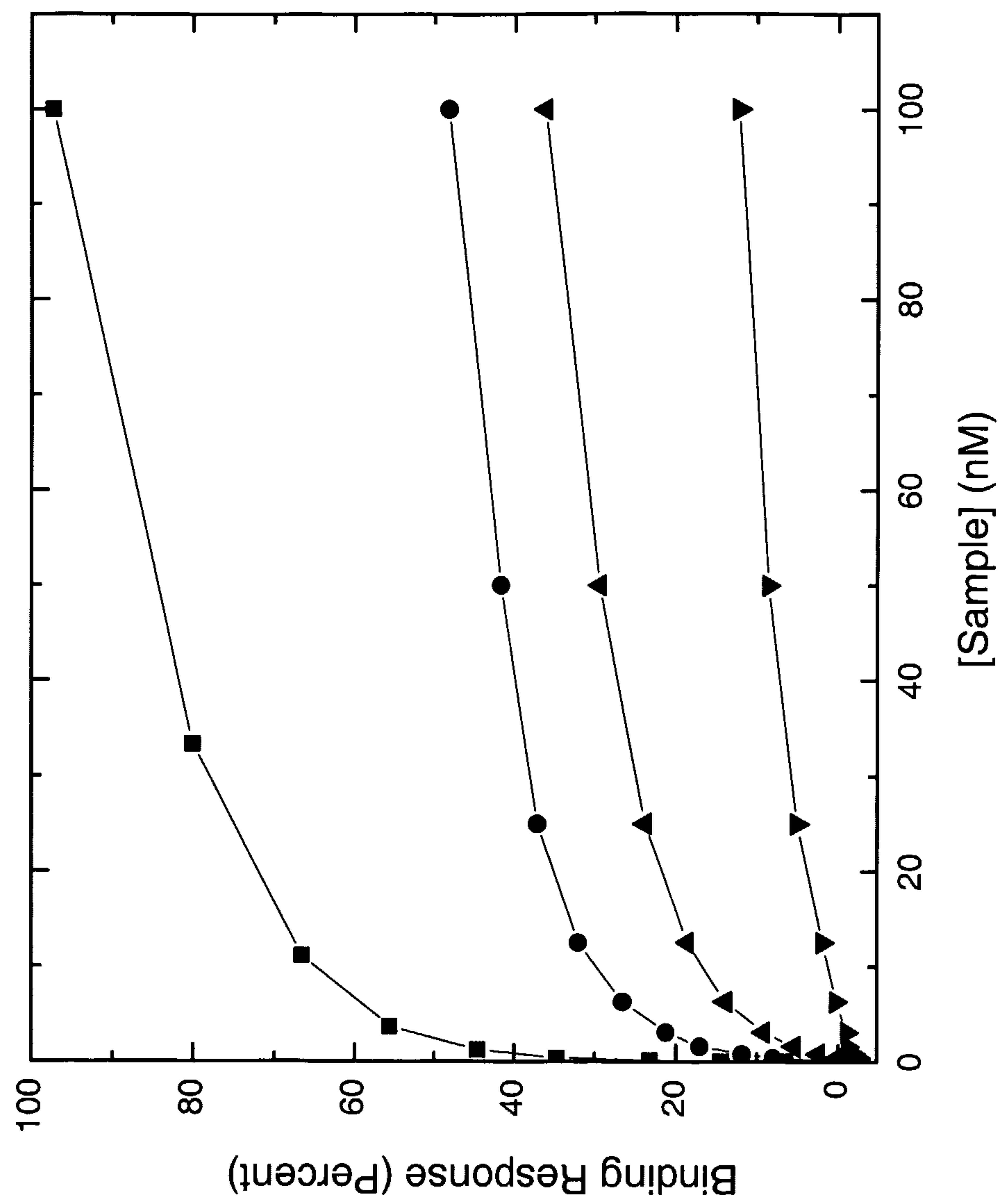
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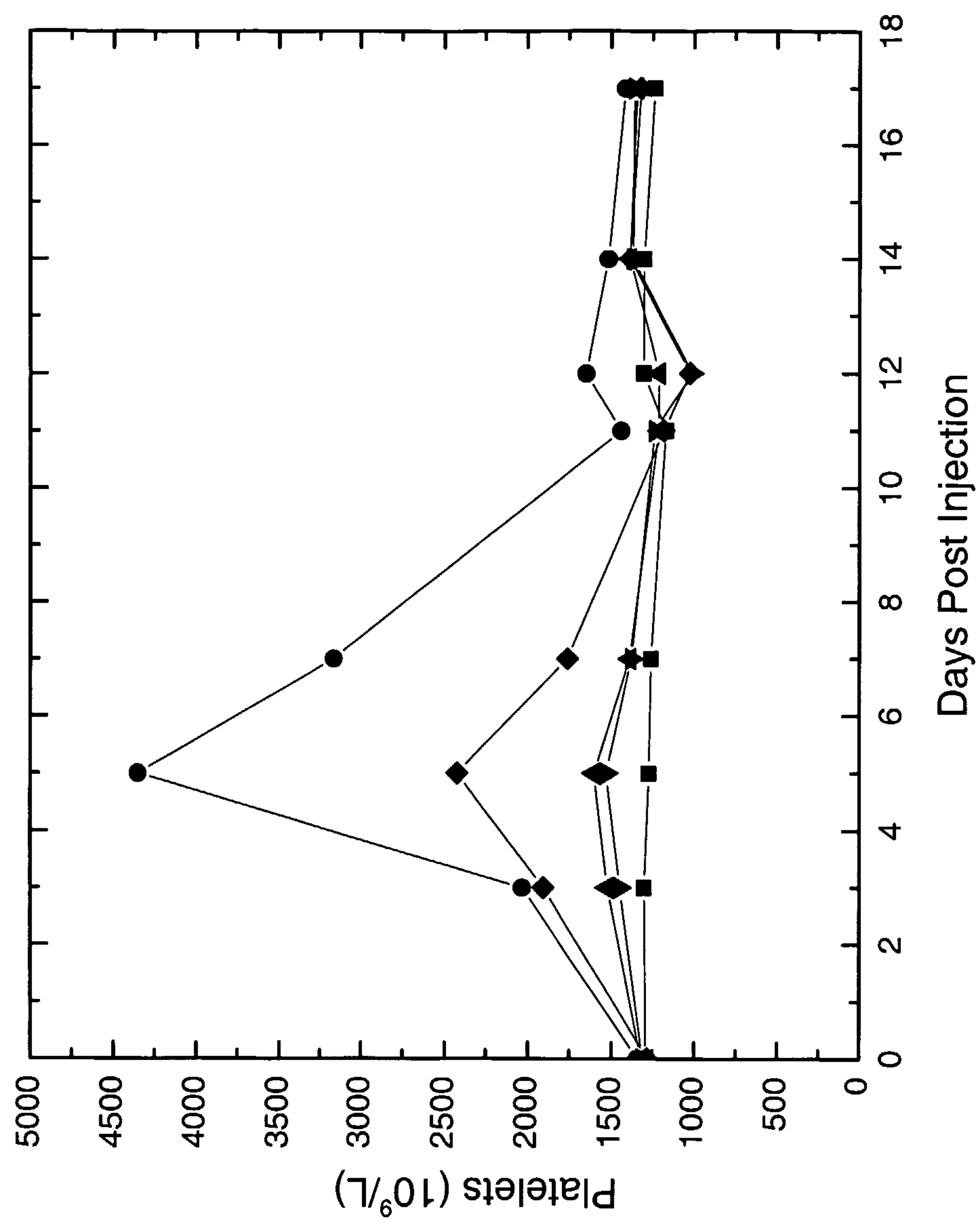


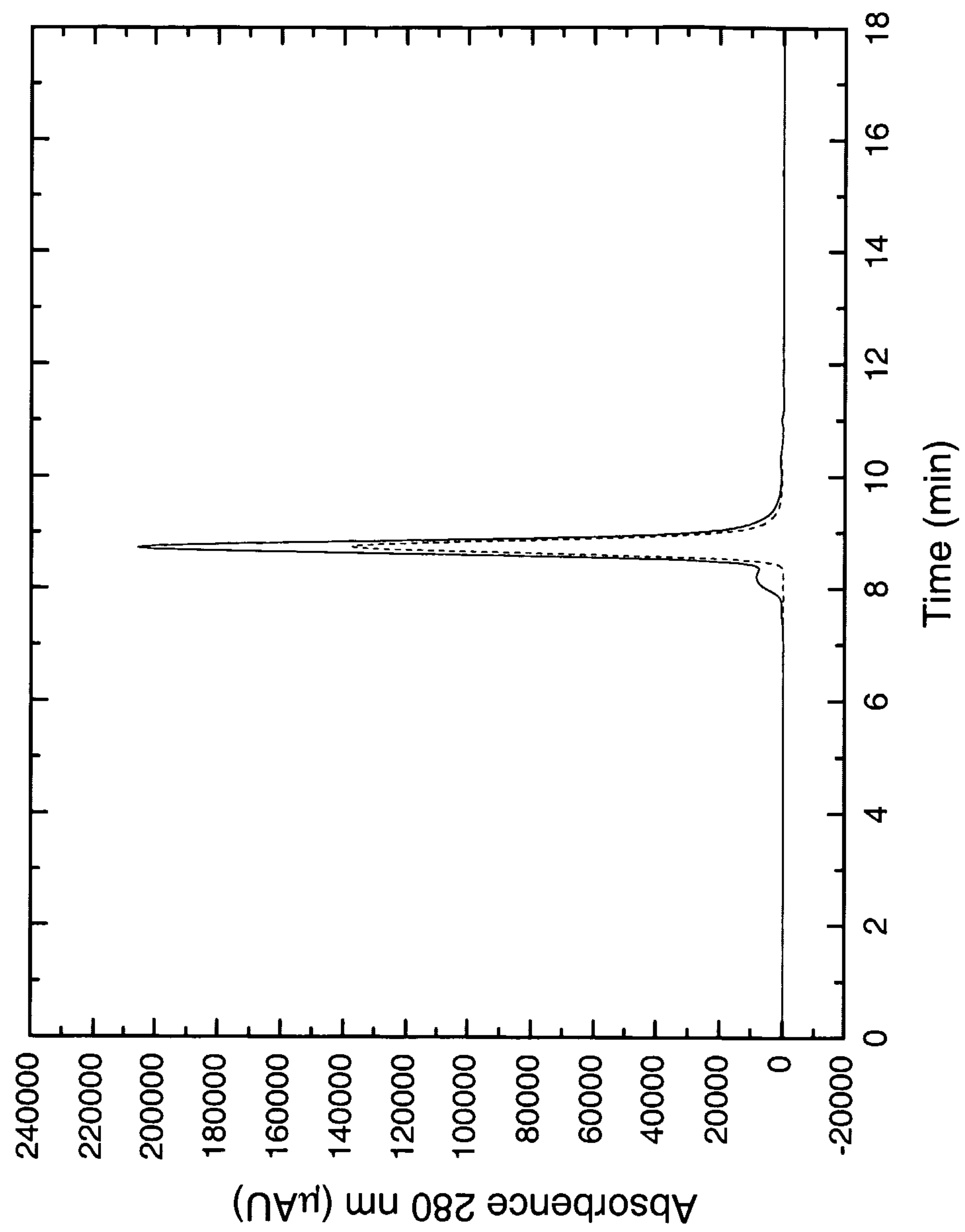
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Figure 7

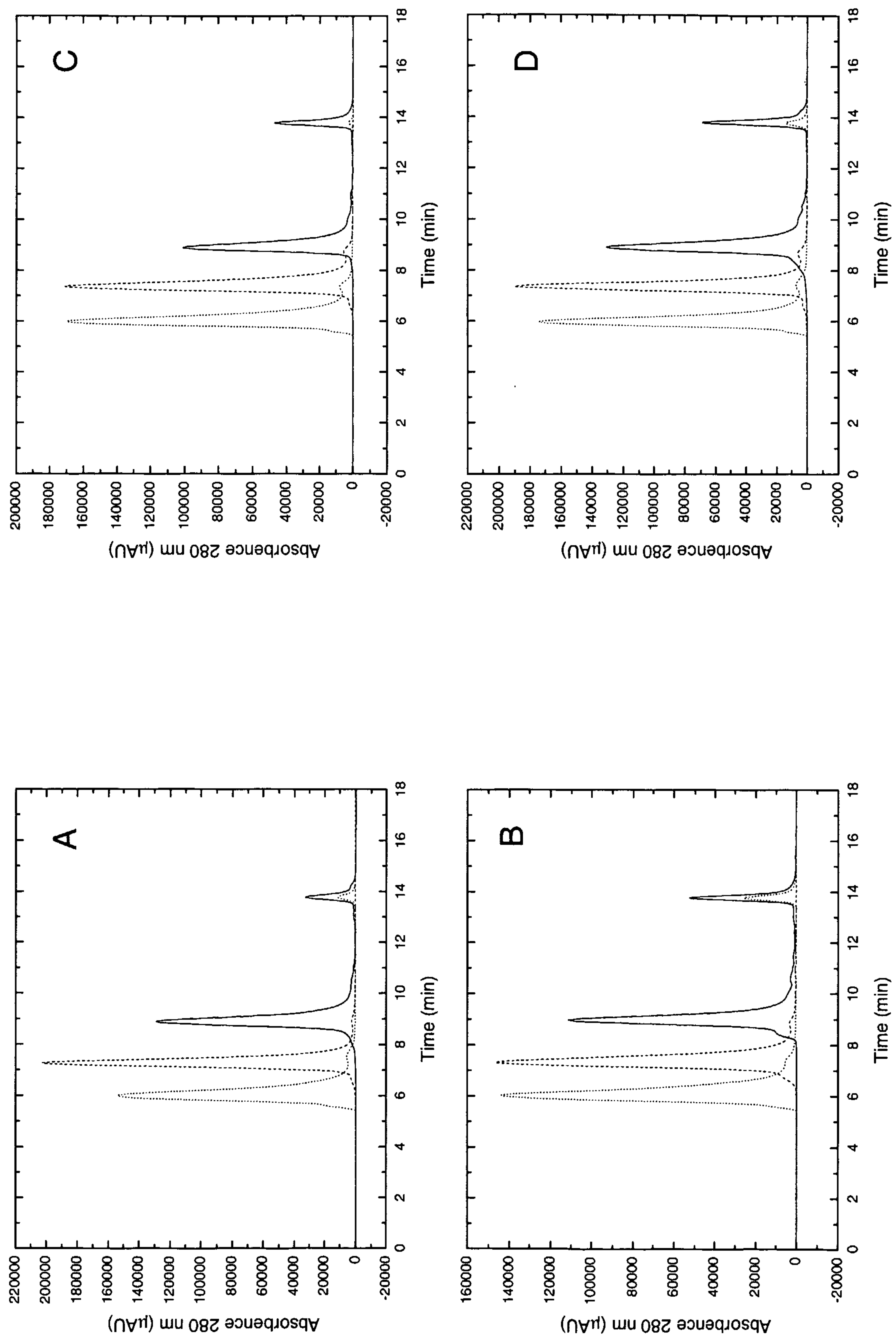


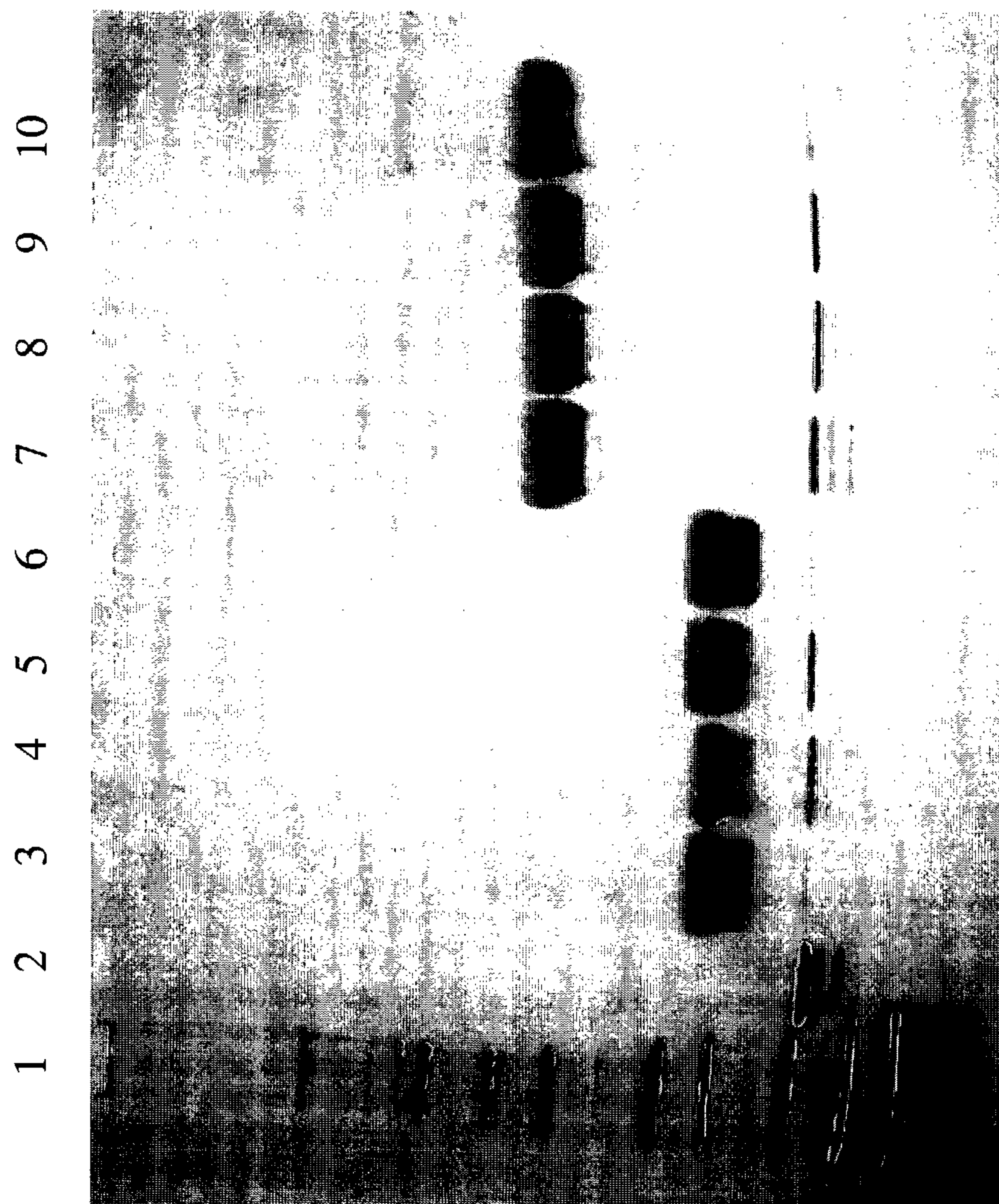
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Figure 9

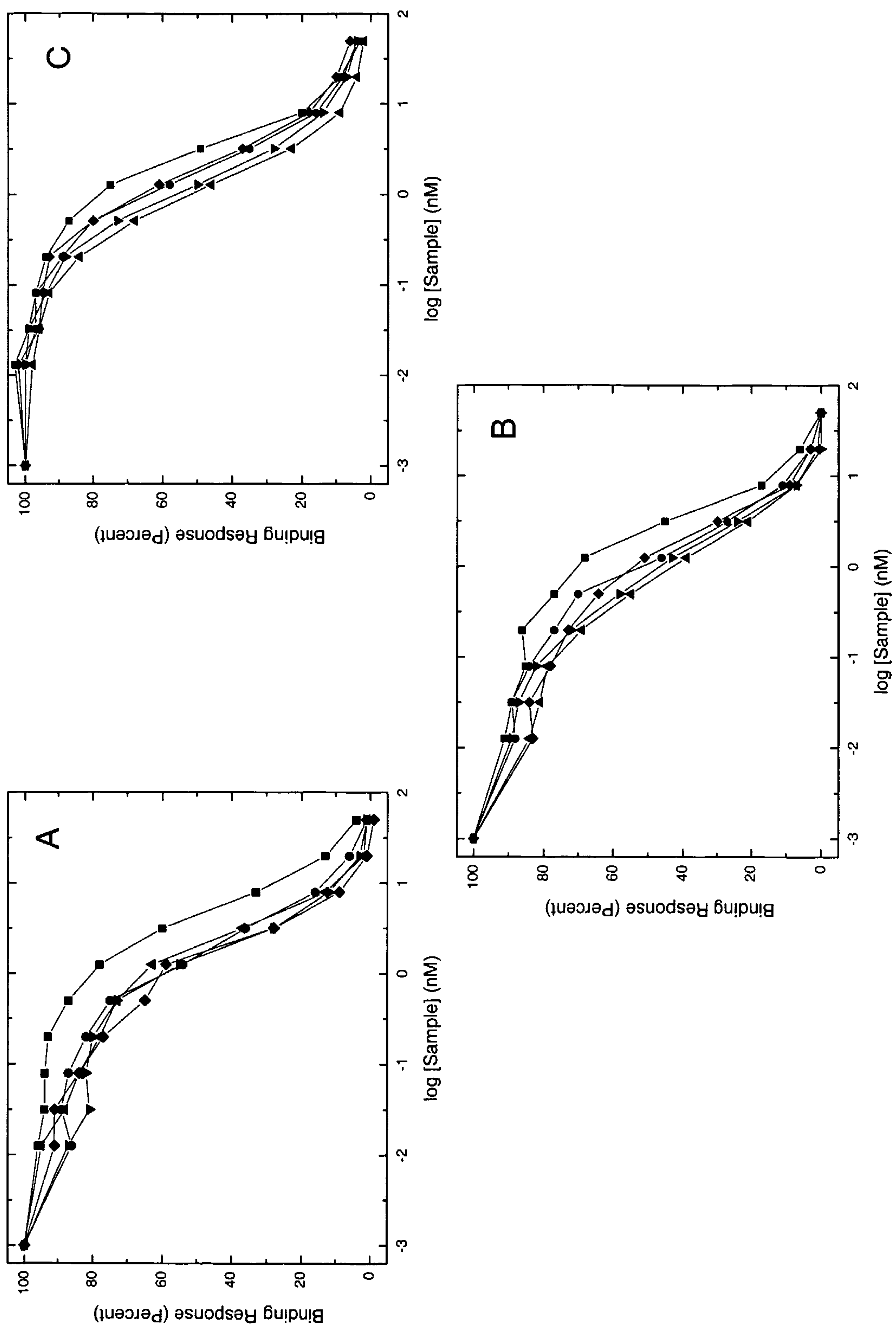


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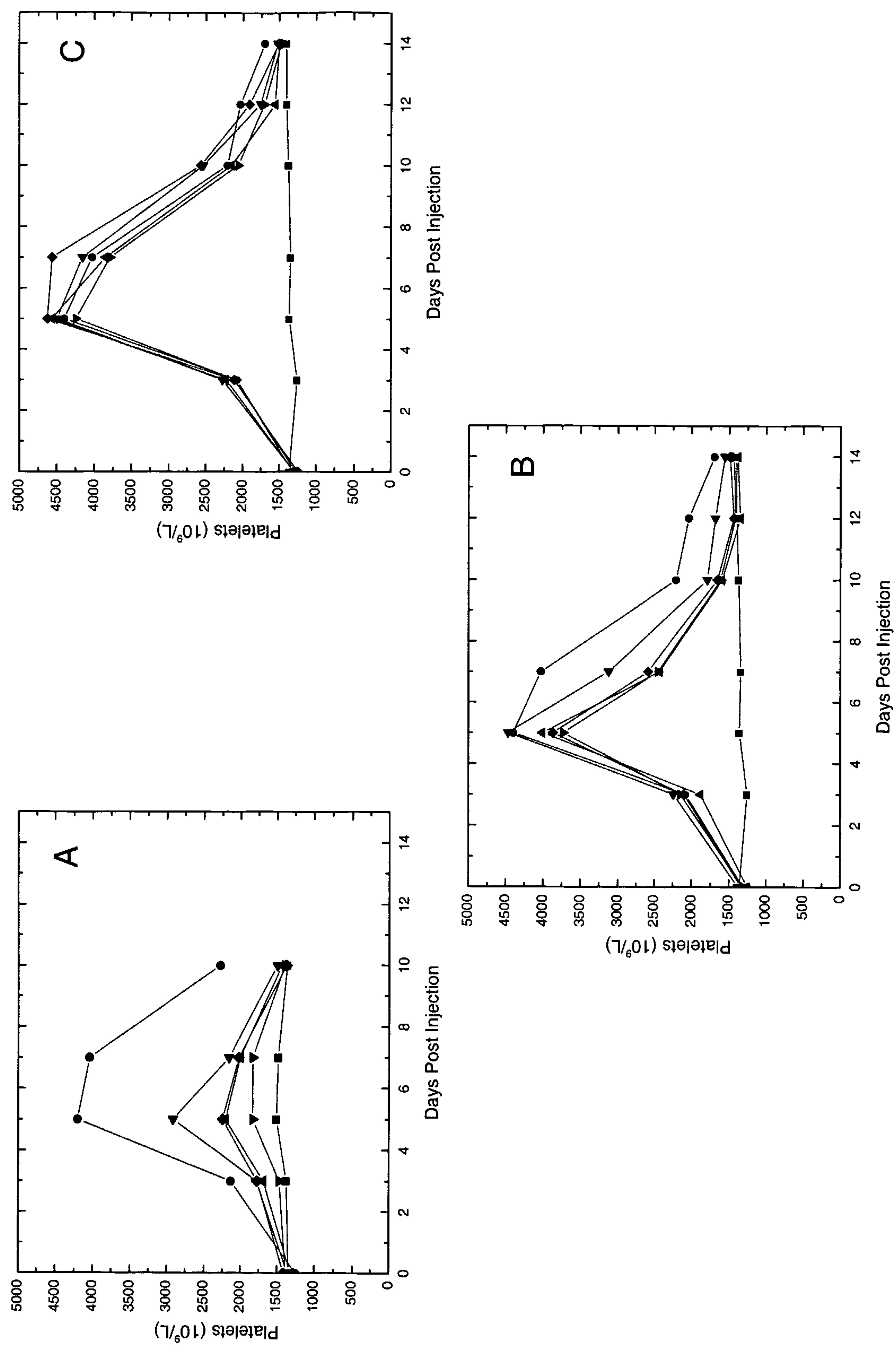


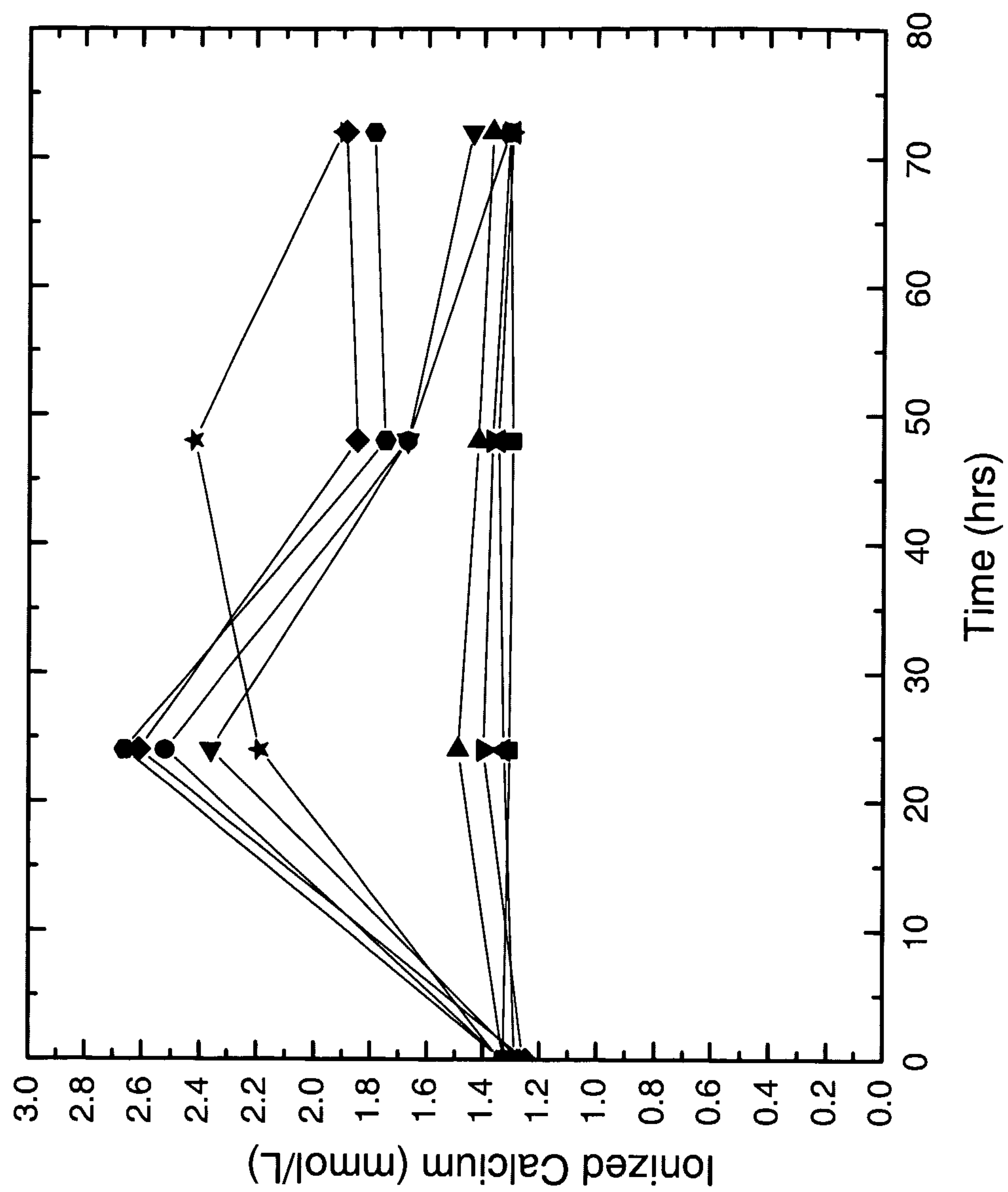
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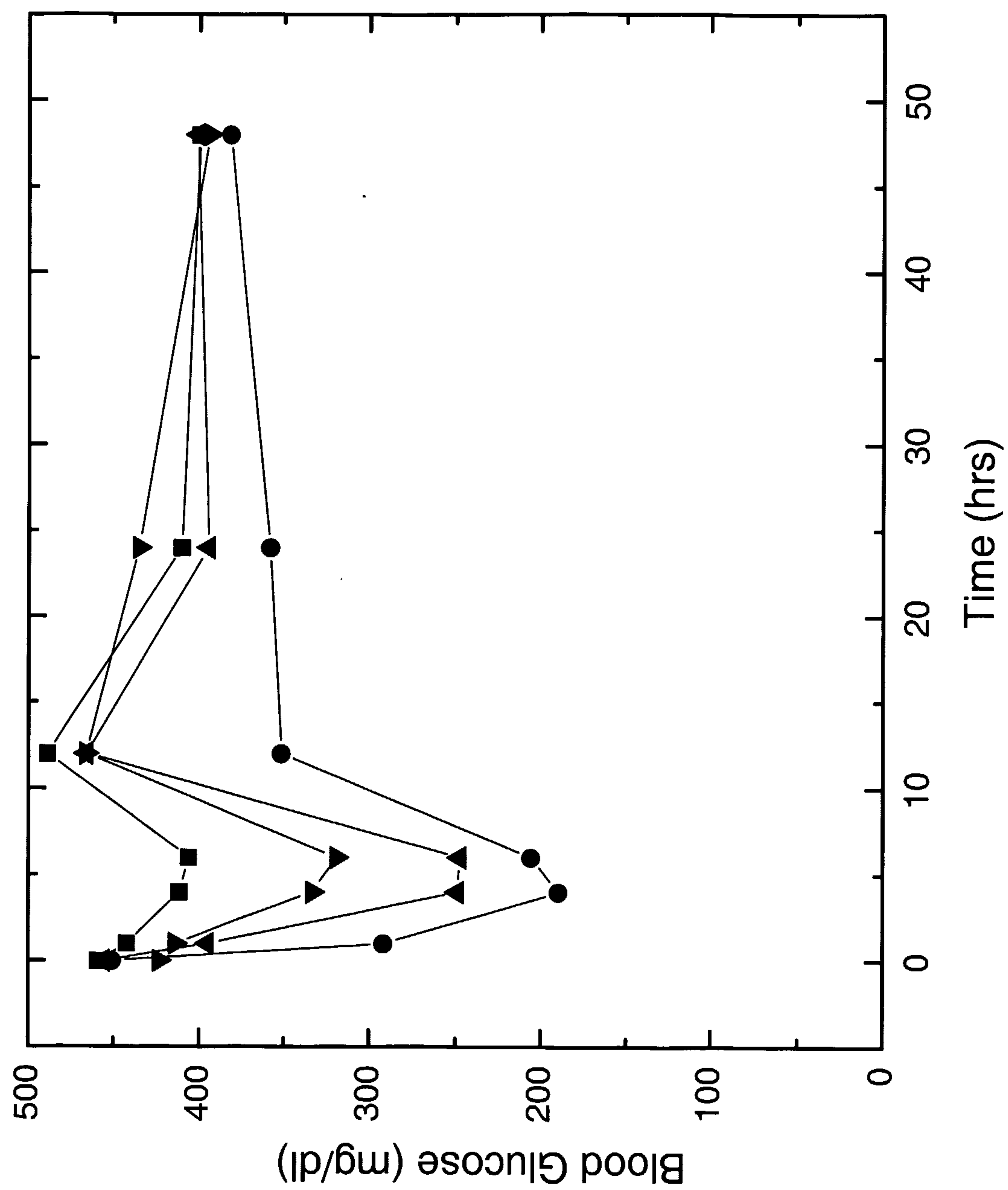
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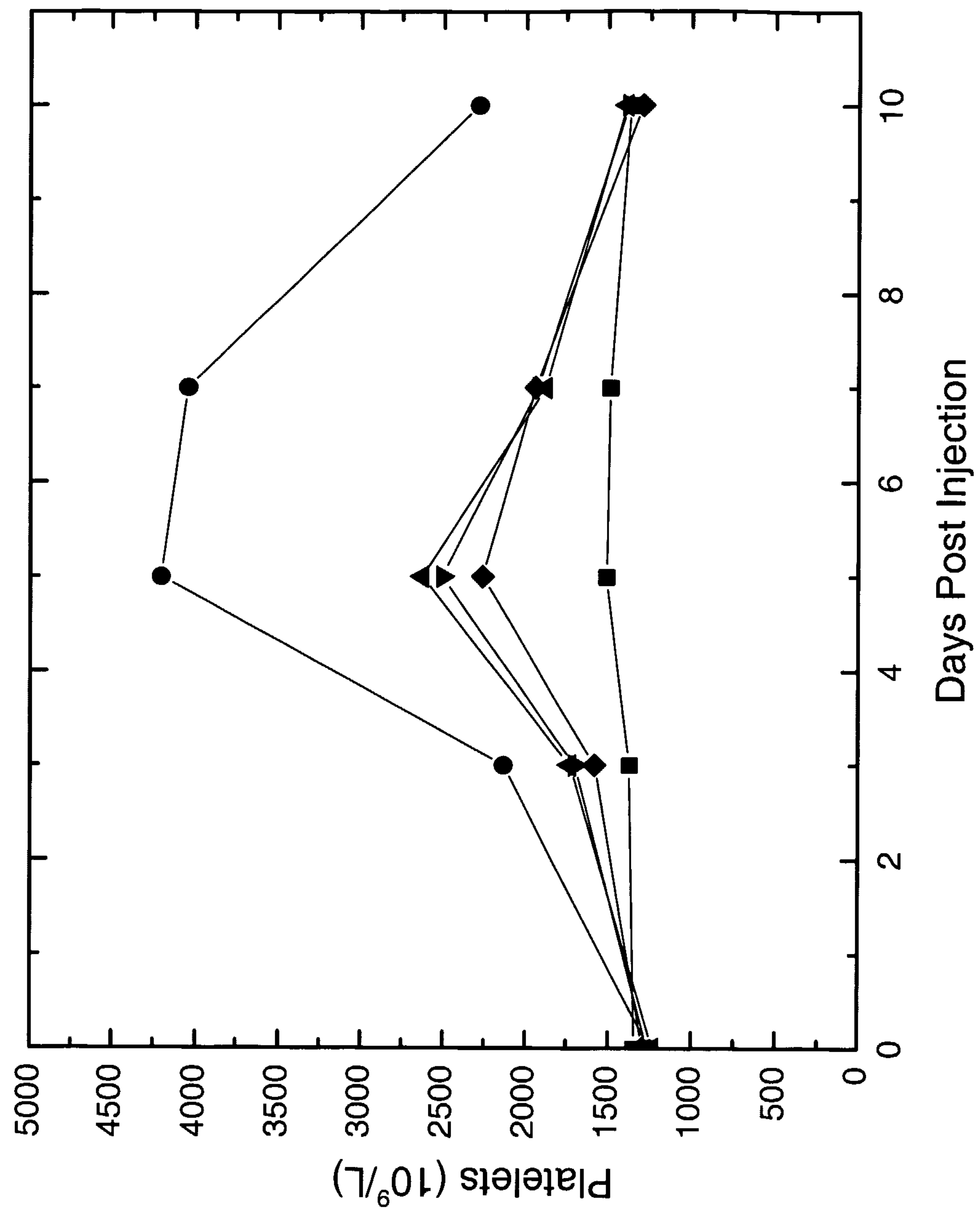
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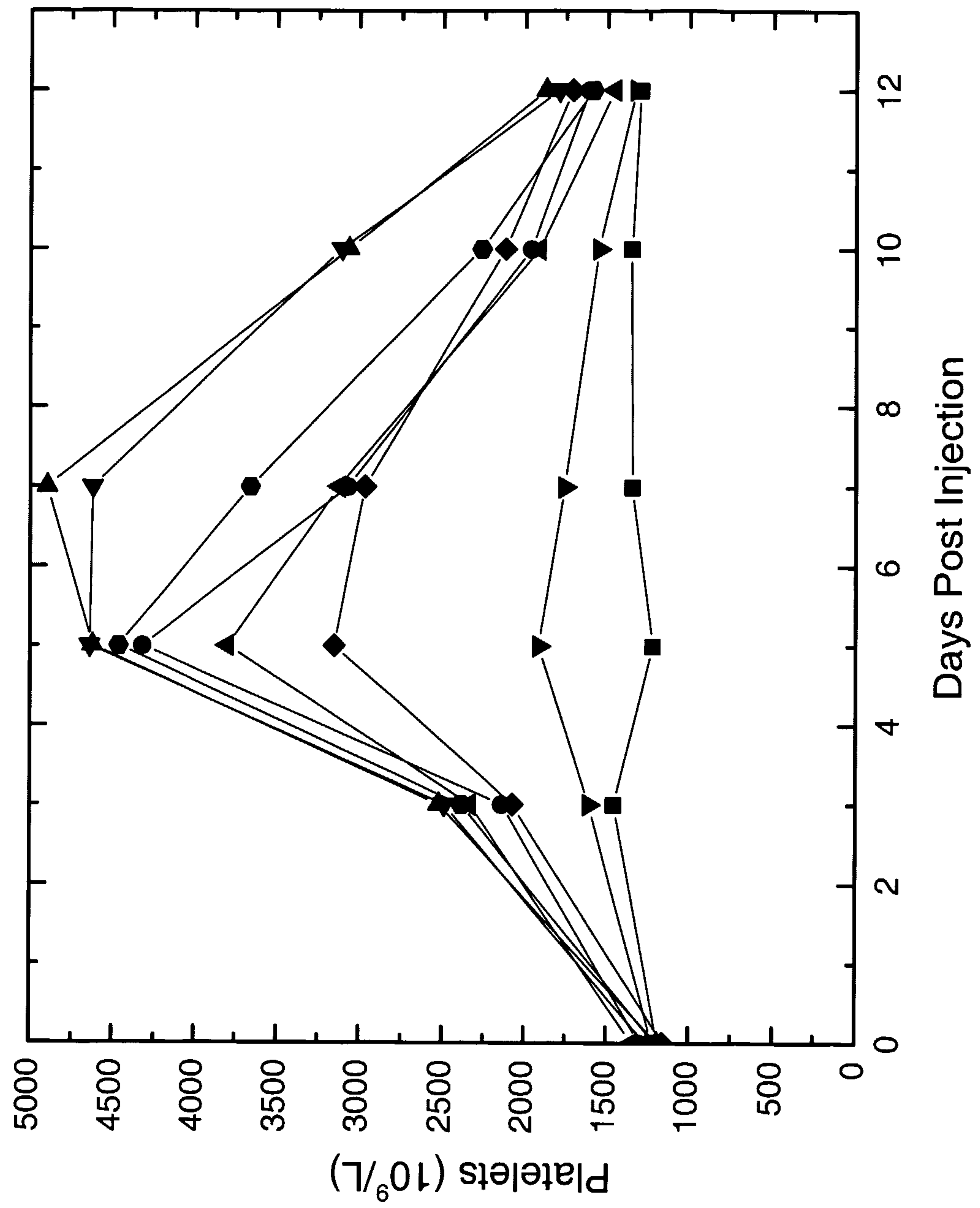
Figure 14

Figure 15

