

Modified Fc Molecules

This application claims the benefit of U.S. Provisional Application No. 60/612,680, filed September 24, 2004, which is hereby incorporated by reference.

Background of the Invention

The success of the drug Enbrel® (etanercept) brought to fruition the promise of therapeutic agents modified with the constant domain of an antibody. Antibodies comprise two functionally independent parts, a variable domain known as "Fab", which binds antigen, and a constant domain known as "Fc", which links to such effector functions as complement activation and attack by phagocytic cells. An Fc has a long serum half-life, whereas an Fab is short-lived. Capon *et al.* (1989), *Nature* 337: 525-31. When constructed together with a therapeutic protein, an Fc domain can provide longer half-life or incorporate such functions as Fc receptor binding, protein A binding, complement fixation and perhaps even placental transfer. *Id.* Table 1 summarizes use of Fc fusion proteins known in the art.

Table 1 – Fc fusion with therapeutic proteins

Form of Fc	Fusion partner	Therapeutic implications	Reference
IgG1	N-terminus of CD30-L	Hodgkin's disease; anaplastic lymphoma; T-cell leukemia	U.S. Patent No. 5,480,981
Murine Fcγ2a	IL-10	anti-inflammatory; transplant rejection	Zheng <i>et al.</i> (1995), <i>J. Immunol.</i> 154: 5590-600
IgG1	TNF receptor	septic shock	Fisher <i>et al.</i> (1996), <i>N. Engl. J. Med.</i> 334: 1697-1702; Van Zee, K. <i>et al.</i> (1996), <i>J. Immunol.</i> 156: 2221-30
IgG, IgA, IgM, or IgE (excluding the first domain)	TNF receptor	inflammation, autoimmune disorders	U.S. Pat. No. 5,808,029, issued September 15, 1998
IgG1	CD4 receptor	AIDS	Capon <i>et al.</i> (1989), <i>Nature</i> 337: 525-31
IgG1, IgG3	N-terminus of IL-2	anti-cancer, antiviral	Harvill <i>et al.</i> (1995), <i>Immunotech.</i> 1: 95-105
IgG1	C-terminus of OPG	osteoarthritis; bone density	WO 97/23614, published July 3, 1997
IgG1	N-terminus of leptin	anti-obesity	WO 98/28427, filed December 11, 1997
Human Ig Cγ1	CTLA-4	autoimmune disorders	Linsley (1991), <i>J. Exp. Med.</i> 174:561-9

A much different approach to development of therapeutic agents is peptide library screening. The interaction of a protein ligand with its receptor often takes place at a relatively large interface. However, as demonstrated for human growth hormone and its receptor, only a few key residues at the interface contribute to most of the binding energy. Clackson *et al.* (1995), *Science* 267: 383-6. The bulk of the protein ligand merely displays the binding epitopes in the right topology or serves functions unrelated to binding. Thus, molecules of only "peptide" length (2 to 40 amino acids) can bind to the receptor protein of a given large protein ligand. Such peptides may mimic the bioactivity of the large protein ligand ("peptide agonists") or, through competitive binding, inhibit the bioactivity of the large protein ligand ("peptide antagonists").

Phage display peptide libraries have emerged as a powerful method in identifying such peptide agonists and antagonists. See, for example, Scott *et al.* (1990), *Science* 249: 386; Devlin *et al.* (1990), *Science* 249: 404; U.S. Pat. No. 5,223,409, issued June 29, 1993; U.S. Pat. No. 5,733,731, issued March 31, 1998; U.S. Pat. No. 5,498,530, issued March 12, 1996; U.S. Pat. No. 5,432,018, issued July 11, 1995; U.S. Pat. No. 5,338,665, issued August 16, 1994; U.S. Pat. No. 5,922,545, issued July 13, 1999; WO 96/40987, published December 19, 1996; and WO 98/15833, published April 16, 1998 (each of which is incorporated by reference). In such libraries, random peptide sequences are displayed by fusion with coat proteins of filamentous phage. Typically, the displayed peptides are affinity-eluted against an antibody-immobilized extracellular domain of a receptor. The retained phages may be enriched by successive rounds of affinity purification and repropagation. The best binding peptides may be sequenced to identify key residues within one or more structurally related families of peptides. See, e.g., Cwirla *et al.* (1997), *Science* 276: 1696-9, in which two distinct families were identified. The peptide sequences may also suggest which residues may be safely replaced by alanine scanning or by mutagenesis at the DNA level. Mutagenesis libraries may be created

and screened to further optimize the sequence of the best binders.

Lowman (1997), Ann. Rev. Biophys. Biomol. Struct. 26: 401-24.

Other methods compete with phage display in peptide research. A peptide library can be fused to the carboxyl terminus of the lac repressor and expressed in E. coli. Another E. coli-based method allows display on the cell's outer membrane by fusion with a peptidoglycan-associated lipoprotein (PAL). Hereinafter, these and related methods are collectively referred to as "E. coli display." Another biological approach to screening soluble peptide mixtures uses yeast for expression and secretion. See Smith et al. (1993), Mol. Pharmacol. 43: 741-8. Hereinafter, the method of Smith et al. and related methods are referred to as "yeast-based screening." In another method, translation of random RNA is halted prior to ribosome release, resulting in a library of polypeptides with their associated RNA still attached. Hereinafter, this and related methods are collectively referred to as "ribosome display." Other methods employ chemical linkage of peptides to RNA; see, for example, Roberts & Szostak (1997), Proc. Natl. Acad. Sci. USA, 94: 12297-303. Hereinafter, this and related methods are collectively referred to as "RNA-peptide screening." Chemically derived peptide libraries have been developed in which peptides are immobilized on stable, non-biological materials, such as polyethylene rods or solvent-permeable resins. Another chemically derived peptide library uses photolithography to scan peptides immobilized on glass slides. Hereinafter, these and related methods are collectively referred to as "chemical-peptide screening." Chemical-peptide screening may be advantageous in that it allows use of D-amino acids and other unnatural analogues, as well as non-peptide elements. Both biological and chemical methods are reviewed in Wells & Lowman (1992), Curr. Opin. Biotechnol. 3: 355-62.

In the case of known bioactive peptides, rational design of peptide ligands with favorable therapeutic properties can be completed. In such an approach, one makes stepwise changes to a peptide sequence and

determines the effect of the substitution upon bioactivity or a predictive biophysical property of the peptide (e.g., solution structure). Hereinafter, these techniques are collectively referred to as "rational design." In one such technique, one makes a series of peptides in which one replaces a single residue at a time with alanine. This technique is commonly referred to as an "alanine walk" or an "alanine scan." When two residues (contiguous or spaced apart) are replaced, it is referred to as a "double alanine walk." The resultant amino acid substitutions can be used alone or in combination to result in a new peptide entity with favorable therapeutic properties.

Structural analysis of protein-protein interaction may also be used to suggest peptides that mimic the binding activity of large protein ligands. In such an analysis, the crystal structure may suggest the identity and relative orientation of critical residues of the large protein ligand, from which a peptide may be designed. See, e.g., Takasaki *et al.* (1997), Nature Biotech. 15: 1266-70. Hereinafter, these and related methods are referred to as "protein structural analysis." These analytical methods may also be used to investigate the interaction between a receptor protein and peptides selected by phage display, which may suggest further modification of the peptides to increase binding affinity.

Conceptually, one may discover peptide mimetics of any protein using phage display and the other methods mentioned above. These methods have been used for epitope mapping, for identification of critical amino acids in protein-protein interactions, and as leads for the discovery of new therapeutic agents. E.g., Cortese *et al.* (1996), Curr. Opin. Biotech. 7: 616-21. Peptide libraries are now being used most often in immunological studies, such as epitope mapping. Kreeger (1996), The Scientist 10(13): 19-20.

Of particular interest here is use of peptide libraries and other techniques in the discovery of pharmacologically active peptides. A number of such peptides identified in the art are summarized in Table 2.

The peptides are described in the listed publications, each of which is hereby incorporated by reference. The pharmacologic activity of the peptides is described, and in many instances is followed by a shorthand term therefore in parentheses. Some of these peptides have been modified (e.g., to form C-terminally cross-linked dimers). Typically, peptide libraries were screened for binding to a receptor for a pharmacologically active protein (e.g., EPO receptor). In at least one instance (CTLA4), the peptide library was screened for binding to a monoclonal antibody.

Table 2—Pharmacologically active peptides

Form of peptide	Binding partner/ protein of interest ^a	Pharmacologic activity	Reference
intrapeptide disulfide-bonded	EPO receptor	EPO-mimetic	Wrighton et al. (1996), <u>Science</u> 273: 458-63; U.S. Pat. No. 5,773,569, issued June 30, 1998 to Wrighton et al.
C-terminally cross-linked dimer	EPO receptor	EPO-mimetic	Livnah et al. (1996), <u>Science</u> 273: 464-71; Wrighton et al. (1997), <u>Nature Biotechnology</u> 15: 1261-5; International patent application WO 96/40772, published Dec. 19, 1996
linear	EPO receptor	EPO-mimetic	Naranda et al. (1999), <u>Proc. Natl. Acad. Sci. USA</u> , 96: 7569-74; WO 99/47151, published September 23, 1999
linear; C-terminally cross-linked dimer	c-Mpl	TPO-mimetic	Cwirla et al. (1997) <u>Science</u> 276: 1696-9; U.S. Pat. No. 5,869,451, issued Feb. 9, 1999; U.S. Pat. No. 5,932,946, issued Aug. 3, 1999; WO 00/24770, published May 4, 2000; U.S. Pat. App. No. 2003/0176352, published Sept. 18, 2003; WO 03/031589, published April 17, 2003
disulfide-linked dimer		stimulation of hematopoiesis ("G-CSF-mimetic")	Paukovits et al. (1984), <u>Hoppe-Seylers Z. Physiol. Chem.</u> 365: 303-

^a The protein listed in this column may be bound by the associated peptide (e.g., EPO receptor, IL-1 receptor) or mimicked by the associated peptide. The references listed for each clarify whether the molecule is bound by or mimicked by the peptides.

			11; Laerum et al. (1988), <u>Exp. Hemat.</u> 16: 274-80
alkylene-linked dimer		G-CSF-mimetic	Bhatnagar et al. (1996), <u>J. Med. Chem.</u> 39: 3814-9; Cuthbertson et al. (1997), <u>J. Med. Chem.</u> 40: 2876-82; King et al. (1991), <u>Exp. Hematol.</u> 19:481; King et al. (1995), <u>Blood</u> 86 (Suppl. 1): 309a
linear	IL-1 receptor	inflammatory and autoimmune diseases ("IL-1 antagonist" or "IL-1ra-mimetic")	U.S. Pat. No. 5,608,035; U.S. Pat. No. 5,786,331; U.S. Pat. No. 5,880,096; Yanofsky et al. (1996), <u>Proc. Natl. Acad. Sci.</u> 93: 7381-6; Akesson et al. (1996), <u>J. Biol. Chem.</u> 271: 30517-23; Wiekzorek et al. (1997), <u>Pol. J. Pharmacol.</u> 49: 107-17; Yanofsky (1996), <u>PNAs</u> , 93:7381-7386.
linear	Facteur thymique serique (FTS)	stimulation of lymphocytes ("FTS-mimetic")	Inagaki-Ohara et al. (1996), <u>Cellular Immunol.</u> 171: 30-40; Yoshida (1984), <u>Int. J. Immunopharmacol.</u> 6:141-6.
intrapeptide disulfide bonded	CTLA4 MAb	CTLA4-mimetic	Fukumoto et al. (1998), <u>Nature Biotech.</u> 16: 267-70
exocyclic	TNF- α receptor	TNF- α antagonist	Takasaki et al. (1997), <u>Nature Biotech.</u> 15:1266-70; WO 98/53842, published December 3, 1998
linear	TNF- α receptor	TNF- α antagonist	Chirinos-Rojas (), <u>J. Imm.</u> , 5621-5626.
intrapeptide disulfide bonded	C3b	inhibition of complement activation; autoimmune diseases ("C3b-antagonist")	Sahu et al. (1996), <u>J. Immunol.</u> 157: 884-91; Morikis et al. (1998), <u>Protein Sci.</u> 7: 619-27
linear	vinculin	cell adhesion processes—cell growth, differentiation, wound healing, tumor metastasis ("vinculin binding")	Adey et al. (1997), <u>Biochem. J.</u> 324: 523-8
linear	C4 binding protein (C4BP)	anti-thrombotic	Linse et al. (1997), <u>J. Biol. Chem.</u> 272: 14658-65
linear	urokinase receptor	processes associated with urokinase interaction with its receptor (e.g., angiogenesis, tumor	Goodson et al. (1994), <u>Proc. Natl. Acad. Sci.</u> 91: 7129-33; International application WO 97/35969, published

		cell invasion and metastasis); ("UKR antagonist")	October 2, 1997
linear	Mdm2, Hdm2	Inhibition of inactivation of p53 mediated by Mdm2 or hdm2; anti-tumor ("Mdm/hdm antagonist")	Picksley et al. (1994), <u>Oncogene</u> 9: 2523-9; Bottger et al. (1997) <u>J. Mol. Biol.</u> 269: 744-56; Bottger et al. (1996), <u>Oncogene</u> 13: 2141-7
linear	p21 ^{WAF1}	anti-tumor by mimicking the activity of p21 ^{WAF1}	Ball et al. (1997), <u>Curr. Biol.</u> 7: 71-80
linear	farnesyl transferase	anti-cancer by preventing activation of ras oncogene	Gibbs et al. (1994), <u>Cell</u> 77:175-178
linear	Ras effector domain	anti-cancer by inhibiting biological function of the ras oncogene	Moodie et al. (1994), <u>Trends Genet</u> 10: 44-48 Rodriguez et al. (1994), <u>Nature</u> 370:527-532
linear	SH2/SH3 domains	anti-cancer by inhibiting tumor growth with activated tyrosine kinases; treatment of SH3-mediated disease states ("SH3 antagonist")	Pawson et al (1993), <u>Curr. Biol.</u> 3:434-432 Yu et al. (1994), <u>Cell</u> 76:933-945; Rickles et al. (1994), <u>EMBO J.</u> 13: 5598-5604; Sparks et al. (1994), <u>J. Biol. Chem.</u> 269: 23853-6; Sparks et al. (1996), <u>Proc. Natl. Acad. Sci.</u> 93: 1540-4; US Pat. No. 5,886,150, issued March 23, 1999; US Pat. No. 5,888,763, issued March 30, 1999
linear	p16 ^{INK4}	anti-cancer by mimicking activity of p16; e.g., inhibiting cyclin D-Cdk complex ("p16-mimetic")	Fähraeus et al. (1996), <u>Curr. Biol.</u> 6:84-91
linear	Src, Lyn	inhibition of Mast cell activation, IgE-related conditions, type I hypersensitivity ("Mast cell antagonist")	Stauffer et al. (1997), <u>Biochem.</u> 36: 9388-94
linear	Mast cell protease	treatment of inflammatory disorders mediated by release of tryptase-6 ("Mast cell protease inhibitors")	International application WO 98/33812, published August 6, 1998
linear	HBV core antigen (HBcAg)	treatment of HBV viral infections ("anti-HBV")	Dyson & Muray (1995), <u>Proc. Natl. Acad. Sci.</u> 92: 2194-8
linear	selectins	neutrophil adhesion; inflammatory diseases ("selectin antagonist")	Martens et al. (1995), <u>J. Biol. Chem.</u> 270: 21129-36; European patent

			application EP 0 714 912, published June 5, 1996
linear, cyclized	calmodulin	calmodulin antagonist	Pierce et al. (1995), <u>Molec. Diversity</u> 1: 259-65; Dedman et al. (1993), <u>J. Biol. Chem.</u> 268: 23025-30; Adey & Kay (1996), <u>Gene</u> 169: 133-4
linear, cyclized-	integrins	tumor-homing; treatment for conditions related to integrin-mediated cellular events, including platelet aggregation, thrombosis, wound healing, osteoporosis, tissue repair, angiogenesis (e.g., for treatment of cancer), and tumor invasion ("integrin-binding")	International applications WO 95/14714, published June 1, 1995; WO 97/08203, published March 6, 1997; WO 98/10795, published March 19, 1998; WO 99/24462, published May 20, 1999; Kraft et al. (1999), <u>J. Biol. Chem.</u> 274: 1979-1985
cyclic, linear	fibronectin and extracellular matrix components of T cells and macrophages	treatment of inflammatory and autoimmune conditions	WO 98/09985, published March 12, 1998
linear	somatostatin and cortistatin	treatment or prevention of hormone-producing tumors, acromegaly, gigantism, dementia, gastric ulcer, tumor growth, inhibition of hormone secretion, modulation of sleep or neural activity	European patent application 0 911 393, published April 28, 1999
linear	bacterial lipopolysaccharide	antibiotic; septic shock; disorders modulatable by CAP37	U.S. Pat. No. 5,877,151, issued March 2, 1999
linear or cyclic, including D-amino acids	pardaxin, mellitin	antipathogenic	WO 97/31019, published 28 August 1997
linear, cyclic	VIP	impotence, neurodegenerative disorders	WO 97/40070, published October 30, 1997
linear	CTLs	cancer	EP 0 770 624, published May 2, 1997
linear	THF-gamma2		Burnstein (1988), <u>Biochem.</u> , 27:4066-71.
linear	Amylin		Cooper (1987), <u>Proc. Natl. Acad. Sci.</u> , 84:8628-32.
linear	Adrenomedullin		Kitamura (1993), <u>BBRC</u> ,

			192:553-60.
cyclic, linear	VEGF	anti-angiogenic; cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis ("VEGF antagonist")	Fairbrother (1998), <u>Biochem.</u> , 37:17754-17764.
cyclic	MMP	inflammation and autoimmune disorders; tumor growth ("MMP inhibitor")	Koivunen (1999), <u>Nature Biotech.</u> , 17:768-774.
	HGH fragment	treatment of obesity	U.S. Pat. No. 5,869,452
	Echistatin	inhibition of platelet aggregation	Gan (1988), <u>J. Biol. Chem.</u> , 263:19827-32.
linear	SLE autoantibody	SLE	WO 96/30057, published October 3, 1996
	GD1alpha	suppression of tumor metastasis	Ishikawa et al. (1998), <u>FEBS Lett.</u> 441 (1): 20-4
	antiphospholipid beta-2-glycoprotein-I (β 2GPI) antibodies	endothelial cell activation, antiphospholipid syndrome (APS), thromboembolic phenomena, thrombocytopenia, and recurrent fetal loss	Blank et al. (1999), <u>Proc. Natl. Acad. Sci. USA</u> 96: 5164-8
linear	T Cell Receptor beta chain	diabetes	WO 96/11214, published April 18, 1996.
		Antiproliferative, antiviral	WO 00/01402, published January 13, 2000.
		anti-ischemic, growth hormone-liberating	WO 99/62539, published December 9, 1999.
		anti-angiogenic	WO 99/61476, published December 2, 1999.
linear		Apoptosis agonist; treatment of T cell-associated disorders (e.g., autoimmune diseases, viral infection, T cell leukemia, T cell lymphoma)	WO 99/38526, published Aug. 5, 1999.
linear	MHC class II	treatment of autoimmune diseases	US Pat. No. 5,880,103, issued March 9, 1999.
linear	androgen R, p75, MJD, DCC, huntingtin	proapoptotic, useful in treating cancer	WO 99/45944, published September 16, 1999.
linear	von Willebrand Factor; Factor VIII	inhibition of Factor VIII interaction; anticoagulants	WO 97/41220, published April 29, 1997.
linear	lentivirus LLP1	antimicrobial	US Pat. No. 5,945,507, issued Aug. 31, 1999.
linear	Delta-Sleep Inducing Peptide	sleep disorders	Graf (1986), <u>Peptides</u> 7:1165.
linear	C-Reactive Protein (CRP)	inflammation and cancer	Barna (1994), <u>Cancer Immunol. Immunother.</u> 38:38 (1994).

linear	Sperm-Activating Peptides	infertility	Suzuki (1992), <u>Comp. Biochem. Physiol.</u> 102B:679.
linear	angiotensins	hematopoietic factors for hematocytopenic conditions from cancer, AIDS, etc.	Lundergan (1999), <u>I. Periodontal Res.</u> 34(4):223-228.
linear	HIV-1 gp41	anti-AIDS	Chan (1998), <u>Cell</u> 93:681-684.
linear	PKC	inhibition of bone resorption	Moonga (1998), <u>Exp. Physiol.</u> 83:717-725.
linear	defensins (HNP-1, -2, -3, -4)	antimicrobial	Harvig (1994), <u>Methods Enz.</u> 236:160-172.
linear	p185 ^{HER2/neu} , C-erbB-2	AHNP-mimetic: anti-tumor	Park (2000), <u>Nat. Biotechnol.</u> 18:194-198.
linear	gp130	IL-6 antagonist	WO 99/60013, published Nov. 25, 1999.
linear	collagen, other joint, cartilage, arthritis-related proteins	autoimmune diseases	WO 99/50282, published Oct. 7, 1999.
linear	HIV-1 envelope protein	treatment of neurological degenerative diseases	WO 99/51254, published Oct. 14, 1999.
linear	IL-2	autoimmune disorders (e.g., graft rejection, rheumatoid arthritis)	WO 00/04048, published Jan. 27, 2000; WO 00/11028, published March 2, 2000.
linear, cyclic	various	inflammatory conditions, autoimmune disease, others	U.S. Pat. No. 6,660,843
linear, cyclic	Ang-2	inhibition of angiogenesis (e.g., for treatment of tumor)	U.S. Pat. App. No. 2003/0229023, published Dec. 11, 2003; WO 03/057134, published 7/17/03; U.S. 2003/0236193, published Dec. 25, 2003
	NGF	chronic pain, migraine, asthma, hyperactive bladder, psoriasis, cancer, other conditions linked to NGF	WO 04/026329, published April 1, 2004
	myostatin		U.S. Serial No. 10/742,379, filed Dec. 19, 2003; PCT/US03/40781, filed Dec. 19, 2003
	BAFF/TALL-1	B-cell mediated autoimmune diseases and cancers (e.g., lupus, B-cell lymphoma)	U.S. 2003/0195156, published Oct. 16, 2003; WO 02/092620, published Nov. 21, 2002
linear	GLP-1	Diabetes, metabolic syndrome	

Peptides identified by peptide library screening were for a long time regarded simply as "leads" in development of therapeutic agents rather than as therapeutic agents themselves. Like other proteins and peptides, they would be rapidly removed in vivo either by renal filtration, cellular clearance mechanisms in the reticuloendothelial system, or proteolytic degradation. Francis (1992), Focus on Growth Factors 3: 4-11. As a result, the art used the identified peptides to validate drug targets or as scaffolds for design of organic compounds that might not have been as easily or as quickly identified through chemical library screening.

- Lowman (1997), Ann. Rev. Biophys. Biomol. Struct. 26: 401-24; Kay et al. (1998), Drug Disc. Today 3: 370-8.

A more recent development is fusion of randomly generated peptides with the Fc domain. See U.S. Pat. No. 6,660,843, issued December 9, 2003 to Feige et al. (incorporated by reference in its entirety). Such molecules have come to be known as "peptibodies." They include one or more peptides linked to the N-terminus, C-terminus, amino acid sidechains, or to more than one of these sites. Peptibody technology enables design of therapeutic agents that incorporate peptides that target one or more ligands or receptors, tumor-homing peptides, membrane-transporting peptides, and the like. Peptibody technology has proven useful in design of a number of such molecules, including linear and disulfide-constrained peptides, "tandem peptide multimers" (i.e., more than one peptide on a single chain of an Fc domain). See, for example, U.S. Pat. No. 6,660,843; U.S. Pat. App. No. 2003/0195156, published Oct. 16, 2003 (corresponding to WO 02/092620, published Nov. 21, 2002); U.S. Pat. App. No. 2003/0176352, published Sept. 18, 2003 (corresponding to WO 03/031589, published April 17, 2003); U.S. Ser. No. 09/422,838, filed October 22, 1999 (corresponding to WO 00/24770, published May 4, 2000); U.S. Pat. App. No. 2003/0229023, published December 11, 2003; WO 03/057134, published July 17, 2003; U.S. Pat. App. No. 2003/0236193, published December 25, 2003 (corresponding to PCT/US04/010989, filed

April 8, 2004); U.S. Ser. No. 10/666,480, filed September 18, 2003
(corresponding to WO 04/026329, published April 1, 2004), each of which
is hereby incorporated by reference in its entirety. The art would benefit
from further technology enabling such rational design of polypeptide
5 therapeutic agents.

Summary of the Invention

The present invention concerns a process in which at least one
biologically active peptide is incorporated as an internal sequence into an
10 Fc domain. Such an internal sequence may be added by insertion (i.e.,
between amino acids in the previously existing Fc domain) or by
replacement of amino acids in the previously existing Fc domain (i.e.,
removing amino acids in the previously existing Fc domain and adding
peptide amino acids). In the latter case, the number of peptide amino acids
15 added need not correspond to the number of amino acids removed from
the previously existing Fc domain; for example, this invention concerns a
molecule in which 10 amino acids are removed and 15 amino acids are
added. In this invention, pharmacologically active compounds are
prepared by a process comprising:

- 20 a) selecting at least one peptide that modulates the activity of a
protein of interest; and
- b) preparing a pharmacologic agent comprising an amino acid
sequence of the selected peptide as an internal sequence of
an Fc domain.

25 This process may be employed to modify an Fc domain that is already
linked through an N- or C-terminus or sidechain to a polypeptide (e.g.,
etanercept) or to a peptide (e.g., as described in U.S. Pat. App. Nos.
2003/0195156, 2003/0176352, 2003/0229023, and 2003/0236193; WO
00/24770; WO 04/026329). The process described throughout may also be
30 employed to modify an Fc domain that is part of an antibody (e.g.,
adalimumab, epratuzumab, infliximab, Herceptin®, and the like). In this

way, different molecules can be produced that have additional functionalities, such as a binding domain to a different epitope or an additional binding domain to the precursor molecule's existing epitope. The peptide can be selected, for example, by phage display (which is preferred), *E. coli* display, ribosome display, RNA-peptide screening, yeast-based screening, chemical-peptide screening, rational design, or protein structural analysis or may be a naturally occurring peptide (e.g. PTH, GLP-1).

The invention further relates to molecules comprising an Fc domain modified to comprise a peptide as an internal sequence (preferably in a loop region) of the Fc domain. Molecules comprising an internal peptide sequence are referred to throughout as "Fc internal peptibodies" or "Fc internal peptide molecules." These molecules are further described herein below.

The Fc internal peptide molecules may include more than one peptide sequence in tandem in a particular internal region, and they may include further peptides in other internal regions. While the putative loop regions are preferred, insertions in any other non-terminal domains of the Fc are also considered part of this invention. Variants and derivatives of the above compounds (described below) are also encompassed by this invention.

The compounds of this invention may be prepared by standard synthetic methods, recombinant DNA techniques, or any other methods of preparing peptides and fusion proteins.

The primary use contemplated for Fc internal peptide molecules is as therapeutic or prophylactic agents. A selected peptide may have activity comparable to—or even greater than—the natural ligand mimicked by the peptide. In addition, certain natural ligand-based therapeutic agents might induce antibodies against the patient's own endogenous ligand. In contrast, the unique sequence of the vehicle-linked peptide avoids this pitfall by having little or typically no sequence identity

with the natural ligand. Furthermore, the Fc internal peptibodies may have advantages in refolding and purification over N- or C-terminally linked Fc molecules. Further still, Fc internal peptibodies may be more stable in both thermodynamically, due to the stabilization of chimeric domains, and chemically, due to increased resistance to proteolytic degradation from amino- and carboxy-peptidases. Fc internal peptibodies may also exhibit improved pharmacokinetic properties.

Although mostly contemplated as therapeutic agents, compounds of this invention may also be useful in screening for such agents. For example, one could use an Fc internal peptibody (e.g., Fc-loop-SH2 domain peptide) in an assay employing anti-Fc coated plates. Fc internal peptibodies may make insoluble peptides soluble and thus useful in a number of assays.

The compounds of this invention may be used for therapeutic or prophylactic purposes by formulating them with appropriate pharmaceutical carrier materials and administering an effective amount to a patient, such as a human (or other mammal) in need thereof. Other related aspects are also included in the instant invention.

Numerous additional aspects and advantages of the present invention will become apparent upon consideration of the figures and detailed description of the invention.

Description of the Figures

Figures 1A, 1B and 1C show loop regions of Fc domains that may be modified in accordance with this invention. In these structural representations of the CH2 and CH3 domains of Fc, the loop regions may be considered any part of the model not shown as β -sheet (flat arrows) or α -helix (cylinder).

Figure 1A shows the monomeric rat IgG2a Fc domain (Protein Database file #1I1C, <http://www.rcsb.org/pdb/>). This figure shows a three dimensional model of rat IgG2a Fc domain monomer from x-ray diffraction crystal structure (pdb #1I1C). Potential Fc loop insertion sites

are shown for both CH2 and CH3 domains with the preferred CH3 domain Fc loop insertion site specifically identified.

Figure 1B shows the monomeric murine IgG1 Fc domain (Protein Database file #1IGY). This figure shows a three-dimensional model of murine IgG1 Fc domain monomer from x-ray diffraction crystal structure (pdb #1IGY). Potential Fc loop insertion sites are shown for both CH2 and CH3 domains with the preferred CH3 domain Fc loop insertion site specifically identified.

Figure 1C shows the monomeric human IgG1 Fc domain (Protein Database file #1H3T). This figure shows a three-dimensional model of human IgG1 Fc domain monomer from x-ray diffraction crystal structure (pdb #1H3T). Potential Fc loop insertion sites are shown for both CH2 and CH3 domains with the preferred CH3 domain Fc loop insertion site specifically identified.

These structures illustrate the high degree of homology in the secondary and tertiary structural conformations within the Fc domains of different IgG subtypes and between species. The x-ray crystal structure coordinates for these structures can be found in the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/>).

Figure 2A shows a sequence of human IgG1 Fc sequence (SEQ ID NO: 599) used for peptibody fusions with predicted loop sequences in boldface. Figure 2A shows, in the context of the human IgG1 sequence used for this invention, the Fc loop regions in boldface (SEQ ID NOS: 621, 622, 624, 625, 627, 628, 630, 632, 634, and 636), which are suggested by the structures shown in Figures 1A, 1B and 1C. Any, or all of the sites shown in boldface may be suitable for full or partial replacement by or insertion of peptide sequences and are considered part of this invention. Specifically preferred internal sites are underlined (SEQ ID NOS: 623, 626, 629, 631, 633, 635, and 637). One preferred site is SEQ ID NO: 631, between Leu₁₃₉ and Thr₁₄₀ in the DELTK (SEQ ID NO: 630) loop. Potential loop sites in

other Ig subtypes are understood in the art based on the alignments provided in Figures 2B and 2C.

Figures 2B and 2C show a sequence alignment of human Fc domains from IgA, IgM and IgG subclasses. Figures 2B and 2C show
 5 exemplary amino acid sequences (SEQ ID NOS: 600 to 607) of human Fc regions from IgA, IgM and IgG subtypes that may be useful in this invention. Also shown in Figures 2B and 2C is a consensus sequence (SEQ ID NO: 608).

Figures 2B and 2C also show in boldface the preferred internal sites
 10 for peptide addition that correspond to those of the Fc sequence shown in Figure 2A (SEQ ID NO: 599). In particular, Figures 2B and 2C show as such preferred sites the following:

- SEQ ID NO: 621 as shown in boldface within SEQ ID NOS: 603 to 608;
- SEQ ID NO: 622 within SEQ ID NOS: 603 to 606 and 608;
- 15 • SEQ ID NO: 638 within SEQ ID NO: 607;
- SEQ ID NO: 624 within SEQ ID NO: 603 to 608;
- SEQ ID NO: 625 within SEQ ID NOS: 603 and 604;
- SEQ ID NO: 639 within SEQ ID NOS: 605 to 608;
- SEQ ID NO: 627 within SEQ ID NOS: 603 to 605, 607, and 608;
- 20 • SEQ ID NO: 640 within SEQ ID NO: 606;
- SEQ ID NO: 628 within SEQ ID NOS: 603, 604, and 608;
- SEQ ID NO: 641 within SEQ ID NO: 605;
- SEQ ID NO: 642 within SEQ ID NO: 606;
- SEQ ID NO: 643 within SEQ ID NO: 607;
- 25 • SEQ ID NO: 630 within SEQ ID NO: 603;
- SEQ ID NO: 644 within SEQ ID NOS: 604 to 608;
- SEQ ID NO: 632 within SEQ ID NOS: 603, 604, 606, 607, and 608;
- SEQ ID NO: 645 within SEQ ID NO: 605;
- SEQ ID NO: 634 within SEQ ID NOS: 603, 604, and 607;
- 30 • SEQ ID NO: 646 within SEQ ID NOS: 605, 606 and 608;

- SEQ ID NO: 636 within SEQ ID NOS: 603, 604, 606, and 608;
- SEQ ID NO: 614 within SEQ ID NO: 605; and
- SEQ ID NO: 620 within SEQ ID NO: 607.

5 The sequence alignments of Figures 2B and 2C suggest two more potential insertion sites at Q₁₆₇/P₁₆₈ and/or G₁₈₃/S₁₈₄ (using the numbering of SEQ ID NO: 599 in Figure 2A). These positions correspond to gaps in the IgG sequences where there are 2 and 3 residue insertions found in the aligned IgA and IgM sequences. Other preferred insertion
10 sites correspond to the sequence in Figure 2A. The preferred insertion sites are underlined Figures 2B and 2C and are as follows:

- H₅₃/E₅₄ in SEQ ID NOS: 603 and 604;
- H₁₀₀/E₁₀₁ in SEQ ID NO: 605;
- H₄₉/E₅₀ in SEQ ID NO: 606;
- 15 • Q₅₀/E₅₁ in SEQ ID NO: 607;
- H₁₁₂/E₁₁₃ in SEQ ID NO: 608;
- Y₈₁/N₈₂ in SEQ ID NOS: 603 and 604;
- F₁₂₈/N₁₂₉ in SEQ ID NO: 605;
- F₇₇/N₇₈ in SEQ ID NO: 606;
- 20 • F₇₈/N₇₉ in SEQ ID NO: 607;
- F₁₄₀/N₁₄₁ in SEQ ID NO: 608;
- N₁₁₀/K₁₁₁ in SEQ ID NOS: 603 and 604;
- N₁₅₇/K₁₅₈ in SEQ ID NO: 605;
- N₁₀₆/K₁₀₇ in SEQ ID NO: 606;
- 25 • N₁₀₇/K₁₀₈ in SEQ ID NO: 607;
- N₁₆₉/K₁₇₀ in SEQ ID NO: 608;
- L₁₄₃/T₁₄₄ in SEQ ID NOS: 603 and 604;
- M₁₉₀/T₁₉₁ in SEQ ID NO: 605;
- M₁₃₉/T₁₄₀ in SEQ ID NO: 606;
- 30 • M₁₄₀/T₁₄₁ in SEQ ID NO: 607;

- M₂₀₄/T₂₀₅ in SEQ ID NO: 608;
- Q₁₇₁P₁₇₂ in SEQ ID NOS: 603 and 604;
- Q₂₁₈/P₂₁₉ in SEQ ID NO: 605;
- Q₁₆₇/P₁₆₈ in SEQ ID NO: 606;
- 5 • Q₁₆₈/P₁₆₉ in SEQ ID NO: 607;
- Q₂₃₂/P₂₃₃ in SEQ ID NO: 608;
- E₁₇₃N₁₇₄ in SEQ ID NOS: 603 and 604;
- E₂₂₀/N₂₂₁ in SEQ ID NO: 605;
- E₁₆₉/N₁₇₀ in SEQ ID NO: 606;
- 10 • E₁₇₀/N₁₇₁ in SEQ ID NO: 607;
- E₂₃₄/N₂₃₅ in SEQ ID NO: 608;
- S₁₈₆/D₁₈₇ in SEQ ID NOS: 603 and 604;
- S₂₃₂/D₂₃₃ in SEQ ID NO: 605;
- S₁₈₁/D₁₈₂ in SEQ ID NO: 606;
- 15 • S₁₈₂/D₁₈₃ in SEQ ID NO: 607;
- S₂₄₆/D₂₄₇ in SEQ ID NO: 608;
- G₁₈₈/S₁₈₉ in SEQ ID NOS: 603 and 604;
- G₂₃₄/S₂₃₅ in SEQ ID NO: 605;
- G₁₈₃/S₁₈₄ in SEQ ID NO: 606;
- 20 • G₁₈₄/S₁₈₅ in SEQ ID NO: 607;
- G₂₄₈/S₂₄₉ in SEQ ID NO: 608;
- G₂₀₅/N₂₀₆ in SEQ ID NOS: 603 and 604;
- G₂₅₂/N₂₅₃ in SEQ ID NO: 605;
- G₂₀₁/N₂₀₂ in SEQ ID NO: 606;
- 25 • G₂₀₂/N₂₀₃ in SEQ ID NO: 607; and
- G₂₆₈/N₂₆₉ in SEQ ID NO: 608.

Figure 2D shows an alignment of human IgG1 Fc domain (Amgen Fc, SEQ ID NO: 609) used for the peptibody platform with rat IgG2A from crystal structure of FcRn/Fc complex (1H1A.pdb, SEQ ID NO: 610). The resulting consensus sequence (SEQ ID NO: 611) is also shown.

Figure 3A shows the amino acid sequence (SEQ ID NO: 612) of a human IgG1 Fc domain having insertion of a myostatin binding peptide (SEQ ID NO: 365). Hereinafter, this molecule is referred to as the "myostatin loop peptibody" or "Fc-loop-myo7." The inserted peptide is shown in boldface and the glycine linkers in italics.

Figure 3B shows the amino acid sequence (SEQ ID NO: 613) of a C-terminally linked peptibody referred to as TN8-19-07. This peptibody incorporates the same peptide sequence as Fc-loop-myo7 (SEQ ID NO: 365). The TN8-19-07 peptide is shown in boldface and the glycine and alanine linkers in italics.

Figure 3C shows the amino acid sequence (SEQ ID NO: 615) of an Fc internal peptibody referred to hereinafter as Fc-loop-EMP. This peptibody incorporates an EPO-mimetic peptide (SEQ ID NO: 2). The inserted peptide is shown in boldface and the glycine linkers in italics. The cysteines that form a disulfide bond are underlined.

Figure 3D shows the amino acid sequence (SEQ ID NO: 616) of an Fc internal peptibody referred to hereinafter as Fc-loop-Amp2. Bioactive peptide (SEQ ID NO: 28) is highlighted in boldface and glycine linkers in italics. There is no disulfide constraint in the AMP-2 peptide insertion.

Figure 3E shows the amino acid sequence (SEQ ID NO: 617) of a C-terminally linked peptibody referred to hereinafter as Fc-loop-AMP2-dimer. This tandem-linked therapeutic peptide dimer shows the therapeutic peptide sequence (SEQ ID NO: 28) in boldface and the linkers in italics. This molecule incorporates a tandem peptide dimer of the same peptide sequence as found in Fc-loop-AMP-2.

Figures 4A and 4B shows the expression in E. coli of Fc-loop-myo7 and TN8-19-07 by SDS-PAGE (4-20%). Samples of the crude cell lysate (lys), the insoluble fraction (insol) and the soluble (sol) fraction for both the Fc-loop-Myo7 (#6951) and TN8-19-07 (#6826) are shown in reducing gels. SeeBlue and molecular weight markers (lane 1), whole cell lysate (lane 2), insoluble fraction (lane 3) and insoluble fraction (lane 4).

Figure 5 shows a reverse phase, high performance liquid chromatography (RP-HPLC) comparison of the unpurified refold reactions of the Fc-loop-Myo7 (#6951) and TN8-19-07 (#6826). Approximately 10 µg of peptibody was loaded directly from a refold reaction to a Vydak C4 column (5 µM, 300 angstrom, 4.6 x 250 mm) and eluted with a linear 40-50% ACN gradient at 0.5 %/min.

Figure 6 shows reversed-phase high performance liquid chromatography (RP-HPLC) comparison of the final, purified pools of Fc-loop TN8-19-07 (#6951) and carboxy-terminal Fc TN8-19-07 (#6826). Loaded 10 µg purified peptibody to Vydak C4 column (5 µM, 300 angstrom, 4.6 x 250 mm) and eluted with a linear 40-50% ACN gradient at 0.5 %/min.

Figure 7 shows the analyses of final purified pools of Fc-loop TN8-19-07 (#6951) and carboxy-terminal Fc TN8-19-07 (#6826) by SDS-PAGE (4-20% gel). Five µg of each sample was loaded as follows: #6951 (lane 1), #6826 (lane 2), SeeBlue + markers (lane M), #6951 reduced (lane 3), #6826 reduced (lane 4).

Figure 8 shows an in vitro cell-based bioassay for measuring myostatin inhibitory compounds. Fc-loop TN8-19-07 (#6951) retains full inhibitory activity relative to the carboxy-terminal TN8-19-07 peptibody (#6826).

Figure 9 shows a western blot analysis of an in vivo stability study for Fc-loop TN8-19-07 (#6951) and the carboxy-terminal TN8-19-07 peptibody (#6826). Sera pools from five mice were evaluated for each time point (0, 4, 24 and 48 hours). Lanes 1-3 are Fc-loop TN8-19-07 standards at 2 ng, 5 ng and 10 ng respectively. Lanes 4 & 5 are the Fc-loop vs. carboxy terminal peptibodies, respectively, at 4 hours. Lanes 6 & 7 are the Fc-loop vs. carboxy terminal peptibodies respectively at 24 hours. Lanes 8 & 9 are the Fc-loop vs. carboxy terminal peptibodies respectively at 48 hours. Lanes 10-12 are the carboxy-terminal peptibody standard at 2 ng, 5 ng and 10 ng, respectively. The gel was a 1 mm 4-12% SDS-PAGE gel

run in MES reducing buffer and the western blot was developed using a goat anti-human IgG Fc-HRP conjugate.

Figure 10A shows the amino acid sequence (SEQ ID NO: 618) of a human IgG1 Fc domain having insertion of an Ang2 binding peptide (SEQ ID NO: 147). Hereinafter, this molecule is referred to as "Ang2 loop peptibody" or "Fc-loop-Ang2." Bioactive peptide is highlighted in boldface and Glycine linkers in italics.

Figure 10B shows the amino acid sequence (SEQ ID NO: 619) of a C-terminally linked peptibody referred to herein as TN8-Con4. This molecule incorporates the same peptide sequence as Fc-loop-ang2 (SEQ ID NO: 147). The bioactive peptide is highlighted in boldface and the glycine and alanine linkers in italics.

Figure 11 shows the expression and distribution in *E. coli* of the Fc-loop TN8-Con4 (#6888) and carboxy-terminal TN8-Con4 (#5564) peptibodies by SDS-PAGE. Samples of the crude cell lysate (lys), the insoluble fraction (insol) and the soluble (sol) fraction for both the Fc-loop-Tn8-Con4 (#6888) and TN8-Con4 (#5564) are shown in reducing gels.

Figure 12 shows a RP-HPLC comparison of Fc-loop Ang2 (#6888) and carboxy-terminal Fc TN8-19-07 (#5564) refold reactions. Loaded 20 μ l refold reaction to Vydak C4 column (5 μ M, 300 angstrom, 4.6 x 250 mm) and eluted with a linear 40-50% ACN gradient at 0.5 %/min.

Figure 13 shows a RP-HPLC comparison of the final purified pools of Fc-loop Ang2 (#6888) and carboxy-terminal Fc TN8-Con4 (#5564). Ten μ g purified peptibody was loaded to a Vydak C4 column (5 μ M, 300 angstrom, 4.6 x 250 mm) and eluted with a linear 40-50% ACN gradient at 0.5 %/min.

Figure 14 shows purified Fc-loop-myo7 and TN8-19-7.

Figure 15 shows Biacore binding analysis of Fc-loop-ang2 and Fc-ang2-tandem.

Figure 16 shows the results of an in vitro enzyme-linked immunosorbent assay (ELISA) for Fc-loop-ang2, TN8-Con4, and Fc-ang2-tandem.

Figure 17 shows the results of a UT7 erythropoietin proliferation assay for Fc-loop-EMP. In the assay, the activity of two different of Fc-loop-EMP molecules is compared to that of epoetin alfa.

Figure 18 shows the expression and distribution in E. coli of the Fc-loop TN8-Amp2 (#6875) peptibody by SDS-PAGE. Samples of the crude cell lysate (lys), the insoluble fraction (insol) and the soluble (sol) fraction for the Fc-loop-Amp2 (#6888) are shown in reducing gels.

Figure 19 shows an analysis of the final purified pool of Fc-loop AMP 2 (#6875) by SDS-PAGE (4-20% gel). Lane 2 was loaded with 5 μ g Fc-loop AMP 2 peptibody; lane 4 with 5 μ g reduced Fc-loop AMP 2 peptibody; lanes 1 and 3 with SeeBlue and two molecular weight markers.

Figure 20 shows an RP-HPLC analysis of the final purified pool of Fc-loop AMP 2 (#6875). Ten μ g purified peptibody was loaded to Vydak C4 column (5 μ M, 300 angstrom, 4.6 x 250 mm) and eluted with a linear 40-50% ACN gradient at 0.5 %/min.

Figure 21 shows a murine in vivo bioassay of Fc-loop AMP 2 and AMG 531 peptibodies. Mice dosed with a single subcutaneous injection of 50 μ g/kg peptibody or carrier alone. See example 9 for assay methodology.

Figure 22 shows several strategies for incorporating 2 bioactive peptides into an Fc-loop peptibody.

Figure 23 shows SDS-PAGE Gels of purified Fc-loop constructs. Samples (2ug/lane) were run +/- reducing buffer on a 4-20% Tris-Glycine SDS-PAGE gel.

Figure 24 shows RP-HPLC of Fc-loop constructs.

Detailed Description of the Invention

Definition of Terms

The terms used throughout this specification are defined as follows, unless otherwise limited in specific instances.

5 When used in connection with an amino acid sequence, the term "comprising" means that a compound may include additional amino acids on either or both of the N- or C- termini of the given sequence.

 "Antibody" or "antibody peptide(s)" refer to an intact antibody, or a binding fragment thereof that competes with the intact antibody for
10 specific binding and includes chimeric, humanized, fully human, and bispecific antibodies. In certain embodiments, binding fragments are produced by recombinant DNA techniques. In additional embodiments, binding fragments are produced by enzymatic or chemical cleavage of intact antibodies. Binding fragments include, but are not limited to, Fab,
15 Fab', F(ab')₂, Fv, and single-chain antibodies.

 The term "native Fc" refers to a molecule or sequence comprising the sequence of a non-antigen-binding fragment resulting from digestion of whole antibody, whether in monomeric or multimeric form, into which a peptide sequence may be added by insertion into or replacement of a
20 loop region. The original immunoglobulin source of the native Fc is preferably of human origin and may be any of the immunoglobulins, although IgG1 and IgG2 are preferred. Native Fc's are made up of monomeric polypeptides that may be linked into dimeric or multimeric forms by covalent (i.e., disulfide bonds) and non-covalent association. The
25 number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (e.g., IgG, IgA, IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, IgGA2). One example of a native Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG (see Ellison et al. (1982), Nucleic Acids Res. 10: 4071-9). The term
30 "native Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms.

The term "Fc variant" refers to a molecule or sequence that is modified from a native Fc but still comprises a binding site for the salvage receptor, FcRn. International applications WO 97/34631 (published 25 September 1997) and WO 96/32478 describe exemplary Fc variants, as well as interaction with the salvage receptor, and are hereby incorporated by reference. Thus, the term "Fc variant" comprises a molecule or sequence that is humanized from a non-human native Fc. Furthermore, a native Fc 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 "Fc variant" comprises a molecule or sequence that lacks one or more native Fc sites or residues that affect or are involved in (1) disulfide bond formation, (2) incompatibility with a selected host cell (3) N-terminal heterogeneity upon expression in a selected host cell, (4) glycosylation, (5) interaction with complement, (6) binding to an Fc receptor other than a salvage receptor, or (7) antibody-dependent cellular cytotoxicity (ADCC). Fc variants are described in further detail hereinafter.

The term "Fc domain" encompasses native Fc and Fc variant molecules and sequences as defined above. As with Fc variants and native Fc's, the term "Fc domain" includes molecules in monomeric or multimeric form, whether digested from whole antibody or produced by other means.

The term "multimer" as applied to Fc domains or molecules comprising Fc domains refers to molecules having two or more polypeptide chains associated covalently, noncovalently, or by both covalent and non-covalent interactions. IgG molecules typically form dimers; IgM, pentamers; IgD, dimers; and IgA, monomers, dimers, trimers, or tetramers. Multimers may be formed by exploiting the sequence and resulting activity of the native Ig source of the Fc or by derivatizing (as defined below) such a native Fc.

The term "dimer" as applied to Fc domains or molecules comprising Fc domains refers to molecules having two polypeptide chains associated covalently or non-covalently. Exemplary dimers within the scope of this invention are as shown in U.S. Pat. No. 6,660,843, Figure 2,
5 which is hereby incorporated by reference.

The terms "derivatizing" and "derivative" or "derivatized" comprise processes and resulting compounds respectively in which (1) the compound has a cyclic portion; for example, cross-linking between cysteinyl residues within the compound; (2) the compound is cross-linked
10 or has a cross-linking site; for example, the compound has a cysteinyl residue and thus forms cross-linked dimers in culture or *in vivo*; (3) one or more peptidyl linkage is replaced by a non-peptidyl linkage; (4) the N-terminus is replaced by -NRR¹, NRC(O)R¹, -NRC(O)OR¹, -NRS(O)₂R¹, -NHC(O)NHR, a succinimide group, or substituted or unsubstituted
15 benzyloxycarbonyl-NH-, wherein R and R¹ and the ring substituents are as defined hereinafter; (5) the C-terminus is replaced by -C(O)R² or -NR³R⁴ wherein R², R³ and R⁴ are as defined hereinafter; and (6) compounds in which individual amino acid moieties are modified through treatment with agents capable of reacting with selected side chains or terminal
20 residues. Derivatives are further described hereinafter.

The term "polypeptide" refers to molecules of greater than 40 amino acids, whether existing in nature or not, provided that such molecules are not membrane-bound. Exemplary polypeptides include IL-1ra, leptin, soluble TNF receptors type 1 and type 2 (sTNF-R1, sTNF-R2),
25 KGF, EPO, TPO, G-CSF, darbepoietin, Fab fragments and the like.

The term "peptide" refers to molecules of 2 to 40 amino acids, with molecules of 3 to 20 amino acids preferred and those of 6 to 15 amino acids most preferred. Exemplary peptides may be randomly generated by any of the methods cited above, carried in a peptide library (e.g., a phage
30 display library), or derived by digestion of proteins.

The term "randomized" as used to refer to peptide sequences refers to fully random sequences (e.g., selected by phage display methods) and sequences in which one or more residues of a naturally occurring molecule is replaced by an amino acid residue not appearing in that position in the naturally occurring molecule. Exemplary methods for identifying peptide sequences include phage display, E. coli display, ribosome display, yeast-based screening, RNA-peptide screening, chemical screening, rational design, protein structural analysis, and the like.

The term "pharmacologically active" means that a substance so described is determined to have activity that affects a medical parameter (e.g., blood pressure, blood cell count, cholesterol level) or disease state (e.g., cancer, autoimmune disorders). Thus, pharmacologically active peptides comprise agonistic or mimetic and antagonistic peptides as defined below.

The terms "-mimetic peptide" and "-agonist peptide" refer to a peptide having biological activity comparable to a protein (e.g., EPO, TPO, G-CSF) 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; see, for example, the G-CSF-mimetic peptides listed in Table 2 hereof and in Table 7 of U.S. Pat. No. 6,660,843, which is hereby incorporated by reference. Thus, the term "EPO-mimetic peptide" comprises any peptides that can be identified or derived as described in Wrighton et al. (1996), Science 273: 458-63, Naranda et al. (1999), Proc. Natl. Acad. Sci. USA 96: 7569-74, or any other reference in Table 2 identified as having EPO-mimetic subject matter. 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 "-antagonist peptide" or "inhibitor peptide" refers to a peptide that blocks or in some way interferes with the biological activity of

the associated protein of interest, or has biological activity comparable to a known antagonist or inhibitor of the associated protein of interest. Thus, the term "TNF-antagonist peptide" comprises peptides that can be identified or derived as described in Takasaki *et al.* (1997), *Nature Biotech.* 15: 1266-70 or any of the references in Table 2 identified as having TNF-antagonistic subject matter. 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" comprises peptides that can be identified or derived as described in Cwirla *et al.* (1997), *Science* 276: 1696-9, U.S. Pat. Nos. 5,869,451 and 5,932,946; U.S. Pat. App. No. 2003/0176352, published Sept. 18, 2003; WO 03/031589, published April 17, 2003 and any other reference in Table 2 identified as having TPO-mimetic subject matter, as well as WO 00/24770, published May 4, 2000, which is hereby incorporated by reference. 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 "ang-2-binding peptide" comprises peptides that can be identified or derived as described in U.S. Pat. App. No. 2003/0229023, published Dec. 11, 2003; WO 03/057134, published 7/17/03; U.S. 2003/0236193, published Dec. 25, 2003; and any other reference in Table 2 identified as having subject matter related to ang-2. 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 "NGF-binding peptide" comprises peptides that can be identified or derived as described in WO 04/026329, published April 1, 2004 and any other reference in Table 2 identified as having subject matter related to NGF. 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 "myostatin-binding peptide" comprises peptides that can be identified or derived as described in U.S. Ser. No. 10/742,379, filed December 19, 2003 and any other reference in Table 2 identified as having subject matter related to myostatin. 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.

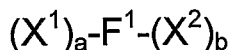
Additionally, physiologically acceptable salts of the compounds of this invention are also encompassed herein. By "physiologically acceptable salts" is meant any salts that are known or later discovered to be pharmaceutically acceptable. Some specific examples are: acetate; trifluoroacetate; hydrohalides, such as hydrochloride and hydrobromide; sulfate; citrate; tartrate; glycolate; and oxalate.

Structure of compounds

In General

In the compositions of matter prepared in accordance with this invention, the peptide may be attached to the vehicle through the peptide's N-terminus or C-terminus. Thus, the vehicle-peptide molecules of this invention may be described by the following formula I:

I



wherein:

F¹ is an Fc domain modified so that it comprises at least one X³ in a loop region;

X¹ and X² are each independently selected from -(L¹)_c-P¹, -(L¹)_c-P¹-(L²)_d-P², -(L¹)_c-P¹-(L²)_d-P²-(L³)_e-P³, and -(L¹)_c-P¹-(L²)_d-P²-(L³)_e-P³-(L⁴)_f-P⁴;

X³ is independently selected from -(L⁵)_c-P⁵, -(L⁵)_c-P⁵-(L⁶)_d-P⁶, -(L⁵)_c-P⁵-(L⁶)_d-P⁶-(L⁷)_e-P⁷, and -(L⁵)_c-P⁵-(L⁶)_d-P⁶-(L⁷)_e-P⁷-(L⁸)_f-P⁸;

P¹, P², P³, and P⁴ are each independently sequences of pharmacologically active polypeptides or pharmacologically active peptides;

P⁵, P⁶, P⁷, and P⁸ are each independently sequences of
 5 pharmacologically active peptides;

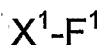
L¹, L², L³, L⁴, L⁵, L⁶, L⁷, and L⁸ are each independently linkers; and
 a, b, c, d, e, and f are each independently 0 or 1.

In preferred embodiments, a and b are both zero--i.e., neither X¹ nor X² groups appear at the N-terminus or C-terminus of the Fc domain.

10 Those of ordinary skill in the art will appreciate that more than one X³ substituent may be present in the Fc domain, and that the multiple X³ substituents may be different; for example, comprising different P⁵ peptides, different linkers attached to the same peptide sequence, and so on. Likewise, X¹ and X² may be the same or different, and the integers c
 15 through f may be different for X¹, X², and X³.

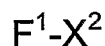
Thus, compound I comprises compounds of the formulae

II



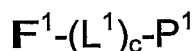
and multimers thereof wherein F¹ is attached at the C-terminus of X¹;

20 III



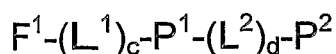
and multimers thereof wherein F¹ is attached at the N-terminus of X²;

IV



25 and multimers thereof wherein F¹ is attached at the N-terminus of -(L¹)_c-P¹; and

V



and multimers thereof wherein F¹ is attached at the N-terminus of -L¹-P¹-
 30 L²-P².

Peptides

Any number of peptides may be used in conjunction with the present invention. Preferred peptides bind to angiopoietin-2 (ang-2), myostatin, nerve growth factor (NGF), tumor necrosis factor (TNF), B cell activating factor (BAFF, also referred to as TALL-1) or mimic the activity of EPO, TPO, or G-CSF. Targeting peptides are also of interest, including tumor-homing peptides, membrane-transporting peptides, and the like. All of these classes of peptides may be discovered by methods described in the references cited in this specification and other references.

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

A particularly preferred group of peptides are those that bind to cytokine receptors. Cytokines have recently been classified according to their receptor code. See Inglot (1997), *Archivum Immunologiae et Therapiae Experimentalis* 45: 353-7, which is hereby incorporated by reference. Among these receptors, most preferred are the CKRs (family I in Table 3). The receptor classification appears in Table 3.

Table 3—Cytokine Receptors Classified by Receptor Code

Cytokines (ligands)		Receptor Type	
family	subfamily	family	subfamily
I. Hematopoietic cytokines	1. IL-2, IL-4, IL-7, IL-9, IL-13, IL-15	I. Cytokine R (CKR)	1. shared γ Cr, IL-9R, IL-4R
	2. IL-3, IL-5, GM-CSF		2. shared GP 140 β R
	3. IL-6, IL-11, IL-		3. 3.shared RP

	12, LIF, OSM, CNTF, Leptin (OB)		130, IL-6 R, Leptin R
	4. G-CSF, EPO, TPO, PRL, GH		4. "single chain" R, GCSF-R, TPO-R, GH-R
	5. IL-17, HVS-IL-17		5. other R ^b
II. IL-10 ligands	IL-10, BCRF-1, HSV-IL-10	II. IL-10 R	
III. Interferons	1. IFN- α 1, α 2, α 4, m, t, IFN- β ^c	III. Interferon R	1. IFNAR
	2. IFN- γ		2. IFNGR
IV. IL-1 and IL-1 like ligands	1. IL-1 α , IL-1 β , IL-1Ra	IV. IL-1R	1. IL-1R, IL-1RAcP
	2. IL-18, IL-18BP		2. IL-18R, IL-18RAcP
V. TNF family	1. TNF- α , TNF- β (LT), FASL, CD40 L, CD30L, CD27 L, OX40L, OPGL, TRAIL, APRIL, AGP-3, BLys, TL5, Ntn-2, KAY, Neutrokin- α	3. NGF/TNF R ^d	TNF-RI, AGP-3R, DR4, DR5, OX40, OPG, TACI, CD40, FAS, ODR
VI. Chemokines	1. α chemokines: IL-8, GRO α , β , γ , IF-10, PF-4, SDF-1	4. Chemokine R	1. CXCR
	2. β chemokines: MIP1 α , MIP1 β , MCP-1,2,3,4, RANTES, eotaxin		2. CCR
	3. γ chemokines: lymphotactin		3. CR
			4. DARC ^e
VII. Growth factors	1.1 SCF, M-CSF, PDGF-AA, AB, BB, KDR, FLT-1, FLT-3L,	VII. RKF	1. TK sub-family
			1.1 IgTK III R, VEGF-RI, VEGF-RII

¹ IL-17R - belongs to CKR family but is unassigned to 4 indicated subfamilies.

² Other IFN type I subtypes remain unassigned. Hematopoietic cytokines, IL-10 ligands and interferons do not possess functional intrinsic protein kinases. The signaling molecules for the cytokines are JAK's, STATs and related non-receptor molecules. IL-14, IL-16 and IL-18 have been cloned but according to the receptor code they remain unassigned.

³ TNF receptors use multiple, distinct intracellular molecules for signal transduction including "death domain" of FAS R and 55 kDa TNF- α R that participates in their cytotoxic effects. NGF/TNF R can bind both NGF and related factors as well as TNF ligands. Chemokine receptors are seven transmembrane (7TM, serpentine) domain receptors. They are G protein-coupled.

⁴ The Duffy blood group antigen (DARC) is an erythrocyte receptor that can bind several different chemokines. IL-1R belongs to the immunoglobulin superfamily but their signal transduction events characteristics remain unclear.

VEGF, SSV- PDGF, HGF, SF	
1.2 FGF α , FGF β	1.2 IgTK IV R
1.3 EGF, TGF- α , VV-F19 (EGF- like)	1.3 Cysteine-rich TK-I
1.4 IGF-I, IGF-II, Insulin	1.4 Cysteine rich TK-II, IGF-RI
1.5 NGF, BDNF, NT-3, NT-4 ^f	1.5 Cysteine knot TK V
2. TGF- β 1, β 2, β 3	2. Serine- threonine kinase subfamily (STKS) ^g

Particular proteins of interest as targets for peptide generation in the present invention include the following:

α v β 3
 α V β 1
 Ang-2
 BAFF/TALL-1
 B7
 B7RP1
 CRP1
 Calcitonin
 CD28
 CETP
 cMet
 Complement factor B
 C4b
 CTLA4
 Glucagon
 Glucagon Receptor
 LIPG
 MPL
 myostatin
 splice variants of molecules preferentially expressed on
 tumor cells; e.g., CD44, CD30
 unglycosylated variants of mucin and Lewis Y surface
 glycoproteins
 CD19, CD20, CD33, CD45
 prostate specific membrane antigen and prostate specific cell
 antigen

⁵ The neurotrophic cytokines can associate with NGF/TNF receptors also.

⁶ STKS may encompass many other TGF- β -related factors that remain unassigned. The protein kinases are intrinsic part of the intracellular domain of receptor kinase family (RKF). The enzymes participate in the signals transmission via the receptors.

matrix metalloproteinases (MMPs), both secreted and
membrane-bound (e.g., MMP-9)
Cathepsins
angiopoietin-2
TIE-2 receptor
heparanase
urokinase plasminogen activator (UPA), UPA receptor
parathyroid hormone (PTH), parathyroid hormone-related
protein (PTHrP), PTH-RI, PTH-RII
Her2
Her3
Insulin

Exemplary peptides for this invention appear in Tables 4 through
20 of U.S. Pat. No. 6,660,843, which are hereby incorporated by reference.
Additional preferred peptides appear in U.S. 2003/0229023, published
Dec. 11, 2003; WO 03/057134, published July 17, 2003; U.S. 2003/0236193,
published Dec. 25, 2003; WO 00/24770, published May 4, 2000; U.S.
2003/0176352, published Sept. 18, 2003; WO 03/031589, published April
17, 2003; U.S. Ser. No. 10/666,480, filed September 18, 2003; WO
04/026329, published April 1, 2004; U.S. Ser. No. 10/742,379, filed
December 19, 2003; PCT/US03/40781, filed December 19, 2003, each of
which are hereby incorporated by reference. Such peptides may be
prepared by methods disclosed in the art.

Particularly preferred peptides appear in the tables below. Single
letter amino acid abbreviations are used. Any of these peptides may be
linked in tandem (i.e., sequentially), with or without linkers. Any peptide
containing a cysteinyl residue may be cross-linked with another Cys-
containing peptide or protein. Any peptide having more than one Cys
residue may form an intrapeptide disulfide bond, as well. Any of these
peptides may be derivatized as described herein. All peptides are linked
through peptide bonds unless otherwise noted.

Table 4—EPO-mimetic peptide sequences

SEQUENCE	SEQ ID NO:
YXCXXGPXTWXCXP	1
GGTYSCHFGPLTWVCKPQGG	2
GGDYHCRMGPITWVCKPLGG	3
GGVYACRMGPITWVCSPLGG	4
VGNYMCHFGPITWVCRPGGG	5
GGLYLCRFGPVTWDCGYKGG	6
GGTYSCHFGPLTWVCKPQGGSSK	7
GGTYSCHGPLTWVCKPQGG	8
VGNYMAHMGPIWVCRPGG	9
GGPHHVYACRMGPITWIC	10
GGTYSCHFGPLTWVCKPQ	11
GGLYACHMGPMWVWCQPLRG	12
TIAQYICYMGPETWECRPSKA	13
YSCHFGPLTWVCK	14
YCHFGPLTWVC	15
GGLYLCRFGPVTWDCGYKGG	16
GGTYSCHFGPLTWVCKPQGG	17
GGDYHCRMGPITWVCKPLGG	18
VGNYMCHFGPITWVCRPGGG	19
GGVYACRMGPITWVCSPLGG	20
VGNYMAHMGPIWVCRPGG	21
GGTYSCHFGPLTWVCKPQ	22
GGLYACHMGPMWVWCQPLRG	23
TIAQYICYMGPETWECRPSKA	24
YSCHFGPLTWVCK	25
YCHFGPLTWVC	26
SCHFGPLTWVCK	27

Table 5—TPO-mimetic peptide sequences

SEQUENCE	SEQ ID NO:
IEGPTLRQWLAARA	28
IEGPTLRQWLAACA	29
IEGPTLRQWLAARA	30
TLREWL	31
GRVRDQVAGW	32
GRVKDQIAQL	33
GVRDQVSWAL	34
ESVREQVMKY	35
SVRSQISASL	36
GVRETVYRHM	37
GVREVIVMHML	38

GRVRDQIWAAL	39
AGVRDQILIWL	40
GRVRDQIMLSL	41
CTLRQWLQGC	42
CTLQEFLEGC	43
CTRTEWLHGC	44
CTLREWLHGGFC	45
CTLREWVFAGLC	46
CTLRQWLILLGMC	47
CTLAEFSLASGVEQC	48
CSLQEFSLHGGYVC	49
CTLREFLDPTTAVC	50
CTLKEWLVSHEVWC	51
REGPTLRQWM	52
EGPTLRQWLA	53
ERGPFWAKAC	54
REGPRCVMWM	55
CGTEGPTLSTWDC	56
CEQDGPTLLEWLKC	57
CELVGPSLMSWLTC	58
CLTGPFVTQWLYEC	59
CRAGPTLLEWLTLC	60
CADGPTLREWISFC	61
GGCTLREWLHGGFCGG	62
GGCADGPTLREWISFCGG	63
GNADGPTLRQWLEGRRPKN	64
LAIEGPTLRQWLHGNGRDT	65
HGRVGPTLREWKTQVATKK	66
TIKGPTLRQWLKSREHTS	67
ISDGPTLKEWLSVTRGAS	68
SIEGPTLREWLTSRTPHS	69
GAREGPTLRQWLEWVRVG	70
RDLDGPTLRQWLPLPSVQ	71
ALRDGPTLKQWLEYRRQA	72
ARQEGPTLKEWLFWVRMG	73
EALLGPTLREWLAWRRAQ	74
MARDGPTLREWLRTYRMM	75
WMPEGPTLKQWLFHGRGQ	76
HIREGPTLRQWLVALRMV	77
QLGHGPTLRQWLSWYRGM	78
ELRQGPTLHEWLQHLASK	79
VGIEGPTLRQWLAQRLNP	80
WSRDGPTLREWLAWRAVG	81
AVPQGPTLKQWLLWRRCA	82

RIREGPTLKEWLAQRRGF	83
RFAEGPTLREWLEQRKLV	84
DRFQGPTLREWLAAIRSV	85
AGREGPTLREWLNMVRVWQ	86
ALQEGPTLRQWLGWGQWG	87
YCDEGPTLKQWLVCGLQ	88
WCKEGPTLREWLRWGFLC	89
CSSGGPTLREWLQCRMVQ	90
CSWGGPTLKQWLQCVRAK	91
CQLGGPTLREWLACRLGA	92
CWEGGPTLKEWLQCLVER	93
CRGGGPTLHQWLSCFRWQ	94
CRDGGPTLRQWLACLQQK	95
ELRSGPTLKEWLVRRLAQ	96
GCRSGPTLREWLACREVQ	97
TCEQGPTLRQWLLCRQGR	98
QGYCDEGPTLKQWLVCGLQHS	99

Table 6 – Ang-2 binding peptide sequences

SEQUENCE	SEQ ID NO.
WDPWT	100
WDPWTC	101
Cz ² WDPWT (wherein z ² is an acidic or neutral polar amino acid residue)	102
Cz ² WDPWTC (wherein z ² is an acidic or neutral polar amino acid residue)	103
PIRQEECDWDPWTCEHMWEV	104
TNIQEECEWDPWTCDHMPGK	105
WYEQDACEWDPWTCEHMAEV	106
NRLQEVCEWDPWTCEHMENV	107
AATQEECEWDPWTCEHMPRS	108
LRHQEGCEWDPWTCEHMFWD	109
VPRQKDCEWDPWTCEHMYVG	110
SISHEECEWDPWTCEHMQVG	111
WAAQEECEWDPWTCEHMGRM	112
TWPQDKCEWDPWTCEHMGST	113
GHSQEECGWDPWTCEHMGTS	114
QHWQEECEWDPWTCDHMPK	115
NVRQEKCEWDPWTCEHMPVR	116
KSGQVECNWDPWTCEHMPRN	117
VKTQEHCDWDPWTCEHMREW	118
AWGQEGCDWDPWTCEHMLPM	119

PVNQEDCEWDPWTCEHMPPM	120
RAPQEDCEWDPWTCAHMDIK	121
HGQNMECEWDPWTCEHMFY	122
PRLQEECVWDPWTCEHMPLR	123
RTTQEKCEWDPWTCEHMESQ	124
QTSQEDCVWDPWTCDHMOVSS	125
QVIGRPCEWDPWTCEHLEGL	126
WAQQEECAWDPWTCDHMOVGL	127
LPGQEDCEWDPWTCEHMOVRS	128
PMNQVECDWDPWTCEHMVPRS	129
FGWSHGCEWDPWTCEHMGST	130
KSTQDDCDWDPWTCEHMOVGP	131
GPRISTCQWDPWTCEHMDQL	132
STIGDMCEWDPWTCAHMOVQD	133
VLGGQGCEWDPWTCRLLQGW	134
VLGGQGCQWDPWTCSHLEDG	135
TTIGSMCEWDPWTCAHMOVGG	136
TKGKSVCQWDPWTCSHMOVSG	137
TTIGSMCQWDPWTCAHMOVGG	138
WVNEVVCEWDPWTCNHWDTP	139
VVQVGMCQWDPWTCKHMLRQ	140
AVGSQTCEWDPWTCAHLVEV	141
QGMKMFCEWDPWTCAHIVYR	142
TTIGSMCQWDPWTCEHMOVGG	143
TSQRVGCEWDPWTCQHLYT	144
QWSWPPCEWDPWTCQTVWPS	145
GTSPSFCQWDPWTCSHMOVQG	146
QEECEWDPWTCEHM	147
QNYKPLDELDATLYEHFIFHYT	148
LNFTPLDELEQTLYEQWTLQQS	149
TKFNPLDELEQTLYEQWTLQHQ	150
VKFKPLDALEQTLYEHWMFQQA	151
VKYKPLDELDEILYEQQTFQER	152
TNFMMPMDDEQLRYEQFILQQG	153
SKFKPLDELEQTLYEQWTLQHA	154
QKFQPLDELEQTLYEQFMLQQA	155
QNFKPMDELEDTLKYQFLFQHS	156
YKFTPLDDLEQTLYEQWTLQHV	157
QEYEPLDELDETLYNQWMFHQR	158
SNFMPLDELEQTLYEQFMLQHQ	159
QKYQPLDELDKTLYDQFMLQQG	160
QKFQPLDELEETLYKQWTLQQR	161
VKYKPLDELDEWLYHQFTLHHQ	162

QKFMPLDELDEILYEQFMFQQS	163
QTFQPLDDLEEYLYEQWIRRYH	164
EDYMPLDALDAQLYEQFILLHG	165
HTFQPLDELEETLYYQWLYDQL	166
YKFNPMDLEEQTLYEFLFQHA	167
TNYKPLDELDATLYEHWILQHS	168
QKFKPLDELEQTLYEQWTLQQR	169
TKFQPLDELDQTLYEQWTLQQR	170
TNFQPLDELDQTLYEQWTLQQR	171
KFNPLDELEETLYEQFTFQQ	172
AGGMRPYDGMLGWPNYDVQA	173
QTWDDPCMHILGPVTWRRCI	174
APGQRPYDGMLGWPTYQRIV	175
SGQLRPCEEIFGCGTQNLAL	176
FGDKRPLECMFGGPIQLCPR	177
GQDLRPCEDMFGCGTKDWYG	178
KRPCEEIFGGCTYQ	179
GFEYCDGMEDPFTFGCDKQT	180
KLEYCDGMEDPFTQGCNDQS	181
LQEWCEGVDPFTFGCEKQR	182
AQDYCEGMEDPFTFGCEMQK	183
LLDYCEGVQDPFTFGCENLD	184
HQEYCEGMEDPFTFGCEYQG	185
MLDYCEGMDDPFTFGCDKQM	186
LQDYCEGVDPFTFGCENQR	187
LQDYCEGVDPFTFGCEKQR	188
FDYCEGVDPFTFGCDNH	189

Table 7--NGF-Binding Peptide Sequences

SEQUENCE	SEQ ID NO.
TGYTEYTEEWPMGFGYQWSF	190
TDWLSDFPFYEYFGLMPPG	191
FMRFNPWKLVEPPQGWYYG	192
VVKAPHFEFLAPPHFHEFPF	193
FSYIWIDETPSNIDRYMLWL	194
VNFPKVPEDVEPWPSLKLY	195
TWHPKTYEEFALPFFVPEAP	196
WHFGTPYIQQQPGVYWLQAP	197
VWNYGPFFMNFDPSTYFLHE	198
WRIHSKPLDYSHVWFPPADF	199
FWDGNQPPDILVDWPWNPPV	200

FYSLEWLKDHSEFFQTVTEW	201
QFMELLKFFNSPGDSSHFL	202
TNVDWISNNWEHMKSFTE	203
PNEKPYQMMSWFPPDWPVPY	204
WSHTEWVPQVWWKPPNHFYV	205
WGEWINDAQVHMHEGFISES	206
VPWEHDHDLWEIISQDWHIA	207
VLHLQDPRGWSNFPPGVLEL	208
IHGCWFTEEGCVWQ	209
YMQCQFARDGCPQW	210
KLQCQYSESGCPTI	211
FLQCEISGGACPAP	212
KLQCEFSTSGCPDL	213
KLQCEFSTQGCPDL	214
KLQCEFSTSGCPWL	215
IQGCWFTEEGCPWQ	216
SFDCDNPWGHVQLQSCFGF	217
SFDCDNPWGHKLQSCFGF	218

Table 8 – Myostatin binding peptide sequences

SEQUENCE	SEQ ID NO:
KDKCKMWHWMCKPP	647
KDLCAMWHWMCKPP	219
KDLCKMWKWMCKPP	220
KDLCKMWHWMCKPK	221
WYPCYEFHFWCYDL	222
WYPCYEGHFWCYDL	223
IFGCKWWDVQCYQF	224
IFGCKWWDVDCYQF	225
ADWCVSPNWFCMVM	226
HKFCPWWALFCWDF	227
KDLCKMWHWMCKPP	228
IDKCAIWGWMCPL	229
WYPCGEFGMWCLNV	230
WFTCLWNCDNE	231
HTPCPWFAPLCVEW	232
KEWCWRWKWMCKPE	233
FETCPSWAYFCLDI	234
AYKCEANDWGCWWL	235
NSWCEDQWHRCWWL	236
WSACYAGHFWCYDL	237
ANWCVSPNWFCMVM	238
WTECYQQEFWCWNL	239

ENTCERWKWMCPPK	240
WLPCHQEGFWCMNF	241
STMCSQWFIWMCNPF	242
IFGCHWWDVDCYQF	243
IYGCKWWDIQCYDI	244
PDWCIDPDWWCKFW	245
QGHCTRWPWMCPPY	246
WQECYREGFWCLQT	247
WFDCYGPFGKWCSP	248
GVRCPKGHLWCLYP	249
HWACGYWPWSCKWV	250
GPACHSPWWWCVFG	251
TTWCISPMWFCSQQ	252
HKFCPPWAIFCWDF	253
PDWCVSPRWYCNMW	254
VWKCHWFGMDCEPT	255
KKHCQIWTWMCAPK	256
WFQCGSTLFWCYNL	257
WSPCYDHYFYCYTI	258
SWMCGFFKEVCMWV	259
EMLCMIHPVFCNPH	260
LKTCNLWPWMCPL	261
VVGCKWYEAWCYNK	262
PIHCTQWAWMCPPT	263
DSNCPWYFLSCVIF	264
HIWCNLAMMKCVEM	265
NLQCIYFLGKCIYF	266
AWRCMWFSDVCTPG	267
WFRCLDADWCTSV	268
EKICQMWSWMCAPP	269
WFYCHLNKSECTEP	270
FWRCAIGIDKCKRV	271
NLGCKWYEVWCFTY	272
IDLCNMWDGMCYPP	273
EMPCNIWGWMCPPV	274
WFRCVLTGIVDWSECFGL	275
GFSCTFGLDEFYVDCSPF	276
LPWCHDQVNADWGFCMLW	277
YPTCSEKFWIYGQTCVLW	278
LGPCPIHHGPWPQYCVYW	279
PFPCEHQISWLGHCLSF	280
HWGCEDLMWSWHPLCRRP	281
LPLCDADMIMPTIGFCVAY	282
SHWCETTFWMNYAKCVHA	283

LPKCTHVPFDQGGFCLWY	284
FSSCWSPVSRQDMFCVFY	285
SHKCEYSGWLQPLCYRP	286
PWWCQDNYVQHMLHCDS	287
WFRCLMNSFDAFQCVSY	288
PDACRDQPWYMFMGCM LG	289
FLACFVEFELCFDS	290
SAYCIITESDPYVLCVPL	291
PSICESYSTMWLPMCQHIN	292
WLDCHDDSWAWTKMCRSH	293
YLNCVMMNTSPFVECVFN	294
YPWCDGFMIIQQGITCMFY	295
FDYCTWLNGFKDWKCWSR	296
LPLCNLKEISHVQACVLF	297
SPECAFARWLGIEQCQRD	298
YPQCFNLHLLIEWTECDWF	299
RWRCEIYDSEFLPKCWFF	300
LVGCDNVWHRCKLF	301
AGWCHVWGEMFGMGCSAL	302
HHECEWMARWMSLDCVGL	303
FPMCGIAGMKDFDFCVWY	304
RDDCTFWPEWLWKLCEP	305
YNFCSYLFVGSKEACQLP	306
AHWCEQGPWRYGNICMAY	307
NLVCGKISAWGDEACARA	308
HNVCTIMGPSMKWFCWND	309
NDLCAMWGWRTIWCQNS	310
PPFCQNDNDMLQSLCKLL	311
WYDCNVPNELLSGLCRLF	312
YGDCDQNHWMWPFTCLSL	313
GWMCHFDLHDWGATCQPD	314
YFHCMFGGHEFEVHCESE	315
AYWCWHGQCVR	316
SEHWTFTDWDGNEWVVRPF	317
MEMLDSLFELLKDMVPISKA	318
SPPEEALMEWLGWQYGKFT	319
SPENLLNDLYILMTKQEWYG	320
FHWEEGIPFHVVTPYSYDRM	321
KRLLEQFMNDLAEVSGHS	322
DTRDALFQEFYEFVRSRLVI	323
RMSAAPRPLTYRDIMDQYWH	324
NDKAHFFEMFMFDVHNFVES	325
QTQAQKIDGLWELLQSI RNQ	326
MLSEFEEFLGNLVHRQEA	327

YTPKMGSEWTSFWHNRIHYL	328
LNDTLLRELKMVLNSLSDMK	329
FDVERDLMRWLEGFMQSAAT	330
HHGWNYLKRGSAPOWFEEAWV	331
VESLHQLQMWLDQKLASGPH	332
RATLLKDFWQLVEGYGDN	333
EELLREFYRFVSAFDY	334
GLLDEFSHFIAEQFYQMPGG	335
YREMSMLEGLLDVLERLQHY	336
HNSSQMLLSELIMLVGSM MQ	337
WREHFLNSDYIRDKLIADG	338
QFPFYVFDDLPAQLEYWIA	339
EFFHWLHNHRSEVNHWLD MN	340
EALFQNFVRDVLTLSEREY	341
QYWEQQWMTYFRENGLHVQY	342
NQRMMLDLWRIMTPMFGRS	343
FLDELKAEISRHYALDDLDE	344
GKLIEGLLNELMQLETMPD	345
ILLDEYKKDWKSWF	346
QGHCTRWPWMCPPYGSGSATGGSGST ASSGSGSATGQGHCTRWPWMCPPY	347
WYPCYEGHFWCYDLGSGSTASSGSGSA TGWYPCYEGHFWCYDL	348
HTPCPWFAPLCVEWVGSGSATGGSGSTA SSGSGSATGHTPCPWFAPLCVEW	349
PDWCIDPDWWCKFWGSGSATGGSGST ASSGSGSATGPDWCIDPDWWCKFW	350
ANWCVSPNWFCMVMGSGSATGGSGST ASSGSGSATGANWCVSPNWFCMVM	351
PDWCIDPDWWCKFWGSGSATGGSGST ASSGSGSATGPDWCIDPDWWCKFW	352
HWACGYWPWSCKWVGSGSATGGSGST ASSGSGSATGHWACGYWPWSCKWV	353
KKHCQIWTWMCAPKGSGSATGGSGST ASSGSGSATGQGHCTRWPWMCPPY	354
QGHCTRWPWMCPPYGSGSATGGSGST ASSGSGSATGKKHCQIWTWMCAPK	355
KKHCQIWTWMCAPKGSGSATGGSGST ASSGSGSATGQGHCTRWPWMCPPY	356
KKHCQIWTWMCAPKGGGGGGGGQGH CTRWPWMCPPY	357
QGHCTRWPWMCPPYGGGGGGGKKHCQ IWTWMCAPK	358
VALHGQCTRWPWMCPPOREG	359
YPEQGLCTRWPWMCPPOTLA	360

GLNQGHCTRWPWMCPPQDSN	361
MITQGGCTRWPWMCPPQPSG	362
AGAQEHCTRWPWMCAPNDWI	363
GVNQGGCTRWRWMCPPNGWE	364
LADHGGCIRWPWMCPPEGWE	365
ILEQAQCTRWPWMCPPQRGG	366
TQTHAQCTRWPWMCPPQWEG	367
VVTQGHCTLWPWMCPPQRWR	368
IYPHDQCTRWPWMCPPQPYP	369
SYWQGGCTRWPWMCPPQWRG	370
MWQQGHCTRWPWMCPPQGWG	371
EFTQWHCTRWPWMCPPQRSQ	372
LDDQWQCTRWPWMCPPQGFS	373
YQTQGLCTRWPWMCPPQSQR	374
ESNQGGCTRWPWMCPPQGGW	375
WDRGPCTRWPWMCPPQANG	376
VGTQGGCTRWPWMCPPYETG	377
PYEQGKCTRWPWMCPPYEVE	378
SEYQGLCTRWPWMCPPQGWK	379
TFSQGHCTRWPWMCPPQGWG	380
PGAHDHCTRWPWMCPPQSR	381
VAEEWHCRRWPWMCPPQDWR	382
VGTQGHCTRWPWMCPPQPAG	383
EEDQAHCRSWPWMCPPQGWV	384
ADTQGHCTRWPWMCPPQHWF	385
SGPQGHCTRWPWMCAPQGWF	386
TLVQGHCTRWPWMCPPQRWV	387
GMAHGKCTRWAWMCPPQSWK	388
ELYHGQCTRWPWMCPPQSWA	389
VADHGHCTRWPWMCPPQGWG	390
PESQGHCTRWPWMCPPQGWG	391
IPAHGHCTRWPWMCPPQRWR	392
FTVHGHCTRWPWMCPPYGWV	393
PDFPGHCTRWRWMCPPQGWE	394
QLWQGPCTQWPWMCPPKGRY	395
HANDGHCTRWQWMCPPQWGG	396
ETDHGLCTRWPWMCPPYGAR	397
GTWQGLCTRWPWMCPPQGWQ	398
VATQGGCTRWPWMCPPQGWG	399
VATQGGCTRWPWMCPPQRWG	400
QREWYPCYGGHLWCYDLHKA	401
ISAWYSCYAGHFWCWDLKQK	402
WTGWYQCYGGHLWCYDLRRK	403
KTFWYPCYDGHFWCYNLKSS	404

ESRWYPCYEGHLWCFDLTET	405
MEMLDSLFELLKDMVPISKA	406
RMEMLESLELLKEIVPMSKAG	407
RMEMLESLELLKEIVPMSKAR	408
RMEMLESLELLKDIVPMSKPS	409
GMEMLESLELLQEIVPMSKAP	410
RMEMLESLELLKDIVPISNPP	411
RIEMLESLELLQEIVPISKA	412
RMEMLQSLLELLKDIVPMSNAR	413
RMEMLESLELLKEIVPTSNGT	414
RMEMLESLELLKEIVPMSKAG	415
RMEMLGSLELLKEIVPMSKAR	416
QMELLDLSELLKEIVPKSQPA	417
RMEMLDSLELLKEIVPMSNAR	418
RMEMLESLELLHEIVPMSQAG	419
QMEMLESLLQLLKEIVPMSKAS	420
RMEMLDSLELLKDMVPMTTGA	421
RIEMLESLELLKDMVPMANAS	422
RMEMLESLLQLLNEIVPMSRAR	423
RMEMLESLELLKELVPMSKGV	424
RIEMLESLELLKDIVPIQKAR	425
RMELLESLELLKDMVPMSDSS	426
RMEMLESLELLQEIVPRAKGA	427
RMEMLDSLELLQLLNEIVPMSHAR	428
RMEMLESLELLKDIVPMSNAG	429
RMEMLQSLLELLKGMVPISKAG	430
RMEMLESLELLKEIVPNSTAA	431
RMEMLQSLLELLKEIVPISKAG	432
RIEMLDSLELLNELVPMSKAR	433
HHGWNLYLRKGSAPQWFEAWV	434
QVESLQQLLMWLDQKLASGPQG	435
RMELLESLELLKEMVPRSKAV	436
QAVSLQHLLMWLDQKLASGPQH	437
DEDSLQQLLMWLDQKLASGPQL	438
PVASLQQLLIWLDQKLAQGPHA	439
EVDELQQLLNWLDHKLASGPLQ	440
DVESLEQLLMWLDHQLASGPHG	441
QVDSLQQLVLLWLEHKLALGPQV	442
GDESLEQLLMWLEQKLALGPHG	443
QIEMLESLLDLRDMVPMSNAF	444
EVDSLQQLLMWLDQKLASGPQA	445
EDESLEQLLIYLDKMLSSGPQV	446
AMDQLHQLLIWLDHKLASGPQA	447
RIEMLESLELLDEIALIPKAW	448

EVVSLQHLLMWLEHKLASGPDG	449
GGESLQQLLMWLDQQLASGPQR	450
GVESLQQLLIFLDHMLVSGPHD	451
NVESLEHLLMMWLERLLASGPYA	452
QVDSLQQLLIWLDHQLASGPKR	453
EVESLQQLLMWLEHKLAAQGPQG	454
EVDSLQQLLMWLDQKLASGPHA	455
EVDSLQQLLMWLDQQLASGPQK	456
GVEQLPQLLMWLEQKLASGPQR	457
GEDSLQQLLMWLDQQLAAGPQV	458
ADDSLQQLLMWLDRLKASGPHV	459
PVDSLQQLLIWLDQKLASGPQG	460
RATLLKDFWQLVVEGYGDN	461
DWRATLLKEFWQLVVEGLGDNLV	462
QSRATLLKEFWQLVVEGLGDKQA	463
DGRATLLTEFWQLVQGLGQKEA	464
LARATLLKEFWQLVVEGLGEKVV	465
GSRDTLLKEFWQLVVGLGDMQT	466
DARATLLKEFWQLVDAYGDRMV	467
NDRAQLLRDFWQLVDGLGVKSW	468
GVRETLLEYELWYLLKGLGANQG	469
QARATLLKEFCQLVGCQGDKLS	470
QERATLLKEFWQLVAGLGQNMR	471
SGRATLLKEFWQLVQGLGEYRW	472
TMRATLLKEFWLFDVGQREMQW	473
GERATLLNDFWQLVDGQGDNTG	474
DERETLLKEFWQLVHGWGDNVA	475
GGRATLLKELWQLLEGQGANLV	476
TARATLLNELVQLVKGYGDKLV	477
GMRATLLQEFWQLVGGQGDNWM	478
STRATLLNDLWQLMKGWAEDRG	479
SERATLLKELWQLVGGWGDNFG	480
VGRATLLKEFWQLVVEGLVGQSR	481
EIRATLLKEFWQLVDEWREQPN	482
QLRATLLKEFLQLVHGLGETDS	483
TQRATLLKEFWQLIEGLGGKHV	484
HYRATLLKEFWQLVDGLREQGV	485
QSRVTLLREFWQLVESYRPIVN	486
LSRATLLNEFWQFVDGQQRDKRM	487
WDRATLLNDFWHLMEELSQKPG	488
QERATLLKEFWRMVEGLGKNRG	489
NERATLLREFWQLVGGYGVNQR	490
YREMSMLEGLLDVLERLQHY	491
HQRDMSMLWELLDVLDGLRQYS	492

TQRDMSMLDGLLEVLDQLRQQR	493
TSRDMSLLWELLEELDRLGHQR	494
MQHDMSMLYGLVELLESLGHQI	495
WNRDMRMLESLEFVLDGLRQQV	496
GYRDMSMLEGLLAVLDRLGPQL	497
TQRDMSMLEGLLEVLDRLGQQR	498
WYRDMSMLEGLLEVLDRLGQQR	499
HNSSQMLLSEIMLVGSMMQ	500
TQNSRQMLLSDFMMLVGSMIQQ	501
MQTSRHILLSEFMMLVGSIMHG	502
HDNSRQMLLSDLLHLVGTMIQQ	503
MENSRQNLLRELIMLVGNMSHQ	504
QDTSRHMLLREFMMLVGEMIQQ	505
DQNSRQMLLSDLMLVGSMIQQ	506
EFFHWLHNHRSEVNHVLDNMN	507
NVFFQWVQKHGRVVYQWLDINV	508
FDFLQWLQNHRSEVEHWLVMDV	509

Table 9--BAFF binding peptide sequences

SEQUENCE	SEQ ID NO:
PGTCFPFPWECTHA	510
WGACWPFPWECFKE	511
VPFCDLLTKHCFEA	512
GSRCYKWDVLTQCFHH	513
LPGCKWDLLIKQWVCDPL	514
SADCYFDILTKSDVCTSS	515
SDDCMYDQLTRMFICSNL	516
DLNCKYDELTYKEWCQFN	517
FHDCKYDLLTRQMVCHGL	518
RNHCFWDHLLKQDICPSP	519
ANQCWWDSLTKKNVCEFF	520
YKGRQQMWDILTRSWVSL	521
QQDVGLWWDILTRAUMPNI	522
QQNAQRVWDLLIRTWVYPQ	523
GWNEAWWDELTKIWWLEQQ	524
RITCDTWDSLKKCVPPQS	525
GAIMQQFWDSLTKTWLRQS	526
WLHSGWWDPLTKHWLQQKV	527
SEWFFWFDPLTRAQQKFR	528
GVWFWWFDPLTKQWTQQAG	529
MQQCKGYDILTKWCVTNG	530
LWSKEVWDILTKSWVSQQA	531
KAAGWWFDWLTKVWVPAP	532

AYQQTWFWDSLTRLWLSTT	533
SGQQHFWWDLLTRSWTPST	534
LGVGQQKWDPLTKQWVSRG	535
VGKMCQQWDPLIKRTVCVG	536
CRQGAKFDDLTKQCLLGR	537
GQAIRHWDVLTKQWVDSQQ	538
RGPCGSWDLLTKHCLDSQQ	539
WQWKQQQWDLLTKQMWWVG	540
PITICRKDLLTKQVVCLD	541
KTCNGKWDLLTKQCLQQQA	542
KCLKGKWDLLTKQCVTEV	543
RCWNGKWDLLTKQCIHPW	544
NRDMRKWDPLIKQWIVRP	545
QAAAAATWDLLTKQWLVP	546
PEGGPKWDPLTKQQFLPPV	547
QQTTPQQKKWDLLTKQWFTRN	548
IGSPCKWDLLTKQMICQQT	549
CTAAGKWDLLTKQCIQQEK	550
VSQCMKWDLLTKQCLQQGW	551
VWGTWKWDLLTKQYLPPQQ	552
GWWEMKWDLLTKQWYRPQQ	553
TAQQVSKWDLLTKQWLPLA	554
QLWGTKWDLLTKQYIQQIM	555
WATSQKWDLLTKQWVQQNM	556
QQRQCAKWDLLTKQCVLFY	557
KTTDCKWDLLTKQRICQQV	558
LLCQQGKWDLLTKQCLKLR	559
LMWFWKWDLLTKQLVPTF	560
QQTWAWKWDLLTKQWIGPM	561
NKELLKWDLLTKQCRGRS	562
GQQKDLKWDLLTKQYVRQS	563
PKPCQQKWDLLTKQCLGSV	564
GQIGWKWDLLTKQWIIQTR	565
VWLDWKWDLLTKQWIHPQQ	566
QQEWEYKWDLLTKQWGWLR	567
HWDSWKWDLLTKQWVVQQA	568
TRPLQQKWDLLTKQWLRVG	569
SDQWQQKWDLLTKQFWWDV	570
QQQTFMKWDLLTKQWIRRH	571
QQGECKWDLLTKQCFPGQ	572
GQQMGWRWDPLIKMCLGPS	573
QQLDGCKWDLLTKQKVCIP	574
HGYWQQKWDLLTKQWVSSE	575
HQQGQCGWDLLTRIYLPCH	576

LHKACKWDL LTKQCWPMQQ	577
GPPGSVWDL LTKIWQQTG	578
ITQQDWRFD TLRWLPLR	579
QQGGFAAWD VLTkmWITVP	580
GHGTPWWDAL TRIWILGV	581
VWPWQQKWDL LTKQVFQD	582
WQQWSWKWDL LTRQYISS	583
NQQT LWKWDL LTKQFITYM	584
PVYQQGWWD TLTkLYWDG	585
WLDGGWRDPL IKRSVQQLG	586
GHQQQFKWDL LTKQWVQSN	587
QQRVGQFWD VLTkMFI TGS	588
QQAQGWSYD ALIKTWIRWP	589
GWMHWKWDP LTKQQALPWM	590
GHPTYKWDL LTKQWILQQM	591
WNNWSLWDPL TKLWLQQQN	592
WQWGWKWDL LTKQWVQQQ	593
GQMGWRWDPL TKMWLGTS	594

Fc Domains

This invention requires the presence of at least one Fc domain modified to comprise a peptide sequence.

- 5 As noted above, both native Fc's and Fc variants are suitable Fc domains for use within the scope of this invention. A native Fc may be extensively modified to form an Fc variant in accordance with this invention, provided binding to the salvage receptor is maintained; see, for example WO 97/34631 and WO 96/32478. In such Fc variants, one may
- 10 remove one or more sites of a native Fc that provide structural features or functional activity not required by the fusion molecules of this invention. One may remove these sites by, for example, substituting or deleting residues, inserting residues into the site, or truncating portions containing the site. The inserted or substituted residues may also be altered amino
- 15 acids, such as peptidomimetics or D-amino acids. Fc variants may be desirable for a number of reasons, several of which are described below. Exemplary Fc variants include molecules and sequences in which:

1. Sites involved in disulfide bond formation are removed. Such removal may avoid reaction with other cysteine-containing proteins present in the host cell used to produce the molecules of the invention. For this purpose, the cysteine-containing segment at the N-terminus may be truncated or cysteine residues may be deleted or substituted with other amino acids (e.g., alanyl, seryl). In particular, one may truncate the N-terminal 20-amino acid segment of SEQ ID NO: 599 or delete or substitute the cysteine residues at positions 7 and 10 of SEQ ID NO: 599. Even when cysteine residues are removed, the single chain Fc domains can still form a dimeric Fc domain that is held together non-covalently.
2. A native Fc is modified to make it more compatible with a selected host cell. For example, one may remove the PA sequence near the N-terminus of a typical native Fc, which may be recognized by a digestive enzyme in E. coli such as proline iminopeptidase. One may also add an N-terminal methionine residue, especially when the molecule is expressed recombinantly in a bacterial cell such as E. coli. The Fc domain of SEQ ID NO: 599 (Figure 2A) is one such Fc variant.
3. A portion of the N-terminus of a native Fc is removed to prevent N-terminal heterogeneity when expressed in a selected host cell. For this purpose, one may delete any of the first 20 amino acid residues at the N-terminus, particularly those at positions 1, 2, 3, 4 and 5.
4. One or more glycosylation sites are removed. Residues that are typically glycosylated (e.g., asparagine) may confer cytolytic response. Such residues may be deleted or substituted with unglycosylated residues (e.g., alanine).
5. Sites involved in interaction with complement, such as the C1q binding site, are removed. For example, one may delete or substitute the EKK sequence of human IgG1. Complement recruitment may not be advantageous for the molecules of this invention and so may be avoided with such an Fc variant.

6. Sites are removed that affect binding to Fc receptors other than a salvage receptor. A native Fc may have sites for interaction with certain white blood cells that are not required for the fusion molecules of the present invention and so may be removed.
- 5 7. The ADCC site is removed. ADCC sites are known in the art; see, for example, Molec. Immunol. 29 (5): 633-9 (1992) with regard to ADCC sites in IgG1. These sites, as well, are not required for the fusion molecules of the present invention and so may be removed.
8. When the native Fc is derived from a non-human antibody, the native
10 Fc may be humanized. Typically, to humanize a native Fc, one will substitute selected residues in the non-human native Fc with residues that are normally found in human native Fc. Techniques for antibody humanization are well known in the art.

Preferred Fc variants include the following. In SEQ ID NO: 599
15 (Figure 2A) the leucine at position 15 may be substituted with glutamate; the glutamate at position 99, with alanine; and the lysines at positions 101 and 103, with alanines. In addition, one or more tyrosine residues can be replaced by phenylalanine residues.

Additional vehicles

20 The invention further embraces molecules covalently modified to include one or more water soluble polymer attachments, such as polyethylene glycol, polyoxyethylene glycol, or polypropylene glycol, as described U.S. Patent Nos.: 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192; and 4,179,337. Still other useful polymers known in the art
25 include monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone)-polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol, as well as mixtures of these polymers. Particularly preferred are
30 peptibodies covalently modified with polyethylene glycol (PEG) subunits. Water-soluble polymers may be bonded at specific positions, for example

at the amino terminus of the peptibodies, or randomly attached to one or more side chains of the polypeptide. The use of PEG for improving the therapeutic capacity for specific binding agents, e.g. peptibodies, and for humanized antibodies in particular, is described in US Patent No. 6, 133, 426 to Gonzales *et al.*, issued October 17, 2000.

Various means for attaching chemical moieties useful as vehicles are currently available, *see*, e.g., Patent Cooperation Treaty ("PCT") International Publication No. WO 96/11953, entitled "N-Terminally Chemically Modified Protein Compositions and Methods," herein incorporated by reference in its entirety. This PCT publication discloses, among other things, the selective attachment of water soluble polymers to the N-terminus of proteins.

A preferred polymer vehicle is polyethylene glycol (PEG). The PEG group may be of any convenient molecular weight and may be linear or branched. The average molecular weight of the PEG will preferably range from about 2 kiloDalton ("kD") to about 100 kD, more preferably from about 5 kDa to about 50 kDa, most preferably from about 5 kD to about 20 kD. The PEG groups will generally be attached to the compounds of the invention via acylation or reductive alkylation through a reactive group on the PEG moiety (e.g., an aldehyde, maleimide, amino, thiol, or ester group) to a reactive group on the inventive compound (e.g., an aldehyde, amino, thiol or ester group).

A useful strategy for the PEGylation of synthetic peptides consists of combining, through forming a conjugate linkage in solution, a peptide and a PEG moiety, each bearing a special functionality that is mutually reactive toward the other. The peptides can be easily prepared with conventional solid phase synthesis (see, for example, Figures 5 and 6 and the accompanying text herein). The peptides are "preactivated" with an appropriate functional group at a specific site. The precursors are purified and fully characterized prior to reacting with the PEG moiety. Ligation of the peptide with PEG usually takes place in aqueous phase and can be

easily monitored by reverse phase analytical HPLC. The PEGylated peptides can be easily purified by preparative HPLC and characterized by analytical HPLC, amino acid analysis and laser desorption mass spectrometry.

5 Polysaccharide polymers are another type of water soluble polymer which may be used for protein modification. Dextrans are polysaccharide polymers comprised of individual subunits of glucose predominantly linked by α 1-6 linkages. The dextran itself is available in many molecular weight ranges, and is readily available in molecular weights from about 1
10 kD to about 70 kD. Dextran is a suitable water soluble polymer for use in the present invention as a vehicle by itself or in combination with another vehicle (e.g., Fc). See, for example, WO 96/11953 and WO 96/05309. The use of dextran conjugated to therapeutic or diagnostic immunoglobulins has been reported; see, for example, European Patent Publication No. 0
15 315 456, which is hereby incorporated by reference. Dextran of about 1 kD to about 20 kD is preferred when dextran is used as a vehicle in accordance with the present invention.

An additional vehicle may also be a protein, polypeptide, peptide, antibody, antibody fragment, or small molecule (e.g., a peptidomimetic
20 compound) capable of binding to a salvage receptor. For example, one could use as a vehicle a polypeptide as described in U.S. Pat. No. 5,739,277, issued April 14, 1998 to Presta et al. Peptides could also be selected by phage display for binding to the FcRn salvage receptor. Such salvage receptor-binding compounds are also included within the meaning of
25 "vehicle" in this invention. Such vehicles should be selected for increased half-life (e.g., by avoiding sequences recognized by proteases) and decreased immunogenicity (e.g., by favoring non-immunogenic sequences, as discovered in antibody humanization).

Linkers

30 Any "linker" group is optional. When present, its chemical structure is not critical, since it serves primarily as a spacer. The linker is

preferably made up of amino acids linked together by peptide bonds. Thus, in preferred embodiments, the linker is made up of from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally occurring amino acids. Some of these amino acids may be glycosylated, as is well understood by those in the art. In a more preferred embodiment, the 1 to 20 amino acids are selected from glycine, alanine, proline, asparagine, glutamine, and lysine. Even more preferably, a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. Thus, preferred linkers are polyglycines (particularly (Gly)₄, (Gly)₅), poly(Gly-Ala), and polyalanines. Other specific examples of linkers are:

(Gly)₃Lys(Gly)₄ (SEQ ID NO: 595);

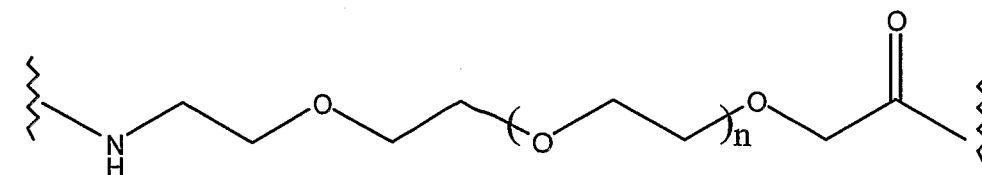
(Gly)₃AsnGlySer(Gly)₂ (SEQ ID NO: 596);

(Gly)₃Cys(Gly)₄ (SEQ ID NO: 597); and

GlyProAsnGlyGly (SEQ ID NO: 598).

To explain the above nomenclature, for example, (Gly)₃Lys(Gly)₄ means Gly-Gly-Gly-Lys-Gly-Gly-Gly-Gly. Combinations of Gly and Ala are also preferred. The linkers shown here are exemplary; linkers within the scope of this invention may be much longer and may include other residues.

Non-peptide linkers are also possible. For example, alkyl linkers such as -NH-(CH₂)_s-C(O)-, wherein s = 2-20 could be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C₁-C₆) lower acyl, halogen (e.g., Cl, Br), CN, NH₂, phenyl, etc. An exemplary non-peptide linker is a PEG linker,



wherein n is such that the linker has a molecular weight of 100 to 5000 kD, preferably 100 to 500 kD. The peptide linkers may be altered to form derivatives in the same manner as described above.

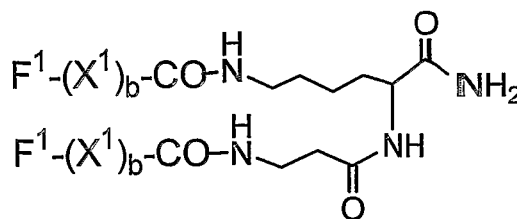
Derivatives

5 The invention also provides "derivatives" that include molecules bearing modifications other than, or in addition to, insertions, deletions, or substitutions of amino acid residues. Preferably, the modifications are covalent in nature, and include for example, chemical bonding with polymers, lipids, other organic, and inorganic moieties. Derivatives of the
10 invention may be prepared to increase circulating half-life of a molecule; to improve targeting capacity for the molecule to desired cells, tissues, or organs; to improve the solubility or absorption of a molecule; or to eliminate or attenuate any undesirable side-effect of a molecule.

Exemplary derivatives include compounds in which:

- 15 1. The compound or some portion thereof is cyclic. For example, the peptide portion may be modified to contain two or more Cys residues (e.g., in the linker), which could cyclize by disulfide bond formation. For citations to references on preparation of cyclized derivatives, see Table 2.
- 20 2. The compound is cross-linked or is rendered capable of cross-linking between molecules. For example, the peptide portion may be modified to contain one Cys residue and thereby be able to form an intermolecular disulfide bond with a like molecule. The compound may also be cross-linked through its C-terminus, as in the molecule
25 shown below.

VII



3. One or more peptidyl [-C(O)NR-] linkages (bonds) is replaced by a non-peptidyl linkage. Exemplary non-peptidyl linkages are -CH₂-carbamate [-CH₂-OC(O)NR-], phosphonate, -CH₂-sulfonamide [-CH₂-S(O)₂NR-], urea [-NHC(O)NH-], -CH₂-secondary amine, and alkylated peptide [-C(O)NR⁶- wherein R⁶ is lower alkyl].
4. The N-terminus is derivatized. Typically, the N-terminus may be acylated or modified to a substituted amine. Exemplary N-terminal derivative groups include -NRR¹ (other than -NH₂), -NRC(O)R¹, -NRC(O)OR¹, -NRS(O)₂R¹, -NHC(O)NHR¹, succinimide, or benzyloxycarbonyl-NH- (CBZ-NH-), wherein R and R¹ are each independently hydrogen or lower alkyl and wherein the phenyl ring may be substituted with 1 to 3 substituents selected from the group consisting of C₁-C₄ alkyl, C₁-C₄ alkoxy, chloro, and bromo.
5. The free C-terminus is derivatized. Typically, the C-terminus is esterified or amidated. For example, one may use methods described in the art to add (NH-CH₂-CH₂-NH₂)₂ to compounds of this invention. Likewise, one may use methods described in the art to add -NH₂ to compounds of this invention. Exemplary C-terminal derivative groups include, for example, -C(O)R² wherein R² is lower alkoxy or -NR³R⁴ wherein R³ and R⁴ are independently hydrogen or C₁-C₈ alkyl (preferably C₁-C₄ alkyl).
6. A disulfide bond is replaced with another, preferably more stable, cross-linking moiety (e.g., an alkylene). See, e.g., Bhatnagar *et al.* (1996), *J. Med. Chem.* 39: 3814-9; Alberts *et al.* (1993) *Thirteenth Am. Pep. Symp.*, 357-9.
7. One or more individual amino acid residues is modified. Various derivatizing agents are known to react specifically with selected sidechains or terminal residues, as described in detail below.

Lysinyl residues and amino terminal residues may be reacted with succinic or other carboxylic acid anhydrides, which reverse the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-

containing residues include imidoesters such as methyl picolinimide; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

5 Arginyl residues may be modified by reaction with any one or combination of several conventional reagents, including phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginyl residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents
10 may react with the groups of lysine as well as the arginine epsilon-amino group.

Specific modification of tyrosyl residues has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most
15 commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl sidechain groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides ($R'-N=C=N-R'$) such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues
20 may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues may be deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues
25 are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Cysteinyl residues can be replaced by amino acid residues or other moieties either to eliminate disulfide bonding or, conversely, to stabilize cross-linking. See, e.g., Bhatnagar *et al.* (1996), *J. Med. Chem.* 39: 3814-9.

30 Derivatization with bifunctional agents is useful for cross-linking the peptides or their functional derivatives to a water-insoluble support matrix or

to other macromolecular vehicles. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-
5 dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates
10 and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Carbohydrate (oligosaccharide) groups may conveniently be attached to sites that are known to be glycosylation sites in proteins.
15 Generally, O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. X is preferably one of the 19 naturally occurring amino acids other than proline. The
20 structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneuraminic acid (referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer
25 acidic properties to the glycosylated compound. Such site(s) may be incorporated in the linker of the compounds of this invention and are preferably glycosylated by a cell during recombinant production of the polypeptide compounds (e.g., in mammalian cells such as CHO, BHK, COS). However, such sites may further be glycosylated by synthetic or
30 semi-synthetic procedures known in the art.

Other possible modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, oxidation of the sulfur atom in Cys, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains. Creighton, Proteins: Structure and Molecule Properties (W. H. Freeman & Co., San Francisco), pp. 79-86 (1983).

Such derivatized moieties preferably improve one or more characteristics including anti-angiogenic activity, solubility, absorption, biological half life, and the like of the compounds. Alternatively, derivatized moieties may result in compounds that have the same, or essentially the same, characteristics and/or properties of the compound that is not derivatized. The moieties may alternatively eliminate or attenuate any undesirable side effect of the compounds and the like.

Compounds of the present invention may be changed at the DNA level, as well. The DNA sequence of any portion of the compound may be changed to codons more compatible with the chosen host cell. For E. coli, which is the preferred host cell, optimized codons are known in the art. Codons may be substituted to eliminate restriction sites or to include silent restriction sites, which may aid in processing of the DNA in the selected host cell. The vehicle, linker and peptide DNA sequences may be modified to include any of the foregoing sequence changes.

Isotope- and toxin-conjugated derivatives. Another set of useful derivatives are the above-described molecules conjugated to toxins, tracers, or radioisotopes. Such conjugation is especially useful for molecules comprising peptide sequences that bind to tumor cells or pathogens. Such molecules may be used as therapeutic agents or as an aid to surgery (e.g., radioimmunoguided surgery or RIGS) or as diagnostic agents (e.g., radioimmunodiagnostics or RID).

As therapeutic agents, these conjugated derivatives possess a number of advantages. They facilitate use of toxins and radioisotopes that would be toxic if administered without the specific binding provided by

the peptide sequence. They also can reduce the side-effects that attend the use of radiation and chemotherapy by facilitating lower effective doses of the conjugation partner.

Useful conjugation partners include:

- 5 • radioisotopes, such as ⁹⁰Yttrium, ¹³¹Iodine, ²²⁵Actinium, and ²¹³Bismuth;
- ricin A toxin, microbially derived toxins such as Pseudomonas endotoxin (e.g., PE38, PE40), and the like;
- partner molecules in capture systems (see below);
- 10 • biotin, streptavidin (useful as either partner molecules in capture systems or as tracers, especially for diagnostic use); and
- cytotoxic agents (e.g., doxorubicin).

One useful adaptation of these conjugated derivatives is use in a capture system. In such a system, the molecule of the present invention would comprise a benign capture molecule. This capture molecule would be able to specifically bind to a separate effector molecule comprising, for example, a toxin or radioisotope. Both the vehicle-conjugated molecule and the effector molecule would be administered to the patient. In such a system, the effector molecule would have a short half-life except when
15 bound to the vehicle-conjugated capture molecule, thus minimizing any toxic side-effects. The vehicle-conjugated molecule would have a relatively long half-life but would be benign and non-toxic. The specific binding portions of both molecules can be part of a known specific binding pair (e.g., biotin, streptavidin) or can result from peptide generation methods
20 such as those described herein.

Such conjugated derivatives may be prepared by methods known in the art. In the case of protein effector molecules (e.g., Pseudomonas endotoxin), such molecules can be expressed as fusion proteins from correlative DNA constructs. Radioisotope conjugated derivatives may be
30 prepared, for example, as described for the BEXA antibody (Coulter). Derivatives comprising cytotoxic agents or microbial toxins may be

prepared, for example, as described for the BR96 antibody (Bristol-Myers Squibb). Molecules employed in capture systems may be prepared, for example, as described by the patents, patent applications, and publications from NeoRx. Molecules employed for RIGS and RID may be prepared, for example, by the patents, patent applications, and publications from NeoProbe.

A process for preparing conjugation derivatives is also contemplated. Tumor cells, for example, exhibit epitopes not found on their normal counterparts. Such epitopes include, for example, different post-translational modifications resulting from their rapid proliferation. Thus, one aspect of this invention is a process comprising:

- a) selecting at least one randomized peptide that specifically binds to a target epitope; and
- b) preparing a pharmacologic agent comprising (i) at least one vehicle (Fc domain preferred), (ii) at least one amino acid sequence of the selected peptide or peptides, and (iii) an effector molecule.

The target epitope is preferably a tumor-specific epitope or an epitope specific to a pathogenic organism. The effector molecule may be any of the above-noted conjugation partners and is preferably a radioisotope.

Variants

Variants are also included within the scope of the present invention. Included within variants are insertional, deletional, and substitutional variants. It is understood that a particular molecule of the present invention may contain one, two or all three types of variants. Insertional and substitutional variants may contain natural amino acids, unconventional amino acids (as set forth below), or both.

In one example, insertional variants are provided wherein one or more amino acid residues, either naturally occurring or unconventional amino acids, supplement a peptide or a peptibody amino acid sequence. Insertions may be located at either or both termini of the protein, or may

be positioned within internal regions of the peptibody amino acid sequence. Insertional variants with additional residues at either or both termini can include, for example, fusion proteins and proteins including amino acid tags or labels. Insertional variants include peptides and
5 peptibodies wherein one or more amino acid residues are added to the peptide or peptibody amino acid sequence, or fragment thereof.

Variants of the invention also include mature peptides and peptibodies wherein leader or signal sequences are removed, and the resulting proteins having additional amino terminal residues, which
10 amino acids may be natural or non-natural. Molecules of this invention (such as peptibodies) with an additional methionyl residue at amino acid position -1 (Met⁻¹-peptibody) are contemplated, as are specific binding agents with additional methionine and lysine residues at positions -2 and -1 (Met⁻²-Lys⁻¹-). Variants having additional Met, Met-Lys, Lys residues (or
15 one or more basic residues, in general) are particularly useful for enhanced recombinant protein production in bacterial host cells.

The invention also embraces variants having additional amino acid residues that arise from use of specific expression systems. For example, use of commercially available vectors that express a desired polypeptide
20 as part of glutathione-S-transferase (GST) fusion product provides the desired polypeptide having an additional glycine residue at amino acid position -1 after cleavage of the GST component from the desired polypeptide. Variants which result from expression in other vector systems are also contemplated, including those wherein poly-histidine
25 tags are incorporated into the amino acid sequence, generally at the carboxy and/or amino terminus of the sequence.

Insertional variants also include fusion proteins wherein the amino and/or carboxy termini of the peptide or peptibody is fused to another polypeptide, a fragment thereof or amino acids which are not generally
30 recognized to be part of any specific protein sequence. Examples of such fusion proteins are immunogenic polypeptides, proteins with long

circulating half lives, such as immunoglobulin constant regions, marker proteins, proteins or polypeptides that facilitate purification of the desired peptide or peptibody, and polypeptide sequences that promote formation of multimeric proteins (such as leucine zipper motifs that are useful in
5 dimer formation/stability).

This type of insertional variant generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion of a second polypeptide. For example, fusion proteins typically employ leader sequences from other species to permit the recombinant
10 expression of a protein in a heterologous host. Another useful fusion protein includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful
15 fusions include linking of functional domains, such as active sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions.

There are various commercially available fusion protein expression systems that may be used in the present invention. Particularly useful
20 systems include but are not limited to the glutathione-S-transferase (GST) system (Pharmacia), the maltose binding protein system (NEB, Beverly, MA), the FLAG system (IBI, New Haven, CT), and the 6xHis system (Qiagen, Chatsworth, CA). These systems are capable of producing recombinant peptides and/or peptibodies bearing only a small number of
25 additional amino acids, which are unlikely to significantly affect the activity of the peptide or peptibody. For example, both the FLAG system and the 6xHis system add only short sequences, both of which are known to be poorly antigenic and which do not adversely affect folding of a polypeptide to its native conformation. Another N-terminal fusion that is
30 contemplated to be useful is the fusion of a Met-Lys dipeptide at the

N-terminal region of the protein or peptides. Such a fusion may produce beneficial increases in protein expression or activity.

Other fusion systems produce polypeptide hybrids where it is desirable to excise the fusion partner from the desired peptide or
5 peptibody. In one embodiment, the fusion partner is linked to the recombinant peptibody by a peptide sequence containing a specific recognition sequence for a protease. Examples of suitable sequences are those recognized by the Tobacco Etch Virus protease (Life Technologies, Gaithersburg, MD) or Factor Xa (New England Biolabs, Beverly, MA).

10 The invention also provides fusion polypeptides which comprises all or part of a peptibody or peptide of the present invention, in combination with truncated tissue factor (tTF). tTF is a vascular targeting agent consisting of a truncated form of a human coagulation-inducing protein that acts as a tumor blood vessel clotting agent, as described U.S.
15 Patent Nos.: 5,877,289; 6,004,555; 6,132,729; 6,132,730; 6,156,321; and European Patent No. EP 0988056. The fusion of tTF to the anti-Ang-2 peptibody or peptide, or fragments thereof facilitates the delivery of anti-Ang-2 to target cells.

 In another aspect, the invention provides deletion variants wherein
20 one or more amino acid residues in a peptide or peptibody are removed. Deletions can be effected at one or both termini of the peptibody, or from removal of one or more residues within the peptibody amino acid sequence. Deletion variants necessarily include all fragments of a peptide or peptibody.

25 In still another aspect, the invention provides substitution variants of peptides and peptibodies of the invention. Substitution variants include those peptides and peptibodies wherein one or more amino acid residues are removed and replaced with one or more alternative amino acids, which amino acids may be naturally occurring or non-naturally occurring.
30 Substitutional variants generate peptides or peptibodies that are "similar" to the original peptide or peptibody, in that the two molecules have a

certain percentage of amino acids that are identical. Substitution variants include substitutions of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, amino acids within a peptide or peptibody, wherein the number of substitutions may be up to ten percent or more, of the amino acids of the peptide or
5 peptibody. In one aspect, the substitutions are conservative in nature, however, the invention embraces substitutions that are also non-conservative and also includes unconventional amino acids.

Identity and similarity of related peptides and peptibodies can be readily calculated by known methods. Such methods include, but are not
10 limited to, those described in Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York (1988); Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey (1994); Sequence
15 Analysis in Molecular Biology, von Heinje, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York (1991); and Carillo et al., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine the relatedness or percent identity
20 of two peptides or polypeptides, or a polypeptide and a peptide, are designed to give the largest match between the sequences tested. Methods to determine identity are described in publicly available computer programs. Preferred computer program methods to determine identity between two sequences include, but are not limited to, the GCG program
25 package, including GAP (Devereux et al., Nucl. Acid. Res., 12:387 (1984); Genetics Computer Group, University of Wisconsin, Madison, WI, BLASTP, BLASTN, and FASTA (Altschul et al., J. Mol. Biol., 215:403-410 (1990)). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (BLAST
30 Manual, Altschul et al. NCB/NLM/NIH Bethesda, MD 20894; Altschul et

al., supra (1990)). The well-known Smith Waterman algorithm may also be used to determine identity.

Certain alignment schemes for aligning two amino acid sequences may result in the matching of only a short region of the two sequences, and this small aligned region may have very high sequence identity even though there is no significant relationship between the two full-length sequences. Accordingly, in certain embodiments, the selected alignment method (GAP program) will result in an alignment that spans at least ten percent of the full length of the target polypeptide being compared, i.e., at least 40 contiguous amino acids where sequences of at least 400 amino acids are being compared, 30 contiguous amino acids where sequences of at least 300 to about 400 amino acids are being compared, at least 20 contiguous amino acids where sequences of 200 to about 300 amino acids are being compared, and at least 10 contiguous amino acids where sequences of about 100 to 200 amino acids are being compared.

For example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, WI), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). In certain embodiments, a gap opening penalty (which is typically calculated as 3X the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 1/10 times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. In certain embodiments, a standard comparison matrix (see Dayhoff et al., Atlas of Protein Sequence and Structure, 5(3)(1978) for the PAM 250 comparison matrix; Henikoff et al., Proc. Natl. Acad. Sci USA, 89:10915-

10919 (1992) for the BLOSUM 62 comparison matrix) is also used by the algorithm.

In certain embodiments, the parameters for a polypeptide sequence comparison include the following:

- 5 Algorithm: Needleman et al., J. Mol. Biol., 48:443-453 (1970);
- Comparison matrix: BLOSUM 62 from Henikoff et al., supra (1992);
- Gap Penalty: 12
- Gap Length Penalty: 4
- Threshold of Similarity: 0

- 10 The GAP program may be useful with the above parameters. In certain embodiments, the aforementioned parameters are the default parameters for polypeptide comparisons (along with no penalty for end gaps) using the GAP algorithm.

- In certain embodiments, the parameters for polynucleotide
- 15 molecule sequence (as opposed to an amino acid sequence) comparisons include the following:

- Algorithm: Needleman et al., supra (1970);
- Comparison matrix: matches = +10, mismatch = 0
- Gap Penalty: 50
- 20 Gap Length Penalty: 3

The GAP program may also be useful with the above parameters. The aforementioned parameters are the default parameters for polynucleotide molecule comparisons.

- Other exemplary algorithms, gap opening penalties, gap extension
- 25 penalties, comparison matrices, thresholds of similarity, etc. may be used, including those set forth in the Program Manual, Wisconsin Package, Version 9, September, 1997. The particular choices to be made will be

apparent to those of skill in the art and will depend on the specific comparison to be made, such as DNA-to-DNA, protein-to-protein, protein-to-DNA; and additionally, whether the comparison is between given pairs of sequences (in which case GAP or BestFit are generally preferred) or between one sequence and a large database of sequences (in which case FASTA or BLASTA are preferred).

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. See Immunology--A Synthesis (2nd Edition, E. S. Golub and D. R. Gren, Eds., Sinauer Associates, Sunderland, Mass. (1991)), which is incorporated herein by reference for any purpose.

The amino acids may have either L or D stereochemistry (except for Gly, which is neither L nor D) and the polypeptides and compositions of the present invention may comprise a combination of stereochemistries. However, the L stereochemistry is preferred. The invention also provides reverse molecules wherein the amino terminal to carboxy terminal sequence of the amino acids is reversed. For example, the reverse of a molecule having the normal sequence $X_1-X_2-X_3$ would be $X_3-X_2-X_1$. The invention also provides retro-reverse molecules wherein, as above, the amino terminal to carboxy terminal sequence of amino acids is reversed and residues that are normally "L" enantiomers are altered to the "D" stereoisomer form.

Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α -, α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include, without limitation: aminoadipic acid, beta-alanine, beta-aminopropionic acid, aminobutyric acid, piperidinic acid, aminocaproic acid, aminoheptanoic acid, aminoisobutyric acid, aminopimelic acid, diaminobutyric acid,

desmosine, diaminopimelic acid, diaminopropionic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, allo-hydroxylysine, hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, sarcosine, N-methylisoleucine, N-methylvaline, norvaline, norleucine, orithine, 4-
 5 hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ -N-methylarginine, and other similar amino acids and amino acids (e.g., 4-hydroxyproline).

Similarly, unless specified otherwise, the left-hand end of single-
 10 stranded polynucleotide sequences is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA
 15 transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences".

It will be appreciated that amino acid residues can be divided into classes based on their common side chain properties:

- 20 1. Neutral Hydrophobic: Alanine (Ala; A), Valine (Val; V), Leucine (Leu; L), Isoleucine (Ile; I), Proline (Pro; P), Tryptophan (Trp; W), Phenylalanine (Phe; F), and Methionine (Met, M).
2. Neutral Polar: Glycine (Gly; G); Serine (Ser; S), Threonine (Thr; T), Tyrosine (Tyr; Y), Cysteine (Cys; C), Glutamine (Glu; Q),
 25 Asparagine (Asn; N), and Norleucine.
3. Acidic: Aspartic Acid (Asp; D), Glutamic Acid (Glu; E);
4. Basic: Lysine (Lys; K), Arginine (Arg; R), Histidine (His; H).

See Lewin, B., Genes V, Oxford University Press (1994), p.11.

Conservative amino acid substitutions may encompass unconventional amino acid residues, which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include, without limitation, peptidomimetics and other reversed or
5 inverted forms of amino acid moieties. Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class.

In making such changes, according to certain embodiments, the hydropathic index of amino acids may be considered. Each amino acid
10 has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. They are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-
15 3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is understood in the art. Kyte et al., J. Mol. Biol., 157:105-131 (1982). It is known that certain amino acids
20 may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, in certain embodiments, the substitution of amino acids whose hydropathic indices are within ± 2 is included. In certain embodiments, those which are within ± 1 are included,
25 and in certain embodiments, those within ± 0.5 are included.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biologically functional peptibody or peptide thereby created is intended for use in immunological embodiments, as in the present case. In
30 certain embodiments, the greatest local average hydrophilicity of a

protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e., with a biological property of the protein.

The following hydrophilicity values have been assigned to these amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 $\pm$ 1); glutamate (+3.0 $\pm$ 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 $\pm$ 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5) and tryptophan (-3.4). In making changes based upon similar hydrophilicity values, in certain embodiments, the substitution of amino acids whose hydrophilicity values are within ± 2 is included, in certain embodiments, those which are within ± 1 are included, and in certain embodiments, those within ± 0.5 are included. One may also identify epitopes from primary amino acid sequences on the basis of hydrophilicity. These regions are also referred to as "epitopic core regions."

Exemplary amino acid substitutions are set forth in Table 10 below.

Table 10
Amino Acid Substitutions

Original Residues	Exemplary Substitutions	Preferred Substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln, Glu, Asp	Gln
Asp	Glu, Gln, Asp	Glu
Cys	Ser, Ala	Ser
Gln	Asn, Glu, Asp	Asn
Glu	Asp, Gln, Asn	Asp
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg

Ile	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, 1,4 Diamino-butyric Acid, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

A skilled artisan will be able to determine suitable variants of the polypeptide as set forth herein using well-known techniques. In certain embodiments, one skilled in the art may identify suitable areas of the molecule that may be changed without destroying activity by targeting regions not believed to be important for activity. In certain embodiments, one can identify residues and portions of the molecules that are conserved among similar peptides or polypeptides. In certain embodiments, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

Additionally, one skilled in the art can review structure-function studies identifying residues in similar polypeptides that are important for activity or structure. In view of such a comparison, one can predict the importance of amino acid residues in a protein that correspond to amino acid residues which are important for activity or structure in similar

proteins. One skilled in the art may opt for chemically similar amino acid substitutions for such predicted important amino acid residues.

One skilled in the art can also analyze the three-dimensional structure and amino acid sequence in relation to that structure in similar polypeptides. In view of such information, one skilled in the art may predict the alignment of amino acid residues of an antibody with respect to its three dimensional structure. In certain embodiments, one skilled in the art may choose not to make radical changes to amino acid residues predicted to be on the surface of the protein, since such residues may be involved in important interactions with other molecules. Moreover, one skilled in the art may generate test variants containing a single amino acid substitution at each desired amino acid residue. The variants can then be screened using activity assays known to those skilled in the art. Such variants could be used to gather information about suitable variants. For example, if one discovered that a change to a particular amino acid residue resulted in destroyed, undesirably reduced, or unsuitable activity, variants with such a change may be avoided. In other words, based on information gathered from such routine experiments, one skilled in the art can readily determine the amino acids where further substitutions should be avoided either alone or in combination with other mutations.

A number of scientific publications have been devoted to the prediction of secondary structure. See Moult J., Curr. Op. in Biotech., 7(4):422-427 (1996), Chou et al., Biochemistry, 13(2):222-245 (1974); Chou et al., Biochemistry, 113(2):211-222 (1974); Chou et al., Adv. Enzymol. Relat. Areas Mol. Biol., 47:45-148 (1978); Chou et al., Ann. Rev. Biochem., 47:251-276 and Chou et al., Biophys. J., 26:367-384 (1979). Moreover, computer programs are currently available to assist with predicting secondary structure. One method of predicting secondary structure is based upon homology modeling. For example, two polypeptides or proteins which have a sequence identity of greater than 30%, or similarity greater than

40% often have similar structural topologies. The recent growth of the protein structural database (PDB) has provided enhanced predictability of secondary structure, including the potential number of folds within a polypeptide's or protein's structure. See Holm et al., Nucl. Acid. Res., 27(1):244-247 (1999). It has been suggested (Brenner et al., Curr. Op. Struct. Biol., 7(3):369-376 (1997)) that there are a limited number of folds in a given polypeptide or protein and that once a critical number of structures have been resolved, structural prediction will become dramatically more accurate.

Additional methods of predicting secondary structure include "threading" (Jones, D., Curr. Opin. Struct. Biol., 7(3):377-87 (1997); Sippl et al., Structure, 4(1):15-19 (1996)), "profile analysis" (Bowie et al., Science, 253:164-170 (1991); Gribskov et al., Meth. Enzym., 183:146-159 (1990); Gribskov et al., Proc. Nat. Acad. Sci., 84(13):4355-4358 (1987)), and "evolutionary linkage" (See Holm, supra (1999), and Brenner, supra (1997)).

In certain embodiments, peptibody variants include glycosylation variants wherein one or more glycosylation sites, such as a N-linked glycosylation site, has been added to the peptibody. An N-linked glycosylation site is characterized by the sequence: Asn-X-Ser or Asn-X-Thr, wherein the amino acid residue designated as X may be any amino acid residue except proline. The substitution or addition of amino acid residues to create this sequence provides a potential new site for the addition of an N-linked carbohydrate chain. Alternatively, substitutions which eliminate this sequence will remove an existing N-linked carbohydrate chain. Also provided is a rearrangement of N-linked carbohydrate chains wherein one or more N-linked glycosylation sites (typically those that are naturally occurring) are eliminated and one or more new N-linked sites are created.

Compounds of the present invention may be changed at the DNA level, as well. The DNA sequence of any portion of the compound may be changed to codons more compatible with the chosen host cell. For E. coli, which is the preferred host cell, optimized codons are known in the art.

- 5 Codons may be substituted to eliminate restriction sites or to include silent restriction sites, which may aid in processing of the DNA in the selected host cell. The vehicle, linker and peptide DNA sequences may be modified to include any of the foregoing sequence changes. Thus, all modifications, substitution, derivitizations, etc. discussed herein apply equally to all
- 10 aspects of the present invention, including but not limited to peptides, peptide dimers and multimers, linkers, and vehicles.

Affinity Maturation

- One embodiment of the present invention includes "affinity
- 15 matured" peptides and peptibodies. This procedure contemplates increasing the affinity or the bio-activity of the peptides and peptibodies of the present invention using phage display or other selection technologies. Based on a consensus sequence (which is generated for a collection of related peptides), directed secondary phage display libraries can be
- 20 generated in which the "core" amino acids (determined from the consensus sequence) are held constant or are biased in frequency of occurrence. Alternatively, an individual peptide sequence can be used to generate a biased, directed phage display library. Panning of such libraries can yield peptides (which can be converted to peptibodies) with
- 25 enhanced binding to the target or with enhanced bio-activity.

Non-Peptide Analogs/Protein Mimetics

- Furthermore, non-peptide analogs of peptides that provide a stabilized structure or lessened biodegradation, are also contemplated. Peptide mimetic analogs can be prepared based on a selected inhibitory
- 30 peptide by replacement of one or more residues by nonpeptide moieties. Preferably, the nonpeptide moieties permit the peptide to retain its natural

confirmation, or stabilize a preferred, e.g., bioactive, confirmation which retains the ability to recognize and bind Ang-2. In one aspect, the resulting analog/ mimetic exhibits increased binding affinity for Ang-2.

One example of methods for preparation of nonpeptide mimetic analogs

5 from peptides is described in Nachman et al., Regul. Pept. 57:359-370

(1995). If desired, the peptides of the invention can be modified, for instance, by glycosylation, amidation, carboxylation, or phosphorylation, or by the creation of acid addition salts, amides, esters, in particular C-terminal esters, and N-acyl derivatives of the peptides of the invention.

10 The peptibodies also can be modified to create peptide derivatives by forming covalent or noncovalent complexes with other moieties.

Covalently-bound complexes can be prepared by linking the chemical moieties to functional groups on the side chains of amino acids comprising the peptibodies, or at the N- or C-terminus.

15 In particular, it is anticipated that the peptides can be conjugated to a reporter group, including, but not limited to a radiolabel, a fluorescent label, an enzyme (e.g., that catalyzes a colorimetric or fluorometric reaction), a substrate, a solid matrix, or a carrier (e.g., biotin or avidin). The invention accordingly provides a molecule comprising a peptibody

20 molecule, wherein the molecule preferably further comprises a reporter group selected from the group consisting of a radiolabel, a fluorescent label, an enzyme, a substrate, a solid matrix, and a carrier. Such labels are well known to those of skill in the art, e.g., biotin labels are particularly contemplated. The use of such labels is well known to those of skill in the art and is described in, e.g., U.S. Patent Nos. 3,817,837; 3,850,752; 3,996,345; and 4,277,437. Other labels that will be useful include but are not limited to radioactive labels, fluorescent labels and chemiluminescent labels. U.S. Patents concerning use of such labels include, for example, U.S. Patent

25 Nos. 3,817,837; 3,850,752; 3,939,350; and 3,996,345. Any of the peptibodies

30 of the present invention may comprise one, two, or more of any of these labels.

Methods of Making

The compounds of this invention largely may be made in transformed host cells using recombinant DNA techniques. To do so, a recombinant DNA molecule coding for the peptide is prepared. Methods of preparing such DNA molecules are well known in the art. For instance, sequences coding for the peptides could be excised from DNA using suitable restriction enzymes. Alternatively, the DNA molecule could be synthesized using chemical synthesis techniques, such as the phosphoramidate method. Also, a combination of these techniques could be used.

The invention also includes a vector capable of expressing the peptides in an appropriate host. The vector comprises the DNA molecule that codes for the peptides operatively linked to appropriate expression control sequences. Methods of effecting this operative linking, either before or after the DNA molecule is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation.

The resulting vector having the DNA molecule thereon is used to transform an appropriate host. This transformation may be performed using methods well known in the art.

Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence. Within these general guidelines,

useful microbial hosts include bacteria (such as E. coli sp.), yeast (such as Saccharomyces sp.) and other fungi, insects, plants, mammalian (including human) cells in culture, or other hosts known in the art.

Next, the transformed host is cultured and purified. Host cells may
5 be cultured under conventional fermentation conditions so that the desired compounds are expressed. Such fermentation conditions are well known in the art. Finally, the peptides are purified from culture by methods well known in the art.

The compounds may also be made by synthetic methods. For
10 example, solid phase synthesis techniques may be used. Suitable techniques are well known in the art, and include those described in Merrifield (1973), Chem. Polypeptides, pp. 335-61 (Katsoyannis and Panayotis eds.); Merrifield (1963), J. Am. Chem. Soc. 85: 2149; Davis et al. (1985), Biochem. Intl. 10: 394-414; Stewart and Young (1969), Solid Phase Peptide Synthesis; U.S. Pat. No. 3,941,763; Finn et al. (1976), The Proteins
15 (3rd ed.) 2: 105-253; and Erickson et al. (1976), The Proteins (3rd ed.) 2: 257-527. Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides.

20 Compounds that contain derivatized peptides or which contain non-peptide groups may be synthesized by well-known organic chemistry techniques.

Uses of the Compounds

In general, The compounds of this invention have pharmacologic
25 activity resulting from their ability to bind to proteins of interest as agonists, mimetics or antagonists of the native ligands of such proteins of interest. The utility of specific compounds is shown in Table 2. The activity of these compounds can be measured by assays known in the art. For the TPO-mimetic and EPO-mimetic compounds, in vivo assays are further
30 described in the Examples section herein.

In addition to therapeutic uses, the compounds of the present invention are useful in diagnosing diseases characterized by dysfunction of their associated protein of interest. In one embodiment, a method of detecting in a biological sample a protein of interest (e.g., a receptor) that is capable of being activated comprising the steps of: (a) contacting the sample with a compound of this invention; and (b) detecting activation of the protein of interest by the compound. The biological samples include tissue specimens, intact cells, or extracts thereof. The compounds of this invention may be used as part of a diagnostic kit to detect the presence of their associated proteins of interest in a biological sample. Such kits employ the compounds of the invention having an attached label to allow for detection. The compounds are useful for identifying normal or abnormal proteins of interest. For the EPO-mimetic compounds, for example, presence of abnormal protein of interest in a biological sample may be indicative of such disorders as Diamond Blackfan anemia, where it is believed that the EPO receptor is dysfunctional.

Therapeutic uses of EPO-mimetic molecules

The EPO-mimetic compounds of the invention are useful for treating disorders characterized by low red blood cell levels. Included in the invention are methods of modulating the endogenous activity of an EPO receptor in a mammal, preferably methods of increasing the activity of an EPO receptor. In general, any condition treatable by erythropoietin, such as anemia, may also be treated by the EPO-mimetic compounds of the invention. These compounds are administered by an amount and route of delivery that is appropriate for the nature and severity of the condition being treated and may be ascertained by one skilled in the art. Preferably, administration is by injection, either subcutaneous, intramuscular, or intravenous.

Therapeutic uses of TPO-mimetic compounds

For the TPO-mimetic compounds, one can utilize such standard assays as those described in WO95/26746 entitled "Compositions and

Methods for Stimulating Megakaryocyte Growth and Differentiation". In vivo assays also appear in the Examples hereinafter.

The conditions to be treated are generally those that involve an existing megakaryocyte/platelet deficiency or an expected
5 megakaryocyte/platelet deficiency (e.g., because of planned surgery or platelet donation). Such conditions will usually be the result of a deficiency (temporary or permanent) of active Mpl ligand in vivo. The generic term for platelet deficiency is thrombocytopenia, and hence the methods and compositions of the present invention are generally available
10 for treating thrombocytopenia in patients in need thereof.

Thrombocytopenia (platelet deficiencies) may be present for various reasons, including chemotherapy and other therapy with a variety of drugs, radiation therapy, surgery, accidental blood loss, and other specific disease conditions. Exemplary specific disease conditions that
15 involve thrombocytopenia and may be treated in accordance with this invention are: aplastic anemia, idiopathic thrombocytopenia, metastatic tumors which result in thrombocytopenia, systemic lupus erythematosus, splenomegaly, Fanconi's syndrome, vitamin B12 deficiency, folic acid deficiency, May-Hegglin anomaly, Wiskott-Aldrich syndrome, and
20 paroxysmal nocturnal hemoglobinuria. Also, certain treatments for AIDS result in thrombocytopenia (e.g., AZT). Certain wound healing disorders might also benefit from an increase in platelet numbers.

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

The TPO-mimetic compounds of this invention may also be useful in
30 stimulating certain cell types other than megakaryocytes if such cells are found to express Mpl receptor. Conditions associated with such cells that express the

Mpl receptor, which are responsive to stimulation by the Mpl ligand, are also within the scope of this invention.

The TPO-mimetic compounds of this invention may be used in any situation in which production of platelets or platelet precursor cells is desired, or in which stimulation of the c-Mpl receptor is desired. Thus, for example, the compounds of this invention may be used to treat any condition in a mammal wherein there is a need of platelets, megakaryocytes, and the like. Such conditions are described in detail in the following exemplary sources:

WO95/26746; WO95/21919; WO95/18858; WO95/21920 and are incorporated herein.

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

Therapeutic uses of Ang-2 binding molecules

Agents that modulate Ang-2 binding activity, or other cellular activity, may be used in combination with other therapeutic agents to enhance their therapeutic effects or decrease potential side effects.

In one aspect, the present invention provides reagents and methods useful for treating diseases and conditions characterized by undesirable or aberrant levels of Ang-2 activity in a cell. These diseases include cancers, and other hyperproliferative conditions, such as hyperplasia, psoriasis, contact dermatitis, immunological disorders, and infertility.

The present invention also provides methods of treating cancer in an animal, including humans, comprising administering to the animal an effective amount of a specific binding agent, such as a peptibody, that inhibits or decreases Ang-2 activity. The invention is further directed to methods of inhibiting cancer cell growth, including processes of cellular proliferation, invasiveness, and metastasis in biological systems. Methods include use of a compound of the invention as an inhibitor of cancer cell growth. Preferably, the methods are employed to inhibit or reduce cancer

cell growth, invasiveness, metastasis, or tumor incidence in living animals, such as mammals. Methods of the invention are also readily adaptable for use in assay systems, e.g., assaying cancer cell growth and properties thereof, as well as identifying compounds that affect cancer cell growth.

5 The cancers treatable by methods of the present invention preferably occur in mammals. Mammals include, for example, humans and other primates, as well as pet or companion animals such as dogs and cats, laboratory animals such as rats, mice and rabbits, and farm animals such as horses, pigs, sheep, and cattle.

10 Tumors or neoplasms include growths of tissue cells in which the multiplication of the cells is uncontrolled and progressive. Some such growths are benign, but others are termed malignant and may lead to death of the organism. Malignant neoplasms or cancers are distinguished from benign growths in that, in addition to exhibiting aggressive cellular
15 proliferation, they may invade surrounding tissues and metastasize. Moreover, malignant neoplasms are characterized in that they show a greater loss of differentiation (greater dedifferentiation), and of their organization relative to one another and their surrounding tissues. This property is also called "anaplasia."

20 Neoplasms treatable by the present invention also include solid tumors, i.e., carcinomas and sarcomas. Carcinomas include those malignant neoplasms derived from epithelial cells that infiltrate (invade) the surrounding tissues and give rise to metastases. Adenocarcinomas are carcinomas derived from glandular tissue, or which form recognizable
25 glandular structures. Another broad category of cancers includes sarcomas, which are tumors whose cells are embedded in a fibrillar or homogeneous substance like embryonic connective tissue. The invention also enables treatment of cancers of the myeloid or lymphoid systems, including leukemias, lymphomas and other cancers that typically do not
30 present as a tumor mass, but are distributed in the vascular or lymphoreticular systems.

The ang-2 binding molecules of this invention are thus useful for the treatment of a wide variety of cancers, including solid tumors and leukemias. Types of cancer or tumor cells amenable to treatment according to the invention include, for example, ACTH-producing tumor;

5 acute lymphocytic leukemia; acute nonlymphocytic leukemia; adenoma; cancer of the adrenal cortex; adenocarcinoma of the breast, prostate, and colon; ameloblastoma; apudoma; bladder cancer; brain cancer; branchioma; breast cancer; all forms of bronchogenic carcinoma of the lung; carcinoid heart disease; carcinoma (e.g., Walker, basal cell,

10 basosquamous, Brown-Pearce, ductal, Ehrlich tumor, Krebs 2, merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhus, bronchiolar, bronchogenic, squamous cell, and transitional cell); malignant carcinoid syndrome; immunoproliferative small lung cell carcinoma; cementoma; cervical cancer; chondroblastoma; chondroma; chondrosarcoma;

15 choristoma; chronic lymphocytic leukemia; chronic myelocytic leukemia; colorectal cancer; chordoma; craniopharyngioma; cutaneous T-cell lymphoma; dysgerminoma; endometrial cancer; esophageal cancer; Ewing's sarcoma; fibroma; fibrosarcoma; gallbladder cancer; giant cell tumors; glioma; hairy cell leukemia; hamartoma; head and neck cancer;

20 hepatoma; histiocytic disorders; histiocytosis; Hodgkin's lymphoma; Kaposi's sarcoma; kidney cancer; lipoma; liposarcoma; liver cancer; lung cancer (small and non-small cell); malignant peritoneal effusion; malignant pleural effusion; melanoma; mesenchymoma; mesonephroma; mesothelioma; multiple myeloma; myosarcoma; myxoma; myxosarcoma;

25 neuroblastoma; non-Hodgkin's lymphoma; odontoma; osteoma; osteosarcoma; ovarian cancer; ovarian (germ cell) cancer; pancreatic cancer; papilloma; penile cancer; plasmacytoma; prostate cancer; reticuloendotheliosis; retinoblastoma; skin cancer; soft tissue sarcoma; squamous cell carcinomas; stomach cancer; teratoma; testicular cancer;

30 thymoma; thyroid cancer; trophoblastic neoplasms; uterine cancer; vaginal cancer; cancer of the vulva; Wilms' tumor.

Further, the following types of cancers may also be treated:

cholangioma; cholesteatoma; cyclindroma; cystadenocarcinoma;
 cystadenoma; granulosa cell tumor; gynandroblastoma; hidradenoma;
 islet cell tumor; Leydig cell tumor; papilloma; Sertoli cell tumor; theca cell
 5 tumor; leiomyoma; leiomyosarcoma; myoblastoma; myoma; myosarcoma;
 rhabdomyoma; rhabdomyosarcoma; ependymoma; ganglioneuroma;
 glioma; medulloblastoma; meningioma; neurilemmoma; neuroblastoma;
 neuroepithelioma; neurofibroma; neuroma; paraganglioma;
 paraganglioma nonchromaffin; angiokeratoma; angiolymphoid
 10 hyperplasia with eosinophilia; angioma sclerosing; angiomatosis;
 glomangioma; hemangioendothelioma; hemangioma;
 hemangiopericytoma; hemangiosarcoma; lymphangioma;
 lymphangiomyoma; lymphangiosarcoma; pinealoma; carcinosarcoma;
 chondrosarcoma; cystosarcoma phyllodes; fibrosarcoma;
 15 hemangiosarcoma; leiomyosarcoma; leukosarcoma; liposarcoma;
 lymphangiosarcoma; myosarcoma; myxosarcoma; ovarian carcinoma;
 rhabdomyosarcoma; sarcoma; neoplasms; neurofibromatosis; and cervical
 dysplasia.

Therapeutic uses of NGF binding molecules

20 The NGF binding molecules may be used in the prevention or treatment
 of NGF-related diseases and disorders. Such indications include but are not
 limited to pain (including, but not limited to, inflammatory pain and associated
 hyperalgesia and allodynia, neuropathic pain and associated hyperalgesia and
 allodynia, diabetic neuropathy pain, causalgia, sympathetically maintained
 25 pain, deafferentation syndromes, acute pain, tension headache, migraine,
 dental pain, pain from trauma, surgical pain, pain resulting from amputation or
 abscess, causalgia, demyelinating diseases, and trigeminal neuralgia). The
 peptides and modified peptides of the invention have therapeutic value for the
 prevention or treatment of other diseases linked to NGF as a causative agent,
 30 including, but not limited to, asthma, urge incontinence (i.e., hyperactive
 bladder), psoriasis, cancer (especially, pancreatic cancer and melanoma),

chronic alcoholism, stroke, thalamic pain syndrome, diabetes, acquired immune deficiency syndrome ("AIDS"), toxins and chemotherapy, general headache, migraine, cluster headache, mixed-vascular and non-vascular syndromes, general inflammation, arthritis, rheumatic diseases, lupus, osteoarthritis, inflammatory bowel disorders, inflammatory eye disorders, inflammatory or unstable bladder disorders, psoriasis, skin complaints with inflammatory components, sunburn, carditis, dermatitis, myositis, neuritis, collagen vascular diseases, chronic inflammatory conditions, asthma, epithelial tissue damage or dysfunction, herpes simplex, disturbances of visceral motility at respiratory, genitourinary, gastrointestinal or vascular regions, wounds, burns, allergic skin reactions, pruritis, vitiligo, general gastrointestinal disorders, colitis, gastric ulceration, duodenal ulcers, vasomotor or allergic rhinitis, or bronchial disorders.

Therapeutic uses of myostatin binding molecules

The myostatin binding agents of the present invention bind to myostatin and block or inhibit myostatin signaling within targeted cells. The present invention provides methods and reagents for reducing the amount or activity of myostatin in an animal by administering an effective dosage of one or more myostatin binding agents to the animal. In one aspect, the present invention provides methods and reagents for treating myostatin-related disorders in an animal comprising administering an effective dosage of one or more binding agents to the animal. These myostatin-related disorders include but are not limited to various forms of muscle wasting, as well as metabolic disorders such as diabetes and related disorders, and bone degenerative diseases such as osteoporosis.

As shown in the Example 8 of U.S. Ser. No. 10/742,379, exemplary peptibodies of the present invention dramatically increases lean muscle mass in the CD1 nu/nu mouse model. This in vivo activity correlates to the in vitro binding and inhibitory activity described below for the same peptibodies.

Muscle wasting disorders include dystrophies such as Duchenne's muscular dystrophy, progressive muscular dystrophy, Becker's type muscular dystrophy, Dejerine-Landouzy muscular dystrophy, Erb's muscular dystrophy, and infantile neuroaxonal muscular dystrophy. For example, blocking myostatin through use of antibodies in vivo improved the dystrophic phenotype of the mdx mouse model of Duchenne muscular dystrophy (Bogdanovich et al. (2002), Nature 420: 28). Use of an exemplary peptibody increases lean muscle mass and increases the ratio of lean muscle to fat in mdx mouse models as described in Example 9 below.

Additional muscle wasting disorders arise from chronic disease such as amyotrophic lateral sclerosis, congestive obstructive pulmonary disease, cancer, AIDS, renal failure, and rheumatoid arthritis. For example, cachexia or muscle wasting and loss of body weight was induced in athymic nude mice by a systemically administered myostatin (Zimmers et al., supra). In another example, serum and intramuscular concentrations of myostatin-immunoreactive protein was found to be increased in men exhibiting AIDS-related muscle wasting and was inversely related to fat-free mass (Gonzalez-Cadavid et al. (1998), PNAS USA 95: 14938-14943). Additional conditions resulting in muscle wasting may arise from inactivity due to disability such as confinement in a wheelchair, prolonged bedrest due to stroke, illness, bone fracture or trauma, and muscular atrophy in a microgravity environment (space flight). For example, plasma myostatin immunoreactive protein was found to increase after prolonged bedrest (Zachwieja et al. J Gravit Physiol. 6(2):11(1999). It was also found that the muscles of rats exposed to a microgravity environment during a space shuttle flight expressed an increased amount of myostatin compared with the muscles of rats which were not exposed (Lalani et al. (2000), J.Endocrin. 167 (3):417-28).

In addition, age-related increases in fat to muscle ratios, and age-related muscular atrophy appear to be related to myostatin. For example, the average serum myostatin-immunoreactive protein increased with age

in groups of young (19-35 yr old), middle-aged (36-75 yr old), and elderly (76-92 yr old) men and women, while the average muscle mass and fat-free mass declined with age in these groups (Yarasheski et al. J Nutr Aging 6(5):343-8 (2002)). It has also been shown that myostatin gene knockout in mice increased myogenesis and decreased adipogenesis (Lin et al. (2002), Biochem Biophys Res Commun 291(3):701-6, resulting in adults with increased muscle mass and decreased fat accumulation and leptin secretion. Exemplary molecules improve the lean muscle mass to fat ratio in aged mdx mice as shown below.

10 In addition, myostatin has now been found to be expressed at low levels in heart muscle and expression is upregulated after cardiomyocytes after infarct (Sharma et al. (1999), J Cell Physiol. 180 (1):1-9). Therefore, reducing myostatin levels in the heart muscle may improve recovery of heart muscle after infarct.

15 Myostatin also appears to influence metabolic disorders including type 2 diabetes, noninsulin-dependent diabetes mellitus, hyperglycemia, and obesity. For example, lack of myostatin has been shown to improve the obese and diabetic phenotypes of two mouse models (Yen et al. supra). In addition, increasing muscle mass by reducing myostatin levels may
20 improve bone strength and reduce osteoporosis and other degenerative bone diseases. It has been found, for example, that myostatin-deficient mice showed increased mineral content and density of the mouse humerus and increased mineral content of both trabecular and cortical bone at the regions where the muscles attach, as well as increased muscle mass
25 (Hamrick et al. (2002), Calcif Tissue Int 71(1): 63-8). In the present invention, an exemplary peptibody increases the lean muscle mass to fat ratio in mdx mouse models as shown below.

30 The present invention also provides methods and reagents for increasing muscle mass in food animals by administering an effective dosage of the myostatin binding agent to the animal. Since the mature C-terminal myostatin polypeptide is identical in all species tested, myostatin

binding agents would be expected to be effective for increasing muscle mass and reducing fat in any agriculturally important species including cattle, chicken, turkeys, and pigs.

The myostatin-binding molecules of the present invention may be
5 used alone or in combination with other therapeutic agents to enhance
their therapeutic effects or decrease potential side effects. The molecules
of the present invention possess one or more desirable but unexpected
combination of properties to improve the therapeutic value of the agents.
These properties include increased activity, increased solubility, reduced
10 degradation, increased half-life, reduced toxicity, and reduced
immunogenicity. Thus the molecules of the present invention are useful
for extended treatment regimes. In addition, the properties of
hydrophilicity and hydrophobicity of the compounds of the invention are
well balanced, thereby enhancing their utility for both in vitro and
15 especially in vivo uses. Specifically, compounds of the invention have an
appropriate degree of solubility in aqueous media that permits absorption
and bioavailability in the body, while also having a degree of solubility in
lipids that permits the compounds to traverse the cell membrane to a
putative site of action, such as a particular muscle mass.

20 The myostatin-binding molecules of the present invention are
useful for treating a "subject" or any animal, including humans, when
administered in an effective dosages in a suitable composition.

In addition, the myostatin-binding molecules of the present
invention are useful for detecting and quantitating myostatin in a number
25 of assays. These assays are described in detail in U.S. Ser. No. 10/742,379.

In general, the myostatin-binding molecules of the present
invention are useful as capture agents to bind and immobilize myostatin
in a variety of assays, similar to those described, for example, in Asai, ed.,
Methods in Cell Biology, 37, Antibodies in Cell Biology, Academic Press,
30 Inc., New York (1993). The myostatin-binding molecule may be labeled in
some manner or may react with a third molecule such as an anti-binding

molecule antibody which is labeled to enable myostatin to be detected and quantitated. For example, a myostatin-binding molecule or a third molecule can be modified with a detectable moiety, such as biotin, which can then be bound by a fourth molecule, such as enzyme-labeled
5 streptavidin, or other proteins. (Akerstrom (1985), J Immunol 135:2589; Chaubert (1997), Mod Pathol 10:585).

Throughout any particular assay, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5
10 minutes to about 24 hours. However, the incubation time will depend upon the assay format, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures.

Therapeutic uses of BAFF-binding molecules. BAFF-binding
15 molecules of this invention may be particularly useful in treatment of B-cell mediated autoimmune diseases. In particular, they may be useful in treating, preventing, ameliorating, diagnosing or prognosing lupus, including systemic lupus erythematosus (SLE), and lupus-associated diseases and conditions. Other preferred indications include B-cell
20 mediated cancers, including B-cell lymphoma.

The compounds of this invention can also be used to treat inflammatory conditions of the joints. Inflammatory conditions of a joint are chronic joint diseases that afflict and disable, to varying degrees, millions of people worldwide. Rheumatoid arthritis is a disease of
25 articular joints in which the cartilage and bone are slowly eroded away by a proliferative, invasive connective tissue called pannus, which is derived from the synovial membrane. The disease may involve peri-articular structures such as bursae, tendon sheaths and tendons as well as extra-articular tissues such as the subcutis, cardiovascular system, lungs, spleen,
30 lymph nodes, skeletal muscles, nervous system (central and peripheral) and eyes (Silberberg (1985), Anderson's Pathology, Kissane (ed.), II:1828).

Osteoarthritis is a common joint disease characterized by degenerative changes in articular cartilage and reactive proliferation of bone and cartilage around the joint. Osteoarthritis is a cell-mediated active process that may result from the inappropriate response of chondrocytes to catabolic and anabolic stimuli. Changes in some matrix molecules of articular cartilage reportedly occur in early osteoarthritis (Thonar *et al.* (1993), *Rheumatic disease clinics of North America*, Moskowitz (ed.), 19:635-657 and Shinmei *et al.* (1992), *Arthritis Rheum.*, 35:1304-1308). TALL-1, TALL-1R and modulators thereof are believed to be useful in the treatment of these and related conditions.

BAFF-binding molecules may also be useful in treatment of a number of additional diseases and disorders, including acute pancreatitis; ALS; Alzheimer's disease; asthma; atherosclerosis; autoimmune hemolytic anemia; cancer, particularly cancers related to B cells; cachexia/anorexia; chronic fatigue syndrome; cirrhosis (e.g., primary biliary cirrhosis); diabetes (e.g., insulin diabetes); fever; glomerulonephritis, including IgA glomerulonephritis and primary glomerulonephritis; Goodpasture's syndrome; Guillain-Barre syndrome; graft versus host disease; Hashimoto's thyroiditis; hemorrhagic shock; hyperalgesia; inflammatory bowel disease; inflammatory conditions of a joint, including osteoarthritis, psoriatic arthritis and rheumatoid arthritis; inflammatory conditions resulting from strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease processes; insulin-dependent diabetes mellitus; ischemic injury, including cerebral ischemia (e.g., brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration); learning impairment; lung diseases (e.g., ARDS); lupus, particularly systemic lupus erythematosus (SLE); multiple myeloma; multiple sclerosis; Myasthenia gravis; myelogenous (e.g., AML and CML) and other leukemias; myopathies (e.g., muscle protein metabolism, esp. in sepsis); neurotoxicity (e.g., as induced by HIV); osteoporosis; pain; Parkinson's disease; Pemphigus;

polymyositis/dermatomyositis; pulmonary inflammation, including autoimmune pulmonary inflammation; pre-term labor; psoriasis; Reiter's disease; reperfusion injury; septic shock; side effects from radiation therapy; Sjogren's syndrome; sleep disturbance; temporal mandibular joint disease; thrombocytopenia, including idiopathic thrombocytopenia and autoimmune neonatal thrombocytopenia; tumor metastasis; uveitis; and vasculitis.

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

production. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

In cases where the inventive compounds are added to compositions of platelets and/or megakaryocytes and related cells, the amount to be included will generally be ascertained experimentally by techniques and assays known in the art. An exemplary range of amounts is 0.1 μ g – 1 mg inventive compound per 10^6 cells.

Pharmaceutical Compositions

In General

The present invention also provides methods of using pharmaceutical compositions of the inventive compounds. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal, transdermal or other forms of administration. In general, the invention encompasses pharmaceutical compositions comprising effective amounts of a compound of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in

dried powder, such as lyophilized form. Implantable sustained release formulations are also contemplated, as are transdermal formulations.

Oral dosage forms

Contemplated for use herein are oral solid dosage forms, which are
5 described generally in Chapter 89 of Remington's Pharmaceutical Sciences
(1990), 18th Ed., Mack Publishing Co. Easton PA 18042, which is herein
incorporated by reference. Solid dosage forms include tablets, capsules,
pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid
encapsulation may be used to formulate the present compositions (as, for
10 example, proteinoid microspheres reported in U.S. Patent No. 4,925,673).
Liposomal encapsulation may be used and the liposomes may be
derivatized with various polymers (e.g., U.S. Patent No. 5,013,556). A
description of possible solid dosage forms for the therapeutic is given in
Chapter 10 of Marshall, K., Modern Pharmaceutics (1979), edited by G. S.
15 Banker and C. T. Rhodes, herein incorporated by reference. In general, the
formulation will include the inventive compound, and inert ingredients
which allow for protection against the stomach environment, and release
of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above
20 inventive compounds. If necessary, the compounds may be chemically
modified so that oral delivery is efficacious. Generally, the chemical
modification contemplated is the attachment of at least one moiety to the
compound molecule itself, where said moiety permits (a) inhibition of
proteolysis; and (b) uptake into the blood stream from the stomach or
25 intestine. Also desired is the increase in overall stability of the compound
and increase in circulation time in the body. Moieties useful as covalently
attached vehicles in this invention may also be used for this purpose.
Examples of such moieties include: PEG, copolymers of ethylene glycol
and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol,
30 polyvinyl pyrrolidone and polyproline. See, for example, Abuchowski and
Davis, Soluble Polymer-Enzyme Adducts, Enzymes as Drugs (1981),

Hocenberg and Roberts, eds., Wiley-Interscience, New York, NY, , pp 367-83; Newmark, et al. (1982), J. Appl. Biochem. 4:185-9. Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are PEG moieties.

5 For oral delivery dosage forms, it is also possible to use a salt of a modified aliphatic amino acid, such as sodium N-(8-[2-hydroxybenzoyl] amino) caprylate (SNAC), as a carrier to enhance absorption of the therapeutic compounds of this invention. The clinical efficacy of a heparin formulation using SNAC has been demonstrated in a Phase II trial
10 conducted by Emisphere Technologies. See US Patent No. 5,792,451, "Oral drug delivery composition and methods".

 The compounds of this invention can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1 mm. The formulation of the material for capsule
15 administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

 Colorants and flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible
20 product, such as a refrigerated beverage containing colorants and flavoring agents.

 One may dilute or increase the volume of the compound of the invention with an inert material. These diluents could include carbohydrates, especially mannitol, α -lactose, anhydrous lactose, cellulose,
25 sucrose, modified dextrans and starch. Certain inorganic salts may also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

 Disintegrants may be included in the formulation of the therapeutic
30 into a solid dosage form. Materials used as disintegrants include but are not limited to starch including the commercial disintegrant based on

starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic
5 exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form
10 a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

15 An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin,
20 vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be
25 added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the compound of this invention into the aqueous environment a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate,
30 dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or

benzethonium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives may also be included in the formulation to enhance uptake of the compound. Additives potentially having this property are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release formulation may be desirable. The compound of this invention could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms e.g., gums. Slowly degenerating matrices may also be incorporated into the formulation, e.g., alginates, polysaccharides. Another form of a controlled release of the compounds of this invention is by a method based on the Oros therapeutic system (Alza Corp.), i.e., the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film coated tablet and the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

Pulmonary delivery forms

- 5 Also contemplated herein is pulmonary delivery of the present protein (or derivatives thereof). The protein (or derivative) is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream. (Other reports of this include Adjei et al., Pharma. Res. (1990) 7: 565-9; Adjei et al. (1990), Internatl. J.
- 10 Pharmaceutics 63: 135-44 (leuprolide acetate); Braquet et al. (1989), J. Cardiovasc. Pharmacol. 13 (suppl.5): s.143-146 (endothelin-1); Hubbard et al. (1989), Annals Int. Med. 3: 206-12 (α 1-antitrypsin); Smith et al. (1989), J. Clin. Invest. 84: 1145-6 (α 1-proteinase); Oswein et al. (March 1990), "Aerosolization of Proteins", Proc. Symp. Resp. Drug Delivery II,
- 15 Keystone, Colorado (recombinant human growth hormone); Debs et al. (1988), J. Immunol. 140: 3482-8 (interferon- γ and tumor necrosis factor α) and Platz et al., U.S. Patent No. 5,284,656 (granulocyte colony stimulating factor).

- Contemplated for use in the practice of this invention are a wide
- 20 range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent
- 25 nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

- 30 All such devices require the use of formulations suitable for the dispensing of the inventive compound. Typically, each formulation is

specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to diluents, adjuvants and/or carriers useful in therapy.

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

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

Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the inventive compound dissolved in
20 water at a concentration of about 0.1 to 25 mg of biologically active protein per mL of solution. The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by
25 atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the inventive compound suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose,
30 such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane,

dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

5 Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing the inventive compound and may also include a bulking agent, such as lactose, sorbitol, sucrose, mannitol, trehalose, or xylitol in amounts which facilitate dispersal of the powder from the device, e.g., 50 to 90% by weight of the formulation.

10 Other delivery forms

Nasal delivery of the inventive compound is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for
15 nasal delivery include those with dextran or cyclodextran. Delivery via transport across other mucous membranes is also contemplated.

Buccal delivery of the inventive compound is also contemplated. Buccal delivery formulations are known in the art for use with peptides.

Dosages

20 The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the daily regimen
25 should be in the range of 0.1-1000 micrograms of the inventive compound per kilogram of body weight, preferably 0.1-150 micrograms per kilogram.

Specific preferred embodiments

The inventors have determined preferred peptide sequences for molecules having many different kinds of activity. The inventors have
30 further determined preferred structures of these preferred peptides combined with preferred linkers and vehicles. Preferred structures for

these preferred peptides listed in Table 11 below. Linker sequences are shown in bold. Active peptide sequences are shown in bold and are underlined.

Table 11 – Preferred embodiments

5

	Sequence/structure	SEQ ID NO:	Activity
1	MDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE	616	Fc-loop-Amp2
51	DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVSVLTVL HQDWLNGKEY		
1 01	KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELG <u>GIEGPTLRQW</u>		
1 51	<u>LAARAGG</u> TKN QVSLTCLVKG FYPSDIAVEW ESNGQPENNY KTTTPVLDSD		
2 01	GSFFLYSKLT VDKSRWQQGN VFSCSVMHEA LHNHYTQKSL SLSPGK*		
1	MDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE	648	Fc-Loop-EMP1 (1 Gly linkers)
51	DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVSVLTVL HQDWLNGKEY		
1 01	KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDEL <u>GGGTYSCHFGPL</u>		
1 51	<u>TWVCKPQGGG</u> TKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD		
2 01	SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK*		
1	MDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE	649	Fc-loop-EMP1 (2Gly linkers)
51	DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVSVLTVL HQDWLNGKEY		
1 01	KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELG <u>GTYSCHFGPL</u>		
1 51	<u>TWVCKPQGGT</u> KKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD		
2 01	SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK*		
1	MDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE	650	Fc-loop-EMP1 (3Gly linkers)
51	DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVSVLTVL HQDWLNGKEY		
1 01	KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELG <u>GGTYSCHFGP</u>		
1 51	<u>LTWVCKPQGG</u> GTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTTPV		
2 01	LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK		
1	MDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE	651	Fc-loop-EMP1 (4Gly linkers)
51	DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVSVLTVL HQDWLNGKEY		
1 01	KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELG <u>GGGTYSCHFG</u>		
1 51	<u>PLTWVCKPQG</u> GGGTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTTP		
2 01	PVLDSGDSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP		
2 51	GK*		
1	MDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE	652	Fc-loop-EMP1 (Cys>Ala variant w/4Gly linkers)
51	DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVSVLTVL HQDWLNGKEY		
1 01	KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELG <u>GGGTYSAHFG</u>		
1 51	<u>PLTWVAKPQG</u> GGGTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTTP		

201	PVLDSGDSFF	LYSKLTVDKS	RWQQGNVFSC	SVMHEALHNH	YTQKSLSLSP		
251	GK*						
1	MDKTHTCPPC	PAPELLGGPS	VFLFPPKPKD	TLNISRTPEV	TCVVVDVSHE	653	Fc-loop-TMP20 (2Gly linkers)
51	DPEVKFNWYV	DGVEVHNAKT	KPREEQYNST	YRVVSVLTVL	HQDWLNGKEY		
101	KCKVSNKALP	APIEKTISKA	KGQPREPQVY	TLPPSRDELG	<u>GQGYCDEGPT</u>		
151	<u>LKQWLVLGL</u>	<u>QHSGG</u> TKNQV	SLTCLVKGFY	PSDIAVEWES	NGQPENNYKT		
201	TPPVLDSDGS	FFLYSKLTV	KSRWQQGNVF	SCSVMHEALH	NHYTQKSLSL		
251	SPGK*						
1	MDKTHTCPPC	PAPELLGGPS	VFLFPPKPKD	TLNISRTPEV	TCVVVDVSHE	654	Fc-loop-TMP20 (Cys>A la variant, w/2Gly linkers)
51	DPEVKFNWYV	DGVEVHNAKT	KPREEQYNST	YRVVSVLTVL	HQDWLNGKEY		
101	KCKVSNKALP	APIEKTISKA	KGQPREPQVY	TLPPSRDELG	<u>GQGYADEGPT</u>		
151	<u>LKQWLVALGL</u>	<u>QHSGG</u> TKNQV	SLTCLVKGFY	PSDIAVEWES	NGQPENNYKT		
201	TPPVLDSDGS	FFLYSKLTV	KSRWQQGNVF	SCSVMHEALH	NHYTQKSLSL		
251	SPGK*						
1	MDKTHTCPPC	PAPELLGGPS	VFLFPPKPKD	TLNISRTPEV	TCVVVDVSHE	655	Fc-loop-GLP1 (2Gly linkers)
51	DPEVKFNWYV	DGVEVHNAKT	KPREEQYNST	YRVVSVLTVL	HQDWLNGKEY		
101	KCKVSNKALP	APIEKTISKA	KGQPREPQVY	TLPPSRDELG	<u>GHAEGTFTSD</u>		
151	<u>VSSYLEGOAA</u>	<u>KEFIAWLKVG</u>	<u>RGGG</u> TKNQVS	LTCLVKGFYP	SDIAVEWESN		
201	GQPENNYKTT	PPVLDSDGSF	FLYSKLTVDK	SRWQQGNVFS	CSVMHEALHN		
251	HYTQKSLSL	PGK*					
1	MDKTHTCPPC	PAPELLGGPS	VFLFPPKPKD	TLNISRTPEV	TCVVVDVSHE	656	Fc-loop-GLP1 (4Gly linkers)
51	DPEVKFNWYV	DGVEVHNAKT	KPREEQYNST	YRVVSVLTVL	HQDWLNGKEY		
101	KCKVSNKALP	APIEKTISKA	KGQPREPQVY	TLPPSRDELG	<u>GGGHAEGTFT</u>		
151	<u>SDVSSYLEGO</u>	<u>AAKEFIAWL</u> V	<u>KGRGGGGG</u> TK	NQVSLTCLVK	GFYPSDIAVE		
201	WESNGQPENN	YKTPPVLDSD	DGSFFLYSKL	TVDKSRWQQG	NVFSCSVMHE		
251	ALHNHYTQKS	LSLSPGK*					
1	MDKTHTCPPC	PAPELLGGPS	VFLFPPKPKD	TLNISRTPEV	TCVVVDVSHE	657	Fc-loop-ANG2 (2Gly linkers)
51	DPEVKFNWYV	DGVEVHNAKT	KPREEQYNST	YRVVSVLTVL	HQDWLNGKEY		
101	KCKVSNKALP	APIEKTISKA	KGQPREPQVY	TLPPSRDELG	<u>GQEECEWDPW</u>		
151	<u>TCEHMG</u> GTKN	QVSLTCLVK	GFYPSDIAVE	ESNGQPENNY	KTPPVLDSD		
201	GSFFLYSKLT	VDKSRWQQGN	VFSCSVMHEA	LHNHYTQKSL	SLSPGK*		
1	MDKTHTCPPC	PAPELLGGPS	VFLFPPKPKD	TLNISRTPEV	TCVVVDVSHE	612	Fc-loop-Myo7 (2Gly linkers)
51	DPEVKFNWYV	DGVEVHNAKT	KPREEQYNST	YRVVSVLTVL	HQDWLNGKEY		
101	KCKVSNKALP	APIEKTISKA	KGQPREPQVY	TLPPSRDELG	<u>GLADHGQCIR</u>		
151	<u>WFWMCPEGW</u>	<u>EGG</u> TKNQVSL	TCLVKGFYPS	DIAVEWESNG	QPENNYKTPP		
201	PVLDSGDSFF	LYSKLTVDKS	RWQQGNVFSC	SVMHEALHNH	YTQKSLSLSP		
251	GK*						

1	MDKTHTCPPC PAPELLGGPS VLFPPKPKD TLMISRTPEV TCVVVDVSHE	658	Fc-loop-ANG1 (4Gly linkers)
51	DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVSVLTVL HQDWLNGKEY		
101	KCKVSNKALP APIEKTISKA KGQPREPOVY TLPPSRDELG <u>GGGGDWTGDM</u>		
151	<u>QVKFDAMMFG PRKEGGGGT</u> KNQVSLTCLV KGFYPSDIAV EWESNGQPEN		
201	NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK		
251	SLSLSPGK		
1	MDKTHTCPPC PAPELLGGPS VLFPPKPKD TLMISRTPEV TCVVVDVSHE	659	Fc-loop-ANG1 (2x peptide w/4Gly linkers)
51	DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVSVLTVL HQDWLNGKEY		
101	KCKVSNKALP APIEKTISKA KGQPREPOVY TLPPSRDELG <u>GGGGDWTGDM</u>		
151	<u>QVKFDAMMFG PRKEGGDWT</u> <u>GDMQVKFDAM MFGPRKEGGG</u> GGTKNQVSLT		
201	CLVKGFYPSD IAVEWESNGQ PENNYKTTPP VLDSGDSFFL YSKLTVDKSR		
251	WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K		

Working examples

The compounds described above may be prepared as described below. These examples comprise preferred embodiments of the invention and are illustrative rather than limiting.

Example 1

Preparation of Fc-loop-ang2

In this example of the invention, the disulphide-constrained peptide TN8-Con4 was inserted into the human IgG1 Fc-loop domain, defined as the sequence D₁₃₇E₁₃₈L₁₃₉T₁₄₀K₁₄₁ (Fig 2A).

TN8-Con4 QEECEWDPWTCEHM (SEQ ID NO: 147)

The peptide insertion is between Fc residues Leu₁₃₉ and Thr₁₄₀ and includes 2 Gly residues as linkers flanking either side of the inserted peptide (Fig. 10A). The Fc-loop TN8-Con4 construct is labeled with Amgen clone #6888. The carboxy-terminal TN8-Con4 fusion peptibody (Fig. 10B) includes a 5 Gly linker and is labeled with Amgen clone #5564.

Both clones #6888 and #5564 were transformed into E. coli by conventional methods known to those familiar in the art. Both clones were found to express at high levels and almost exclusively in the insoluble inclusion body fraction (Fig. 11). The isolated inclusion body fraction (1 g) was solubilized in 6 M guanidine-HCl, 50 mM Tris, 8 mM

DTT, pH 9 (10 ml) at room temperature with mixing, for 1 hour. The denatured and reduced peptibodies were refolded from the solubilized inclusion bodies by a 1:25 (v/v) dilution into a refold buffer consisting of 2 M urea, 50 mM Tris, 4 mM cysteine, 1 mM cystamine, pH 8.5. The
5 solubilized peptibodies were added drop wise to the refold buffer at 4 °C with stirring. The refold reactions were allowed to stir for 48 hours, and then aliquots were evaluated by SDS-PAGE and reverse-phase HPLC. The Fc-loop TN8-Con4 (#6888) is considerably more homogeneous in the refold reaction than the carboxy-terminal Fc TN8-Con4 peptibody (#5564)
10 as shown by RP-HPLC (Fig. 12).

Purification of the refolded Fc-loop TN8-Con4 and carboxy-terminal Fc TN8-Con4 was achieved using a 2-column process. First a recombinant Protein-A column was equilibrated in 2 M urea, 50 mM Tris, pH 8.5 and loaded with the filtered peptibody refold reaction. The column
15 was then washed with 2 column volumes of equilibration buffer, followed by 2 column volumes of PBS. The peptibody fraction was eluted with 50mM NaOAc, pH3 and quickly neutralized by a 1:4 dilution into 10 mM NaOAc, 50 mM NaCl, pH 5. The diluted Protein-A eluate was again filtered and loaded to an SP Sepharose HP cation exchange column
20 (Pharmacia) equilibrated in 10 mM NaOAc, 50 mM NaCl, pH 5. The peptibody fractions were then eluted with a linear 50-500 mM NaCl gradient, pooled and concentrated to about 2 mg/ml. The final pools of Fc-loop TN8-Con4 (#6888) and the carboxy-terminal Fc TN8-Con4 (#5564) were evaluated by RP-HPLC (Fig. 13) and SDS-PAGE (Fig. 14). Both RP-
25 HPLC and SDS-PAGE demonstrate that improved product homogeneity is achieved with the Fc-loop TN8-Con4 (#6888) relative to the comparable carboxy-terminal fused peptibody (#5564).

Both the Fc-loop TN8-Con4 and carboxy-terminal fused TN8-Con4 peptibodies were further evaluated in an in vitro ELISA for competitive
30 inhibition of the angiopoietin 2 receptor. In this format the peptibody competes with an angiopoietin 2 receptor-Fc fusion for binding to

immobilized angiopoietin 2. Binding of the angiopoietin 2 receptor-Fc fusion is monitored by fluorescence using an enzyme-linked immunodetection method and reported as an inhibition constant at 50% inhibition (IC_{50}). This experiment shows that the Fc-loop TN8-Con4 is
 5 fully active relative to the carboxy-terminal Fc TN8-Con4 (Fig. 15).

Stability in vivo of the Fc-loop TN8-Con4 peptibody was compared to the carboxy-terminal TN8-Con4 peptibody in mice. In this study groups of 15 mice were dosed subcutaneously with either peptibody construct at 5 mg/kg. At 4 hours after injection, 5 mice were sacrificed
 10 and serum collected. At 24 hours, another 5 mice were harvested and likewise at 48 hours. Each individual serum sample was evaluated by western blot for detectable human IgG-Fc peptibody. Since all the serum within each 5-mouse group was very similar, the groups were pooled to allow representative samples to be run together on a single gel/blot. The
 15 result of that analysis (Fig. 16) clearly shows that both the Fc-loop TN8-Con4 peptibody and the carboxy-terminal TN8-Con4 peptibody persists in the pooled mouse sera throughout the 48-hour time course with no apparent loss. This result demonstrates that the Fc-loop designed peptibodies are not destabilized in vivo by the peptide insertion.

20

Example 2

Preparation of Fc-loop-myo7

In another embodiment of this invention, a novel, disulphide-constrained peptide TN8-19-7 (U.S. Pat App 2004-0181033-A1, which is incorporated by reference) of the sequence:

25 **TN8-19-07** **LADHGQCIRWPWMCPPEGWE** (SEQ ID NO: 365)

was engineered between Leu139 and Thr140 as an internal fusion in the putative Fc-loop sequence DELTK of an IgG1 Fc sequence (Fig 2A). An additional two Gly residues were also added at each end of the TN8-19-07 peptide as flanking linkers. The final Fc-loop TN8-19-07 sequence is given
 30 in Figure 3A and is labeled clone # 6951. Alternatively, a carboxy terminal fusion of TN8-19-07 with the same IgG1 Fc sequence was prepared to

serve as a control (Fig 3B) and labeled clone #6826. The carboxy-terminal fusion included five Gly residues between the Fc and TN8-19-07 to serve as a linker.

Both clones #6951 and #6826 were transformed into *E. coli* by conventional methods used by those familiar in the art, and were found to express at high levels and almost exclusively in the insoluble inclusion body fraction (Fig. 4A and 4B). The isolated inclusion body fraction (1 g) was solubilized in 6 M guanidine-HCl, 50 mM Tris, 8 mM DTT, pH 9 (10 ml) at room temperature with mixing for 1 hour. The denatured and reduced peptibodies were refolded from the solubilized inclusion body fraction by a 1:25 (v/v) dilution into 2 M urea, 50 mM Tris, 4 mM cysteine, 1 mM cystamine, pH 8.5. The solubilized peptibodies were added drop-wise to the refold buffer at 4 °C with stirring. The refold reactions were allowed to stir for 48 hours, and then aliquots were evaluated by SDS-PAGE and reverse-phase HPLC. The Fc-loop TN8-19-07 (#6951) was found to be considerably more homogeneous by RP-HPLC (Fig. 5) in the refold reaction than the carboxy-terminal Fc-TN8-19-07 peptibody (#6826).

Purification was achieved by a 2-column process. First a recombinant Protein-A column was equilibrated in 2 M urea, 50 mM Tris, pH 8.5 and loaded with the filtered peptibody refold reaction. The column was then washed with 2 column volumes of equilibration buffer, followed by 2 column volumes of PBS. The peptibody fraction was eluted with 50 mM NaOAc, pH 3 and quickly neutralized by a 1:4 dilution into 10 mM NaOAc, 50 mM NaCl, pH 5. The diluted Protein-A eluate was again filtered and loaded to an SP Sepharose HP cation exchange column (Pharmacia) equilibrated in 10 mM NaOAc, 50 mM NaCl, pH 5. The peptibody fractions were then eluted with a linear 50-500 mM NaCl gradient, pooled and concentrated to about 2 mg/ml. The final pools of Fc-loop TN8-19-07 (#6951) and the carboxy-terminal Fc TN8-19-07 (#6826) were evaluated by RP-HPLC (Fig. 6) and SDS-PAGE (Fig. 7). Both RP-HPLC and SDS-PAGE demonstrate that improved homogeneity in the

final product is achieved with the Fc-loop TN8-19-07 (#6951) relative to the comparable carboxy-terminal fused peptibody (#6826).

An in vitro cell-based assay, which measures the inhibition of myostatin signaling activity, was used to determine the bioactivity of the Fc-loop TN8-19-07 (#6951) compared to the carboxy-terminal fusion (#6826). In this assay, both constructs were titrated against 4 nM myostatin and evaluated for their ability to inhibit the myostatin signaling activity as measured by a luciferase reporter system. The relative peptibody activities are reported as the effective concentration for 50% inhibition (EC₅₀). This experiment shows that the Fc-loop TN8-19-07 peptibody (#6951) retains full in vitro bioactivity (Fig. 8).

Stability in vivo of the Fc-loop TN8-19-07 peptibody was compared to the carboxy-terminal TN8-19-07 peptibody in mice. In this study, groups of 15 mice were dosed subcutaneously with either peptibody construct at 5 mg/kg. At 4 hours post injection 5 mice were sacrificed and serum collected. At 24 hours another 5 mice were harvested and likewise at 48 hours. Each individual serum was evaluated by western blot for detectable human IgG-Fc peptibody. Since all the serum within each 5-mouse group was very similar, the groups were pooled to allow representative samples to be run together on a single gel/blot. The result of that analysis (Fig. 9) clearly shows that the Fc-loop TN8-19-07 peptibody persists in the pooled mouse sera throughout the 48-hour time course with no apparent loss. In contrast, the concentration of the carboxy-terminal TN8-19-07 peptibody diminishes steadily through the course of the study until it is nearly undetectable at the 48-hour time point. This result suggests that the Fc-loop design approach may confer additional in vivo stability to the TN8-19-07 peptibody.

Example 3

Preparation of TN8-Con4

This molecule was prepared as described above in Example 1 and in U.S. Pat. App. No. 2003/0236192 (also PCT/US04/10989), which is hereby incorporated by reference.

Example 4

5

Preparation of Fc-ang2-tandem

This molecule was prepared as described in U.S. 2003/0236193, published Dec. 25, 2003 (also PCT/US04/10989, filed April 8, 2004), which is hereby incorporated by reference.

Example 5

10

Preparation of TN8-19-7

This molecule was prepared as described above in example 2 and in U.S. Serial No. 10/742,379, filed Dec. 19, 2003 (also PCT/US03/40781, filed Dec. 19, 2003), which is hereby incorporated by reference.

Example 6

15

Preparation of Fc-loop-EMP

This molecule was prepared as previously described in example 1.

Example 7

Preparation of Fc-loop-Amp2

20 In another embodiment of this invention a linear, non-constrained peptide, AMP 2 was inserted into the human IgG1 Fc -loop domain, defined as the sequence D₁₃₇E₁₃₈L₁₃₉T₁₄₀K₁₄₁ (Fig. 2A).

AMP-2: IEGPTLRQWLAARA (SEQ ID NO: 28)

The Fc insertion is between Leu₁₃₉ and Thr₁₄₀ and includes 2 Gly residues as linkers flanking either side of the inserted peptide (Fig. 3D).

25 The Fc-loop AMP 2 construct is labeled as Amgen clone #6875.

The Fc-loop AMP 2 clone (#6875) was transformed into E. coli by conventional methods known to those in the art and was found to express at high levels and almost exclusively in the insoluble inclusion body fraction (Fig. 17). The isolated inclusion body fraction (1g) was solubilized
30 in 6 M guanidine-HCl, 50 mM Tris, 8 mM DTT, pH 9 (10 ml) at room temperature with mixing, for 1 hour. The denatured and reduced

peptibody was refolded from the solubilized inclusion body fraction by a 1:25 (v/v) dilution into 2 M urea, 50 mM Tris, 4 mM cysteine, 1 mM cystamine, pH 8.5. The solubilized peptibody was added drop wise to the refold buffer at 4 °C with stirring. The refold reactions were allowed to stir for 48 hours, and then aliquots were evaluated by SDS-PAGE and reversed-phase HPLC.

Purification was achieved using a 2-column process. First a recombinant Protein-A column was equilibrated in 2 M urea, 50 mM Tris, pH 8.5 and loaded with the filtered peptibody refold reaction. The column was then washed with 2 column volumes of equilibration buffer, followed by 2 column volumes of PBS. The peptibody fraction was eluted with 50 mM NaOAc, pH 3 and quickly neutralized by a 1:4 dilution into 10 mM NaOAc, 50 mM NaCl, pH 5. The diluted Protein-A eluate was again filtered and loaded to an SP Sepharose HP cation exchange column (Pharmacia) equilibrated in 10 mM NaOAc, 50 mM NaCl, pH 5. The peptibody fractions were then eluted with a linear 50-500 mM NaCl gradient, pooled and concentrated to about 2 mg/ml. The final pools of Fc-loop AMP 2 (#6875) were evaluated by SDS-PAGE (Fig. 19) and RP-HPLC (Fig. 20).

The final preparation of Fc-loop AMP 2 was tested in an *in vivo* mouse bioassay against a carboxy-terminal peptibody fusion of two AMP 2 sequences linked in tandem (Fc tandem AMP2). In this comparison, the Fc-tandem-AMP2 has a total valence of four AMP 2 peptides compared to the Fc-loop AMP 2 with only two peptides. The mice received a single subcutaneous injection of 50 µg/kg of either peptibody while their platelet levels were monitored over 15 days (Fig. 21). While the Fc-tandem-AMP2 induced a significant initial platelet increase, the total response was complete by day 9. In contrast, the Fc-loop AMP 2 elicited a much smaller response that peaked at day 8 and persisted for 15 days. These results suggest that the efficacious half-life of the Fc-loop AMP 2 peptibody may be much greater than the conventional carboxy-terminal fused peptibody.

The difference in overall amplitude of the response may be a consequence of the greater valence of Fc-tandem-Amp2.

Example 8

Preparation of Amp2

5 This molecule was prepared as described above in example 7, and in U.S. Pat. No. 6,660,843.

Example 9

10 **In Vitro cell-based assay and the measurement of myostatin-signaling activity (Figure 8)**

To quantitate myostatin activity and its blockade, a luciferase reporter system was developed, referred to as pMARE-Luc, which senses Myostatin/Activin signaling strength. The pMARE-luc vector was constructed by subcloning a Smad-responsive CAGA tandem repeat
15 sequence into a basic reporter plasmid pLuc-MCS containing a minimal promoter element (TATA box). The pMARE-luc vector was stably transfected into a skeletal muscle-derived C2C12 cell line (murine).

Characterization of myostatin responses of the stable clones led to the identification of C2C12-based clonal reporter cell lines that were
20 capable of detecting both myostatin and Activin signaling activities in 96-well format in a highly sensitive and reproducible manner.

Example 10

25 **In Vitro HTRF (homogeneous time-resolved fluorescence) Ang-2 binding assay (Figure 15).**

Starting from a concentration of 100 nM, Fc-loop peptibody and proper controls were serially diluted in HTRF buffer 3-fold, 9-times across a 96-well plate. Dilutions were then mixed with the following reagents on
30 a 96-well black, round-bottomed assay plate: Streptavidin-Europium (1.6 nM), Biotinylated human angiopoietin-2 (8.0 nM), human Tie2-Fc-APC (10 nM). Assay plate was then incubated at room temperature with shaking

for 2 hours. Plate next read on a Rubystar microplate reader (BMG Labtechnologies Inc.). Results were converted to %inhibition, and IC50s were then calculated by analyzing the %inhibition values in the program GRAFIT 5.0 (IC50, 0-100% parameter).

5

Example 11

In Vivo AMP-2 efficacy assay (Figure 21)

Female BDF1 mice are injected subcutaneously with either carrier fluid (1 x PBS with 0.1% BSA), 50 mcg/kg of Fc-tandem-AMP2, or 50 mcg/kg of Fc-loop-AMP2. The injection volume is 0.2 mL. Blood is collected from each mouse via a puncture of the retro orbital sinus into a heparinized capillary tube, and then transferred to microtainers containing EDTA. Complete blood counts (CBC) including differential white blood cell counts are obtained using an ADVIA120 blood analyzer calibrated for mouse blood (Bayer Corp., Tarrytown, NY). Standard bleed days are 0, 3, 5, 7 and 10. Platelet counts are plotted as a function of time post-injection.

10
15

Example 12

UT-7 EPO Proliferation Assay for EMP Activity (Figure 18)

The UT-7Epo proliferation assay uses human megakaryoblastic leukemia cell line that responds to murine EPO (mEPO) and human EPO (huEPO) or other EPO like molecules for growth and survival.

Growth factors are serially diluted from 1000 ng/ml to 0.488 ng/ml in triplicate, in 100 µl of 10% FBS- Iscoves Modified Dulbelcco's medium (IMDM) across the 96 well plate. 15000 cells/well are added to the 96 well plate in 100 µl of 10% FBS IMDM. The total volume per well is 200 µl of media with 15000 cells per well. Cells and media alone are the zero control. Cells are incubated in a humidified chamber at 37 °C.

25

After incubation for 72 hours with the growth factor to be examined, viable cells are determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium, inner salt) incorporation (5+/-1 hr at 37 °C) and growth is measured by O.D.

30

(490 nm absorbance), limits not > 4.0 O.D. Reference: Yasusada Miura, Keita Kirito, and Norio Komatsu (1998), "Regulation of Both Erythroid and Megakaryocytic Differentiation of a Human Leukemia Cell Line, UT-7," Acta Haematologica, 99:180-184.

5

Example 13 Alternate Fc-Loop Insertion Sites

Having proven the feasibility of Fc-Loop insertion-style peptibodies with the L139/T140 insertion site using several different peptides, additional loops were surveyed in the Fc crystal structure. Using Fc-domain homology modeling, twelve different potential insertion sites were selected based on solvent accessible surface exposure, steric constraints within the loop, proximity to the Fc dimer interface and juxtaposition to sites of known effector function, such as the FcRn binding interface. The Fc-Loop sites that were identified as potential insertion sites are detailed and ranked. See Figure 2A and Table 12 below.

Table 12

Specific insertion sites for the human IgG1 Fc sequence

Domain	Loop	Insertion	Comments
CH2	P ₂₅ -P ₂₆	Not preferred	Tight turn
CH2	D ₄₆ -E ₅₃	H ₄₉ /E ₅₀ - 1 st E ₅₀ / D ₅₁ - 2 nd	No homology H/E site
CH2	V ₆₅ -A ₆₈	Not preferred	FcRn interactive
CH2	E ₇₄ -T ₈₀	Y ₇₇ /N ₇₈ - 1 st N ₇₈ /S ₇₉ - 2 nd	Low homology Y/N site
CH2	V ₈₉ -E ₉₉	Not preferred	FcRn interactive
CH2-CH3 linker	N ₁₀₆ -P ₁₂₇	K ₁₀₇ /A ₁₀₈ - 1 st N ₁₀₆ /K ₁₀₇ - 2 nd	Exposed turn
CH3	D ₁₃₇ -K ₁₄₁	L ₁₃₉ /T ₁₄₀ - 1 st E ₁₃₈ /L ₁₃₉ - 2 nd	Successfully tested L ₁₃₉ /T ₁₄₀
CH3	N ₁₆₅ -N ₁₇₇	E ₁₆₉ /N ₁₇₀ - 1 st N ₁₇₀ /N ₁₇₁ - 2 nd	Avoid tight turn N ₁₆₅ -P ₁₆₈ . No homology E/N site
CH3	T ₁₇₅ -S ₁₈₄	S ₁₈₁ /D ₁₈₂ - 1 st V ₁₇₈ /L ₁₇₉ - 2 nd	No homology V/L site. S/D site poss. better exposed
CH3	K ₁₉₅ -V ₂₀₃	G ₂₀₁ /N ₂₀₂ - 1 st N ₂₀₂ /V ₂₀₃ - 2 nd	α/β content. G/N site exposed. N/V site low

			homology
CH3	NA	Q ₁₆₇ /P ₁₆₈	IgA, IgM insertion site
CH3	NA	G ₁₈₃ /S ₁₈₄	IgA, IgM insertion site

Of these potential insertion sites, six were expressed using the TN8-19-7 peptide insert (Example 2) and evaluated for refolding efficiency and in vitro activity. An additional construct was added which contained an asymmetric linker system Gly4/Gly6 engineered into the original loop insertion site (L139/T140) previously described. In all, seven new Fc Loop Myostatin constructs were refolded, purified, and tested for activity.

The seven new Fc-Loop TN8-19-7 constructs that were tested included insertions in both CH2 and CH3 domains of human IgG1 Fc. Specifically, these insertions were: G201/N202 (CH3), E169/N170 (CH3), S181/D182 (CH3), H49/E50 (CH2), L139/T140 (G4-6) (CH3), Y77/N78 (CH2), and K107/A108 (CH2-CH3 linker domain). These constructs were transformed into E. coli by conventional methods known in the art, and were found to express almost exclusively in the insoluble inclusion body fraction. Interestingly, the H49/E50, L139/T140 (G4-6) and Y77/N78 constructs appeared to have the highest levels of expression. The E169/N170, S181/D182, K107/108 showed moderate expression, and the G201/202 construct showed some expression, but very little.

Those Fc-Loop TN8-19-7 constructs which expressed well in E. coli were purified by first solubilizing the isolated inclusion body fractions in 6 M Guanidine-HCl, 50 mM Tris, 8 mM DTT pH 9.0 (10 mL per 1g inclusion body) at room temperature with mixing for 1 hour. Then, a variety of refolding conditions were evaluated for each of the denatured and reduced Fc-Loop TN8-19-7 constructs to identify optimal refolding conditions. The three G201/N202, E169/N170, and S181/D182 constructs did not refold well under any of the conditions tested. Of the remaining four Fc-Loop TN8-19-7 constructs, L139/T140 (G4-6) refolded the best, while the remaining three H49/E50, Y77/N78 and K107/A108 refolded with sufficient yield to pursue further purification.

Using optimized refold conditions, the denatured and reduced Fc-Loop TN8-19-7 constructs were refolded from the solubilized inclusion body fractions by a 1:25 (v/v) dilution into 4 M Urea, 50 mM Tris-HCl, 0.16 M Arg-HCl, 20% glycerol, 3 M Cystine, 5 mM Cystamine pH 8.5. The solubilized peptibodies were added drop-wise to the refold buffer at 4°C with stirring. The refold reactions were allowed to stir for 72 hours, and subsequently purified chromatographically. Final purification was achieved by a 2-column chromatographic process, as described in Example 2.

The final pools of L139/T140 (G4-6), H49/E50, Y77/N78, and K107/108 were evaluated by RP-HPLC and SDS-PAGE, as illustrated in Figures 23 and 24. The yields from these four constructs are tabulated in Table 13.

Table 13
Expression and purification yields
of recoverable Fc-loop constructs

Insertion Site	Grams IB per Grams Paste	mg product per gram of IB
H49/E50 (CH2 domain)	0.182	2.08
L139/T140 (G4-6) (CH3 domain)	0.156	14.58
Y77/N78 (CH2 domain)	0.162	10.26
K107/A108	0.140	0.22

Among the four analogs purified the L139/T140 (G4-6) insertion site analog was produced with the best purity and in the highest yield.

The purified Fc-loop insertion analogs were further analyzed for functional myostatin receptor binding activity using an in vitro cell based inhibition assay, as described in Example 9. The results are shown in Table 14.

Table 14

***In vitro* cell based myostatin inhibition assay results**

Fc-Loop insertion analogs	IC ₅₀ (nM)
H49/E50	13.94
L139/T140 (G4-6)	0.8727
Y77/N78	17.06
Fc-Loop #6951 (139/140)	0.8335

The purified Fc-loop insertion analogs were further analyzed for FcRn binding using a Biacore assay system. Sample K107/A108 was not tested due to insufficient sample remaining after analysis. The IC₅₀ values determined using the *in vitro* cell based Myostatin inhibition assay, were similar for the L139/T140 (G4-6) insertion and the original Fc-Loop #6951, whereas the Fc-loop insertions at H49/E50 and Y77/N78 showed a reduced ability to inhibit myostatin (Figure 30). The Biacore FcRn binding experiments showed that H49/E50 and Y77/N78 bound the Fc receptor comparably to the control (Fc-Loop-1xTN8-19-7, #6951) with an EC₅₀ of about 680 nM, but L139/T140 (G4-6) had a lower and more favorable EC₅₀ at around 220 nM.

In summary, of the six new insertion sites evaluated, three failed to refold efficiently. Among the three new insertion site analogs recovered, K107/A108 folded poorly, H49/E50 (CH2 domain) refolded marginally well, and the two remaining insertions at Y77/N78 (CH2 domain) and the original insertion site of L139/T140 (CH3 domain) with an extended, asymmetric linker folded with a higher efficiency. Interestingly, all of these novel insertion site analogs refolded with significantly lower yield than the original Fc-loop construct (#6951) with TN8-19-7 in position L139/T140 using symmetric Gly2 linkers.

When tested for retention of FcRn binding capacity, all the Fc-loop molecules appeared similar in affinity with the possible exception of the extended, asymmetric linker construct, which seemed slightly better. This was consistent with the design paradigm to minimize steric interactions between the inserted peptide and the FcRn binding interface.

While all the purified Fc-loop constructs were active by the in vitro, cell-based functional assay (Table 14), the original insertion site (L139/T140) and the extended, asymmetric linker insertion at the same site appeared to be the most potent.

5 This work demonstrates that multiple loop domains within the human IgG1 Fc, as identified in Figure 23, will tolerate insertion of bioactive peptides while preserving the activity of both the peptide and Fc effector functions such as FcRn binding. Peptide insertion analogs utilizing these Fc-loop domains can vary significantly in refolding
10 efficiency and peptide activity. Each peptide/insertion combination can be individually optimized to maximize recovery and potency.

More preferable would be peptide insertions targeting the underlined sub-domains in Figure 23. Most preferable are the insertion site (L139/T140) and two additional loops in the CH2 domain (H49/E50
15 and Y77/N78).

Abbreviations

Abbreviations used throughout this specification are as defined
20 below, unless otherwise defined in specific circumstances.

Ac	acetyl (used to refer to acetylated residues)
AcBpa	acetylated p-benzoyl-L-phenylalanine
ACN	acetonitrile
ADCC	antibody-dependent cellular cytotoxicity
25 Aib	aminoisobutyric acid
bA	beta-alanine
Bpa	p-benzoyl-L-phenylalanine
BrAc	bromoacetyl (BrCH ₂ C(O))
BSA	Bovine serum albumin
30 Bzl	Benzyl
Cap	Caproic acid

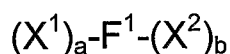
	CTL	Cytotoxic T lymphocytes
	CTLA4	Cytotoxic T lymphocyte antigen 4
	DARC	Duffy blood group antigen receptor
	DCC	Dicyclohexylcarbodiimide
5	Dde	1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)ethyl
	EDTA	ethylene diamine tetraacetic acid
	EMP	Erythropoietin-mimetic peptide
	ESI-MS	Electron spray ionization mass spectrometry
	EPO	Erythropoietin
10	Fmoc	fluorenylmethoxycarbonyl
	G-CSF	Granulocyte colony stimulating factor
	GH	Growth hormone
	HCT	hematocrit
	HGB	hemoglobin
15	hGH	Human growth hormone
	HOBt	1-Hydroxybenzotriazole
	HPLC	high performance liquid chromatography
	IL	interleukin
	IL-R	interleukin receptor
20	IL-1R	interleukin-1 receptor
	IL-1ra	interleukin-1 receptor antagonist
	Lau	Lauric acid
	LPS	lipopolysaccharide
	LYMPH	lymphocytes
25	MALDI-MS	Matrix-assisted laser desorption ionization mass spectrometry
	Me	methyl
	MeO	methoxy
	MES	(2-[N-Morpholino]ethanesulfonic acid)
30	MHC	major histocompatibility complex
	MMP	matrix metalloproteinase

	MMPI	matrix metalloproteinase inhibitor
	NaOAc	sodium acetate
	1-Nap	1-naphthylalanine
	NEUT	neutrophils
5	NGF	nerve growth factor
	Nle	norleucine
	NMP	N-methyl-2-pyrrolidinone
	PAGE	polyacrylamide gel electrophoresis
	PBS	Phosphate-buffered saline
10	Pbf	2,2,4,6,7-pendamethyldihydrobenzofuran-5-sulfonyl
	PCR	polymerase chain reaction
	Pec	pipecolic acid
	PEG	Poly(ethylene glycol)
	pGlu	pyroglutamic acid
15	Pic	picolinic acid
	PLT	platelets
	pY	phosphotyrosine
	PTFE	polytetrafluoroethylene
	RBC	red blood cells
20	RBS	ribosome binding site
	RP-HPLC	reversed phase HPLC
	RT	room temperature (25 °C)
	Sar	sarcosine
	SDS	sodium dodecyl sulfate
25	STK	serine-threonine kinases
	t-Boc	tert-Butoxycarbonyl
	tBu	tert-Butyl
	TGF	tissue growth factor
	THF	thymic humoral factor
30	TK	tyrosine kinase
	TMP	Thrombopoietin-mimetic peptide

	TNF	Tissue necrosis factor
	TPO	Thrombopoietin
	TRAIL	TNF-related apoptosis-inducing ligand
	Trt	trityl
5	UK	urokinase
	UKR	urokinase receptor
	VEGF	vascular endothelial cell growth factor
	VIP	vasoactive intestinal peptide
	WBC	white blood cells
10		

What is claimed is:

1. A composition of matter of the formula



and multimers thereof, wherein:

- 5 F^1 is an Fc domain modified so that it comprises at least one X^3 in a loop region;

X^1 and X^2 are each independently selected from $-(L^1)_c-P^1$, $-(L^1)_c-P^1-(L^2)_d-P^2$, $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3$, and $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3-(L^4)_f-P^4$;

- X^3 is independently selected from $-(L^5)_c-P^5$, $-(L^5)_c-P^5-(L^6)_d-P^6$, $-(L^5)_c-P^5-(L^6)_d-P^6-(L^7)_e-P^7$, and $-(L^5)_c-P^5-(L^6)_d-P^6-(L^7)_e-P^7-(L^8)_f-P^8$;

P^1 , P^2 , P^3 , and P^4 are each independently sequences of pharmacologically active polypeptides or pharmacologically active peptides;

- P^5 , P^6 , P^7 , and P^8 are each independently sequences of pharmacologically active peptides;

L^1 , L^2 , L^3 , L^4 , L^5 , L^6 , L^7 , and L^8 are each independently linkers; and

a , b , c , d , e , and f are each independently 0 or 1.

2. The composition of matter of Claim 1 wherein a and b are each 0.
3. The composition of matter of Claim 1 wherein the Fc domain
- 20 comprises an IgG Fc domain.
4. The composition of matter of Claim 3 wherein the Fc domain comprises a sequence selected from SEQ ID NOS: 599 and 603 to 607.
5. The composition of matter of Claim 1 wherein the Fc domain comprises an IgG1 Fc domain.
- 25 6. The composition of matter of Claim 5 wherein the IgG1 Fc domain comprises SEQ ID NO: 599 and X^3 is inserted into or replaces all or part of a sequence selected from SEQ ID NOS: 621, 622, 624, 625, 627, 628, 630, 632, 634, and 636.
7. The composition of matter of Claim 6 wherein X^3 is inserted into or
- 30 replaces all or part of a sequence selected from SEQ ID NOS: 623, 626, 629, 631, 633, 635, and 637.

8. The composition of matter of Claim 7 wherein X³ is inserted at Leu₁₃₉/Thr₁₄₀.
9. The composition of matter of Claim 5 wherein the IgG1 Fc domain comprises SEQ ID NO: 603 and X³ is inserted into or replaces all or part
5 of a sequence selected from SEQ ID NOS: 621, 622, 624, 625, 627, 628, 630, 632, 634, and 636.
10. The composition of matter of Claim 9 wherein X³ is inserted at H₅₃/E₅₄, Y₈₁/N₈₂, N₁₁₀/K₁₁₁, L₁₄₃/T₁₄₄, Q₁₇₁/P₁₇₂, E₁₇₃/N₁₇₄, S₁₈₆/D₁₈₇, G₁₈₈/S₁₈₉, or G₂₀₅/N₂₀₆.
- 10 11. The composition of matter of Claim 5 wherein the IgG1 Fc domain comprises SEQ ID NO: 604 and X³ is inserted into or replaces all or part of a sequence selected from SEQ ID NOS: 621, 622, 624, 625, 627, 628, 632, 634, 636, and 644.
12. The composition of matter of Claim 11 wherein X³ is inserted at
15 H₅₃/E₅₄, Y₈₁/N₈₂, N₁₁₀/K₁₁₁, L₁₄₃/T₁₄₄, Q₁₇₁/P₁₇₂, E₁₇₃/N₁₇₄, S₁₈₆/D₁₈₇, G₁₈₈/S₁₈₉, or G₂₀₅/N₂₀₆.
13. The composition of matter of Claim 1 wherein the Fc domain comprises an IgG3 Fc domain.
14. The composition of matter of Claim 13 wherein the IgG3 Fc domain
20 comprises SEQ ID NO: 605 and X³ is inserted into or replaces all or part of a sequence selected from SEQ ID NOS: 614, 621, 622, 624, 627, 639, 641, 644, 645, and 646.
15. The composition of matter of Claim 14 wherein X³ is inserted at
25 H₁₀₀/E₁₀₁, F₁₂₈/N₁₂₉, N₁₅₇/K₁₅₈, M₁₉₀/T₁₉₁, Q₂₁₈/P₂₁₉, E₂₂₀/N₂₂₁, S₂₃₂/D₂₃₃, G₂₃₄/S₂₃₅, or G₂₅₂/N₂₅₃.
16. The composition of matter of Claim 1 wherein the Fc domain comprises an IgG2 Fc domain.
17. The composition of matter of Claim 16 wherein the Fc domain
30 comprises SEQ ID NO: 606 and X³ is inserted into or replaces all or part of a sequence selected from SEQ ID NOS: 621, 622, 624, 632, 636, 639, 640, 642, 644, and 646.

18. The composition of matter of Claim 17 wherein X³ is inserted at
H₄₉/E₅₀, F₇₇/N₇₈, N₁₀₆/K₁₀₇, M₁₃₉/T₁₄₀, Q₁₆₇/P₁₆₈, E₁₆₉/N₁₇₀, S₁₈₁/D₁₈₂,
G₁₈₃/S₁₈₄, or G₂₀₁/N₂₀₂.
19. The composition of matter of Claim 1 wherein the Fc domain
5 comprises an IgG4 Fc domain.
20. The composition of matter of Claim 19 wherein the Fc domain
comprises SEQ ID NO: 607 and X³ is inserted into or replaces all or part
of a sequence selected from SEQ ID NOS: 620, 621, 624, 627, 632, 634,
638, 639, 643, and 644.
- 10 21. The composition of matter of Claim 20 wherein X³ is inserted at
Q₅₀/E₅₁, F₇₈/N₇₉, N₁₀₇/K₁₀₈, M₁₄₀/T₁₄₁, Q₁₆₈/P₁₆₉, E₁₇₀/N₁₇₁, S₁₈₂/D₁₈₃,
G₁₈₄/S₁₈₅, or G₂₀₂/N₂₀₃.
22. The composition of matter of Claim 1 wherein the Fc domain
comprises SEQ ID NO: 608 and X³ is inserted into or replaces all or part
15 of a sequence selected from SEQ ID NOS: 621, 622, 628, 624, 627, 632,
636, 639, 644, and 646.
23. The composition of matter of Claim 22 wherein X³ is inserted at
H₁₁₂/E₁₁₃, F₁₄₀/N₁₄₁, N₁₆₉/K₁₇₀, M₂₀₄/T₂₀₅, Q₂₃₂/P₂₃₃, E₂₃₄/N₂₃₅,
S₂₄₆/D₂₄₇, G₂₄₈/S₂₄₉, or G₂₆₈/N₂₆₉.
- 20 24. The composition of matter of Claim 1 wherein X³ comprises an
angiotensin-2 (ang-2) binding peptide sequence.
25. The composition of matter of Claim 24 wherein the ang-2 binding
peptide sequence is selected from SEQ ID NOS: 100 to 189.
26. The composition of matter of Claim 25 wherein the ang-2 binding
25 peptide sequence is SEQ ID NO: 147.
27. The composition of matter of Claim 26 wherein F¹ comprises an IgG1
Fc domain.
28. The composition of matter of Claim 27 having the sequence of SEQ ID
NO: 618.
- 30 29. The composition of matter of Claim 1 wherein X³ comprises a
myostatin binding peptide sequence.

30. The composition of matter of Claim 29 wherein the myostatin binding peptide sequence is selected from SEQ ID NOS: 218 to 509.
31. The composition of matter of Claim 30 wherein the myostatin binding peptide sequence is SEQ ID NO: 365.
- 5 32. The composition of matter of Claim 31 wherein F¹ comprises an IgG1 Fc domain.
33. The composition of matter of Claim 32 having the sequence SEQ ID NO: 612.
34. The composition of matter of Claim 1 wherein X³ comprises an
10 erythropoietin-mimetic (EPO-mimetic) peptide sequence.
35. The composition of matter of Claim 34 wherein the EPO-mimetic peptide sequence is selected from SEQ ID NOS: 1 to 27.
36. The composition of matter of Claim 35 wherein the EPO-mimetic peptide sequence is SEQ ID NO: 2.
- 15 37. The composition of matter of Claim 36 wherein F¹ comprises an IgG1 Fc domain
38. The composition of matter of Claim 37 having the sequence of SEQ ID NO: 615.
39. The composition of matter of Claim 1 wherein X³ comprises a
20 thrombopoietin-mimetic (TPO-mimetic) peptide sequence.
40. The composition of matter of Claim 39 wherein the TPO-mimetic peptide sequence is selected from SEQ ID NOS: 28 to 99.
41. The composition of matter of Claim 40 wherein the TPO-mimetic peptide sequence is SEQ ID NO: 28.
- 25 42. The composition of matter of Claim 41 wherein F¹ comprises an IgG1 Fc domain
43. The composition of matter of Claim 42 having the sequence SEQ ID NO: 616.
44. The composition of matter of Claim 1 wherein X³ comprises a nerve
30 growth factor (NGF) binding peptide sequence.

45. The composition of matter of Claim 44 wherein the NGF binding peptide sequence is selected from SEQ ID NOS: 190 to 218.
46. The composition of matter of Claim 1 wherein X³ comprises a B cell activating factor (BAFF) binding peptide sequence.
- 5 47. The composition of matter of Claim 46 wherein the BAFF binding peptide sequence is selected from SEQ ID NOS: 510 to 594.
48. A DNA encoding a composition of matter of Claim 1.
49. An expression vector comprising the DNA of Claim 46.
50. A host cell comprising the expression vector of Claim 47.
- 10 51. The cell of Claim 48, wherein the cell is an E. coli cell.
52. A process for preparing a pharmacologically active compound, which comprises:
- a. selecting at least one randomized peptide that modulates the activity of a protein of interest; and
- 15 b. preparing a pharmacologic agent comprising an amino acid sequence of the selected peptide as an internal sequence of an Fc domain..
53. The process of Claim 50, wherein the internal region of the Fc domain is a loop region.
- 20 54. The process of Claim 50, wherein the peptide is selected in a process comprising one or more techniques selected from yeast-based screening, rational design, protein structural analysis, or screening of a phage display library, an E. coli display library, a ribosomal library, or a chemical peptide library.
- 25 55. The process of Claim 50, wherein the preparation of the pharmacologic agent is carried out by:
- a. preparing a gene construct comprising a nucleic acid sequence encoding an Fc domain wherein a sequence the selected peptide is inserted into or replaces one or more amino acids within the Fc
- 30 domain; and
- b. expressing the gene construct.

56. The process of Claim 53, wherein the gene construct is expressed in an E. coli cell.
57. The process of Claim 50, wherein the protein of interest is a cell surface receptor.
- 5 58. The process of Claim 50, wherein the protein of interest has a linear epitope.
59. The process of Claim 50, wherein the protein of interest is a cytokine receptor.
60. The process of Claim 50 wherein the Fc domain is an IgG Fc domain.
- 10 61. A modified antibody, comprising an Fc domain modified so that it comprises at least one X³ in a loop region, wherein:
- X³ is independently selected from -(L⁵)_c-P⁵, -(L⁵)_c-P⁵-(L⁶)_d-P⁶, -(L⁵)_c-P⁵-(L⁶)_d-P⁶-(L⁷)_e-P⁷, and -(L⁵)_c-P⁵-(L⁶)_d-P⁶-(L⁷)_e-P⁷-(L⁸)_f-P⁸;
- P⁵, P⁶, P⁷, and P⁸ are each independently sequences of
- 15 pharmacologically active peptides;
- L⁵, L⁶, L⁷, and L⁸ are each independently linkers; and
- c, d, e, and f are each independently 0 or 1.
62. A process for preparing a modified antibody, which comprises:
- a) selecting at least one peptide that modulates the activity of a
- 20 protein of interest; and
- b) preparing an antibody comprising an amino acid sequence of the selected peptide in a loop region of an Fc domain of the antibody.